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Cilia
epithelium
mucin
newborn
oxygen toxicity
trachea

Toxic Effects of Oxygen on Cultured Human Neonatal Respiratory Epithelium

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Extract

Explants of tracheal epithelium from each of six human neonates were exposed to both 80% and 20% oxygen under otherwise identical culture conditions. Cessation of ciliary movement and carbon particle transport occurred after 48–96 hr of exposure to 80% oxygen, but not after 168 hr of exposure to 20% oxygen.

This alteration of ciliary function was related temporally to squamous metaplasia, or to degeneration and sloughing of cells from the surface epithelium. Explants secreted more mucin and lysozyme during the first 24–72 hr of culture in 80% oxygen. Thereafter, diminished secretion was observed, apparently related to loss of goblet cells from the surface epithelium and failure to discharge the secretory products of submucosal glands. These findings indicate that high oxygen concentrations at atmospheric pressure alone can cause marked alterations of structure and function in neonatal large airways epithelium. Onset of these changes corresponds to the time when the earliest clinical and cytologic evidence of bronchopulmonary dysplasia has been detected, suggests that similar oxygen-induced changes are produced *in vivo*. Loss of mucociliary function may be an important pathogenetic component of bronchopulmonary dysplasia.

Speculation

Organ culture of human large airways epithelium appears to be a useful model for study of pulmonary oxygen toxicity. Studies of oxygen concentrations and duration of exposure tolerated by human respiratory epithelium, the cellular mechanisms of oxygen-induced alteration, and the use of pharmacologic agents to prevent or delay onset of toxic changes may be facilitated by using this *in vitro* technique.

Introduction

Neonatal lungs which have been exposed to high concentrations of oxygen (HiO) show marked alteration of epithelial and vascular tissues, especially at the level of terminal airways [13]. No direct evidence demonstrating oxygen-induced changes in large airways of neonates has been presented. Cytologic studies by Northway and Rosan [14] do suggest an early disruption of ciliated epithelium somewhere in the airways of neonates who were exposed to HiO. However, these infants were intubated and ventilated with positive pressure, often for prolonged periods. It is difficult to differentiate the effects of HiO from these and other potential insults to lung tissue. We have recently developed an organ-culture system for the maintenance of human respiratory epithelium [1] which allows direct observation of the effects of HiO in controlled

situations. Changes in mucociliary transport, surface epithelial morphology, and mucin secretion of explants exposed to 80% oxygen in this culture system are reported.

Materials and Methods

The tissue source for large airways epithelium was trachea which was removed from six neonates between 1 and 3 hr after death. Gestational ages at birth for the six trachea donors ranged from 24–40 weeks, postnatal survival from less than 24 hr to 28 days. Causes of death included extreme prematurity [2], hypoplastic left ventricle [2], asphyxiating thoracic dystrophy, and trisomy 13–15. None of the subjects had been intubated or exposed to oxygen concentrations higher than 40%.

Tracheas were transported to the laboratory at room temperature in Hank's balanced salt solution containing 100 μg/ml gentamicin and 1 μg/ml amphotericin [17]. Extraneous tissue was removed and each trachea was cut into four full length, full thickness strips. Two strips were placed in each of two preweighed plastic petri dishes (50 by 12 mm) containing 2.0 ml single strength Medium 199 (Earle's base) to which had been added 100 μg/ml gentamicin and 10 μg/ml nystatin [18]. In two experiments, both 1×10^6 dpm/ml ^{3}H-6-glucosamine and 8×10^6 dpm/ml $^{35}SO_4$ were added to the media. Tissue weights were calculated from the difference of petri dish weights before and after addition of tissue. The two petri dishes were then placed in separate incubators. Both contained 5% CO_2 and a water-saturated environment at 35°. One incubator contained 20% oxygen, the other 80% oxygen. The high oxygen concentration was continuously monitored and adjusted within a $\pm 1\%$ range by an IMI oxygen controller [19].

Petri dishes were removed from the incubators at 24-hr intervals. At these times, the surface was illuminated obliquely with a high intensity light source and ciliary movement observed through a dissecting microscope [6]. Sterile India ink diluted with nine parts sterile saline was placed on explant surfaces to confirm the presence or absence of effective ciliary movement. Medium was then removed from each dish after gentle irrigation of explants to remove the existing mucus layer, and immediately replaced with fresh medium. Harvested media were stored at −20° until completion of the experiment.

Before culture, and after 96 and 168 hr of incubation, pieces of tissue selected randomly were cut from tracheal strips and fixed in Bouin's solution or buffered neutral formalin. These tissues were subsequently processed for histologic and histochemical examination. In addition to routine staining with hematoxylin and eosin, tissue sections were stained sequentially with Alcian blue (AB) at pH 2.5 and periodic acid-Schiff (PAS) [12].

Samples of media designated for analysis were thawed, dialyzed individually against four changes, 300 volumes each, of distilled water over a 72- –96-hr period, lyophilized, and taken up in a fixed volume of distilled water. Determinations performed on these samples include: (*1*) lysozyme by the radial diffusion method of Osserman and Lawlor [15], (*2*) sialic acid by the thiobarbituric acid assay of Warren [16], (*3*) fucose by the H_2SO_4-cysteine method of Dische and Danilchenko [5], and (*4*) blood group activity by a microtitrametric method [7]. Tritium and $^{35}SO_4$ were counted simultaneously in Bray's solution at 59% efficiency for ^{35}S with a 2.4% contribution of ^3H, and at 39% efficiency for ^3H with a 24.4% contribution of ^{35}S.

Chromatographic and electrophoretic studies were performed on explant secretions which had been reduced with 5 mM dithiothreitol in the presence of 8 M urea, alkylated, and then dialyzed extensively with dilute buffer [2]. Chromatographic fractionation of these secretions was carried out on a column (2.5 by 90 cm) of Bio-Gel A-5 equilibrated with a solution containing 50 mM NaCl and 10 mM Tris-HCl (pH 7.0). Samples (5 ml) which contained approximately 10 mg protein were eluted by upward flow with the same solution. Discontinuous polyacrylamide gel electrophoresis of reduced and alkylated secretions was carried out by the method of Davis [4]. After electrophoresis, gels were cut into 1.0-mm slices, each slice was solubilized in 0.2 ml 30% hydrogen peroxide, and this solution counted as described above.

Results

In the presence of 20% oxygen ciliary motility of explant surfaces was not perceptibly altered after 7 days of culture. Transport of carbon particles proceeded at a rate between 1.0 and 1.5 cm/min on strips of trachea cultured in 20% oxygen at all intervals up to 7 days after initiation of culture. In contrast, slowing of ciliary movement and focal inactivity of cilia were noted consistently after 48 hr of exposure to 80% oxygen. All ciliary activity had ceased after 72 or 96 hr in 80% oxygen (Table I). No movement of carbon particles was noted after 48–96 hr of culture in 80% oxygen.

Table I. Effect of 80% oxygen on ciliary function of cultured human tracheal epithelium

Oxygen concentration, %	Number of experiments	Cessation of all ciliary movement, hr	Cessation of particle transport, hr
20	6	Did not cease	Did not cease
80	6	72–96	48–96

Excellent preservation of ciliated and goblet cells in the surface epithelium was observed after both 96 and 168 hr of culture in 20% oxygen (Figs. 1, 2, and 3). Focal necrosis of deep submucosal glands was noted in these explants and is considered to be the result of hypoxia created by an excessive oxygen gradient between the surface and deeper portions of the explants.

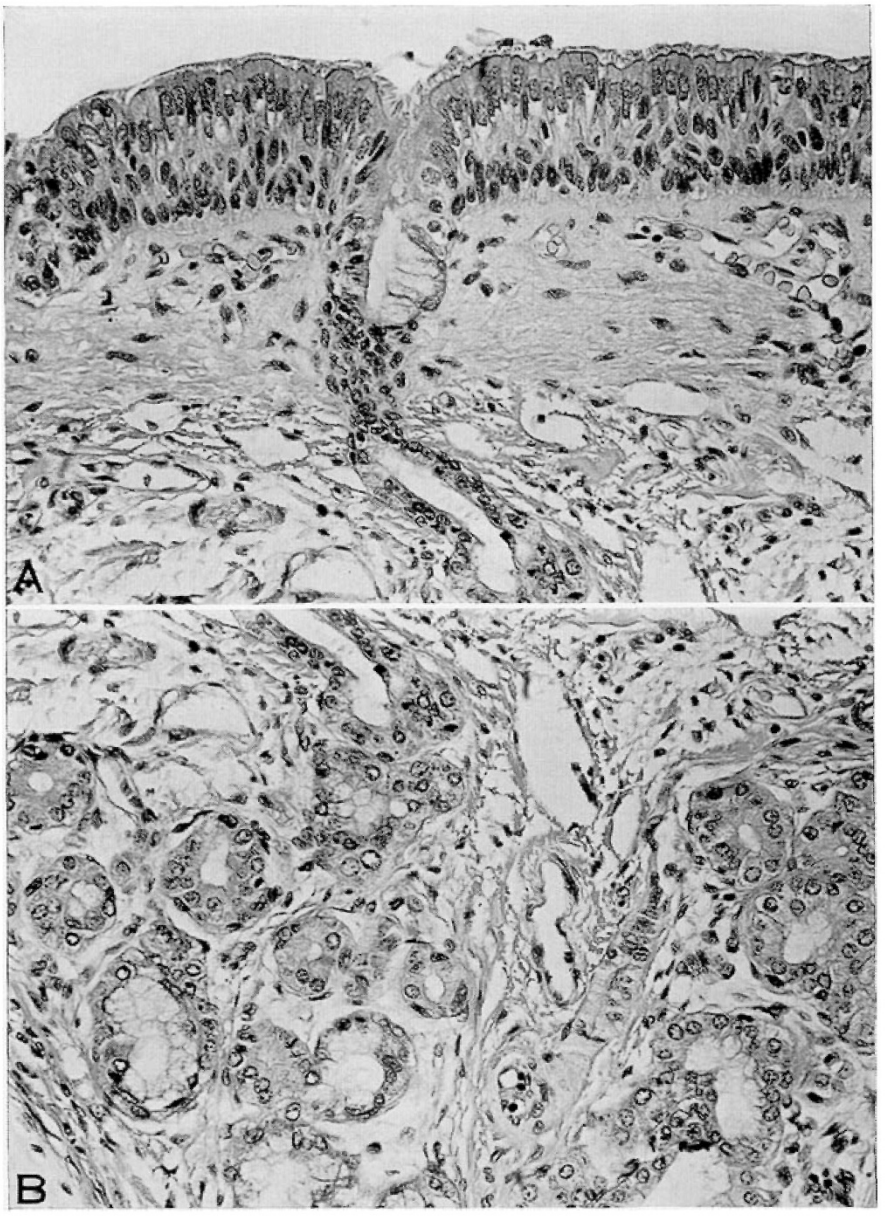

Fig. 1. A: Preculture. This photomicrograph and all others (Figs. 2–5) show preculture or explant tracheal tissue from a single experiment. The tissue donor was an infant with asphyxiating thoracic dystrophy who died before 24 hr of age. The pseudostratified ciliated columnar epithelium is histologically normal. Cilia can be seen. × 250; hematoxylin and eosin. *B:* Preculture. Submucosal glands are well preserved. Both serous acini and mucus acini with so-called serous demilunes are seen. Little retained luminal secretion is present. × 250; hematoxylin and eosin.

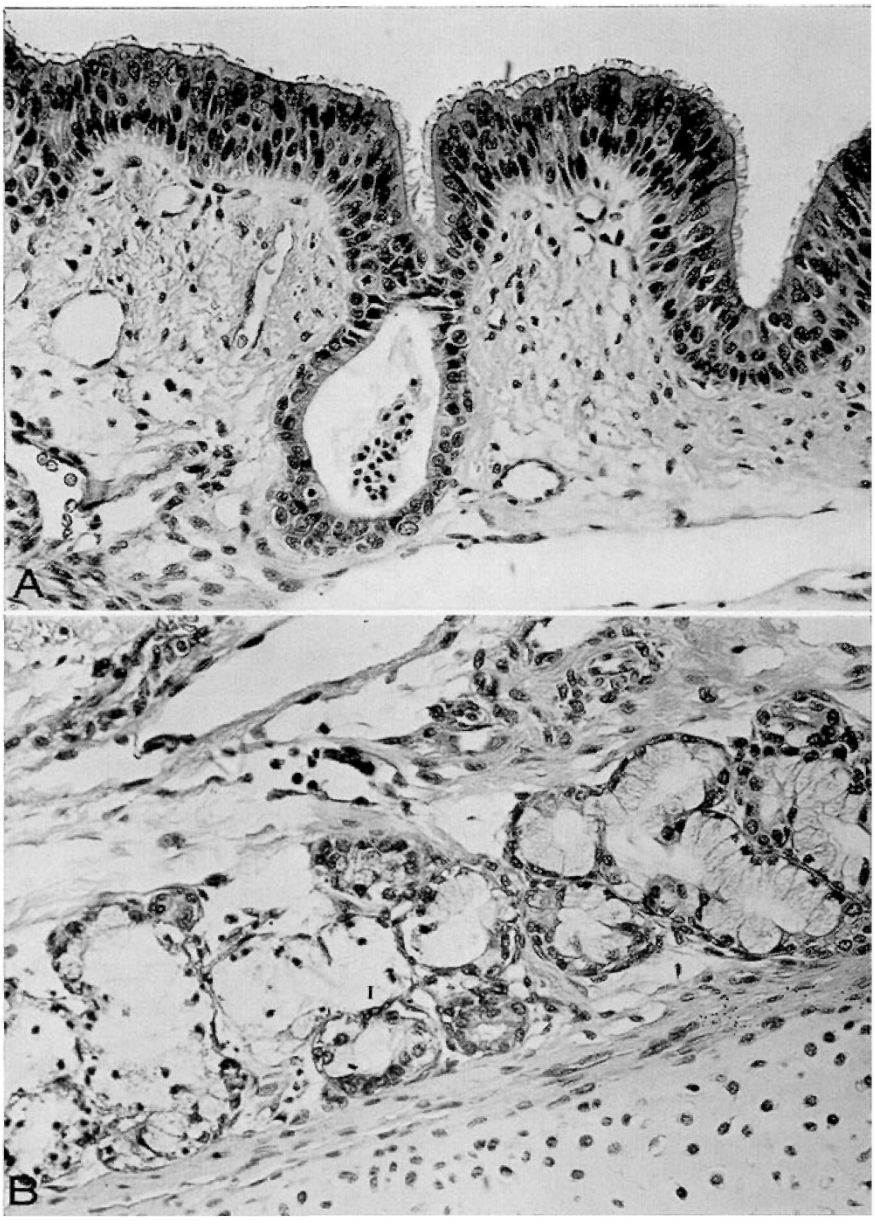

Fig. 2. A: 96-hr culture, 20% oxygen. The pseudostratified ciliated columnar epithelium, including the basal cell layer, is well preserved. Degenerating epithelial cells are seen within a duct lumen. × 250; hematoxylin and eosin. *B:* 96-hr culture, 20% oxygen. The gland structures at the right of the photograph are well preserved. Those to the left show degeneration, exfoliation, and pyknosis. This demonstrates the improved preservation of superficial and peripheral glands of explants cultured in 20% oxygen. × 250; hematoxylin and eosin.

More superficial submucosal glands were well preserved after both intervals in culture. Material staining with AB-PAS was present in both the goblet cells of surface epithelium and in cells and lumina of intact submucosal glands after 4 and 7 days of exposure to 20% oxygen.

Marked histologic changes were noted in explants cultured in 80% oxygen for these intervals (Figs. 4 and 5). At 96 hr, and even more impressively at 168 hr, the pseudostratified architecture of surface epithelium was disrupted. Ciliated surface cells became round and appeared to be in the process of desquamation. Many surface cells were no longer ciliated. In some areas metaplasia of surface epithelium to a stratified squamous type occurred. In other areas only a single layer of basal cells remained. No goblet cells were seen. Sub-

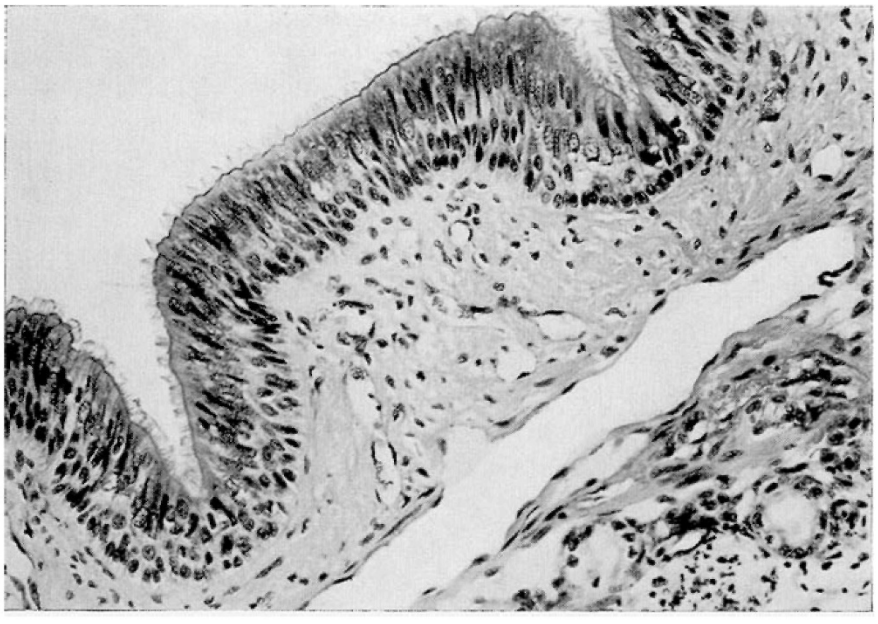

Fig. 3. One hundred sixty-eight-hour culture, 20% oxygen. The surface epithelium remains intact in its original pseudostratified, ciliated form. At the lower right margin several poorly preserved submucosal gland acini can be seen. × 250; hematoxylin and eosin.

mucosal gland cells, on the other hand, were uniformly intact. Many of these glands were dilated and had accumulated large amounts of intracellular and intraluminal AB-PAS-positive material.

Two of these experiments also were designed to assess the secretion of mucins and other macromolecules by explants in both 20% and 80% oxygen. In these experiments both ^{3}H-6-glucosamine and $^{35}SO_4$ were added to the culture medium. At least 80% of the nondialyzable label in spent culture medium behaved chromatographically and electrophoretically like high molecular weight mucin. This major portion of labeled macromolecules, after reduction and alkylation, was eluted from a column of Bio-Gel A-5 with the void volume. The void volume material stained intensely with PAS and failed to penetrate 7.5% polyacrylamide gels after electrophoresis at pH 8.4 for 3 hr. The remaining 20% of the nondialyzable label was incorporated into smaller macromolecules which penetrated 7.5% polyacrylamide gels and were probably nonmucin glycoproteins. On the basis of these data, quantitation of radioactivity in dialyzed media was used to estimate the production and discharge of mucins by explants.

Discharge of labeled mucin (tritiated and $^{35}SO_4$-labeled macromolecules) in the presence of 80% oxygen equaled or exceeded mucin discharge in the presence of 20% oxygen for the first 48–72 hr in culture (Fig. 6). Thereafter, discharge of labeled mucin was consistently greater in the presence of 20% oxygen. Similarly, titers of secreted blood group active mucin in the media were identical after 24–48 hr in culture, but declined more rapidly thereafter in the presence of 80% oxygen. Secretion of macromolecular fucose and sialic acid, and of the nonmucin protein lysozyme, was also diminished after 2–3 days in 80% oxygen (Fig. 7).

Discussion

An adverse effect of HiO at atmospheric pressure on human tracheobronchial epithelium has never been documented under controlled experimental conditions. Postmortem studies of lungs exposed to HiO demonstrate marked changes of bronchiolar epithelium [13], but observations of changes in larger airways epithelium have not been communicated. An additional problem with interpretation of previous studies is that the tissues have usually been simultaneously exposed to prolonged positive pressure ventilation and other potential sources of injury such as endotracheal intubation and suction, altered humidity, infection, and drugs. Observed changes, therefore, have not been unequivocally oxygen-induced [3]. In one study with a design more appropriate for the determination of oxygen-induced changes, cat tracheal mucus flow was markedly impaired after a brief exposure to 100% oxy-

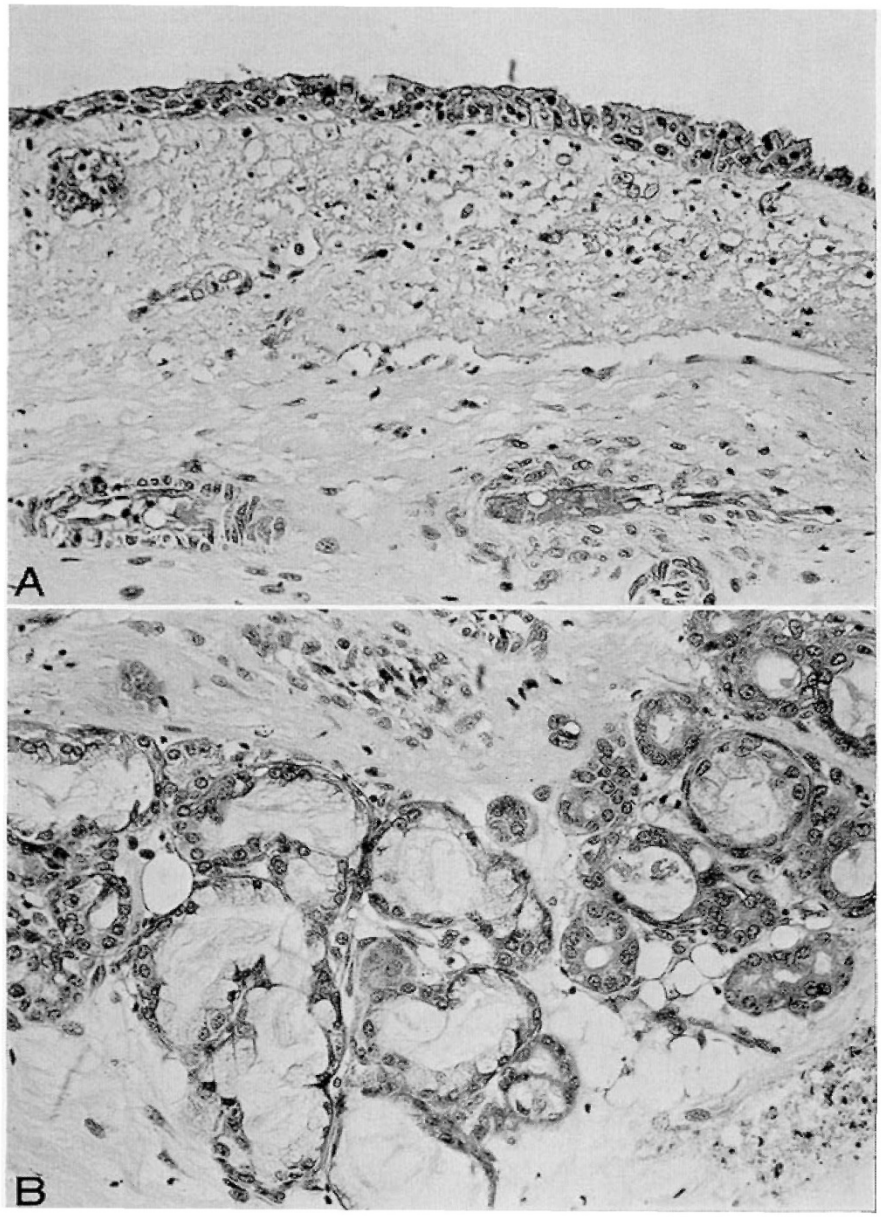

Fig. 4. A: 96-hr culture, 80% oxygen. The surface epithelium is largely squamous; only a few ciliated cells are present. Within the epithelium individual cell pyknosis and degeneration are present. The submucosa is edematous and contains many degenerating leukocytes. × 250; hematoxylin and eosin. *B:* 96-hr culture, 80% oxygen. Submucosal glands are well preserved. Both serous and mucus gland elements can be seen. Many gland lumina contain fibrillar secretory material. × 250; hematoxylin and eosin.

gen [9]. Other workers, however, were unable to confirm this observation in dog tracheae [11]. These results emphasize the hazards of reaching general conclusions on the basis of studies conducted in one mammalian species.

The organ culture system used in the present study offers several advantages. (*1*) Oxygen toxicity studies can be carried out on human tissue. (*2*) Tissue from one source can be exposed simultaneously to two oxygen concentrations under otherwise uniform conditions. (*3*) The effect of oxygen can be studied sequentially for a relatively long duration, *i.e.,* 7 days or more. Long term observations in this series of controlled *in vitro* experiments demonstrate conclusively that oxygen is toxic to human large airways epithelium.

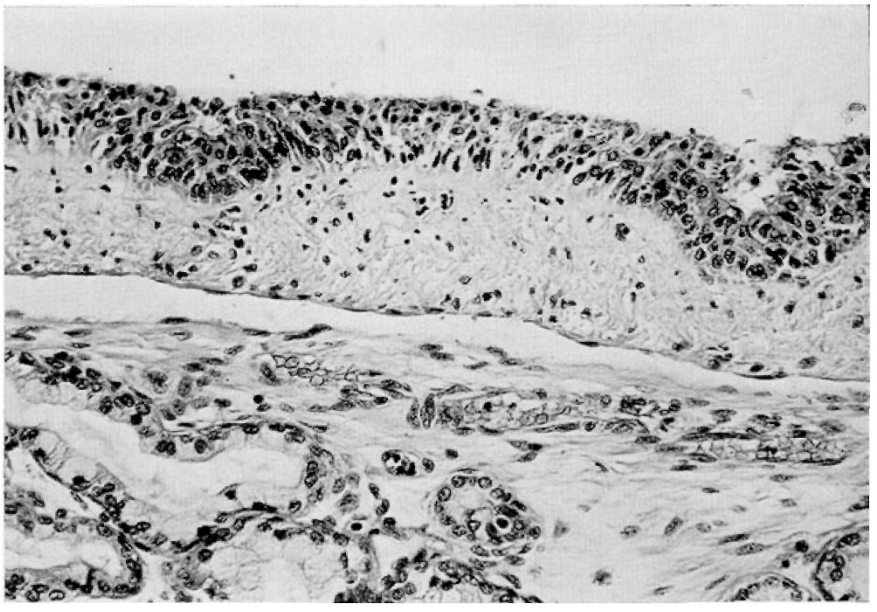

Fig. 5. One hundred sixty-eight hour culture, 80% oxygen. The surface epithelium is squamous and exfoliating. No ciliated cells can be seen; cell layers are poorly organized. The submucosal glands are well preserved, predominantly mucus in character, dilated, and show retained luminal secretion. × 250; hematoxylin and eosin.

It is not yet certain that this observation can be extrapolated to the *in vivo* situation. However, several pieces of evidence do indicate that the changes of oxygen toxicity may be similar *in vitro* and *in vivo*. Ciliated cells appear in tracheal secretions of the neonate within 3 days after initiation of therapy with HiO [14]. Manifestations of bronchopulmonary dysplasia, both clinical and radiographic, generally are observed after 4 or 5 days of exposure to HiO. This time sequence is consistent with the appearance of changes in ciliary motility and epithelial morphology of cultured respiratory epithelium exposed to 80% oxygen.

The clinical implications of these observed changes may be of therapeutic importance. These changes in intact airways would eliminate mucociliary function, a primary defense mechanism of the lung. Expected sequelae would be accumulation of secretions, defective clearance of particles, and predisposition to lower respiratory tract infection. These sequelae may in turn cause severe obstructive changes in conducting airways. Under these circumstances the early use of therapy designed to assist in the clearance of secretions would be indicated.

The alteration of mucin secretion by cultured respiratory epithelium after exposure to HiO is also potentially important. Increased secretion of mucin and lysozyme by explants for the first 24–48 hr of culture in HiO may have resulted from better preservation of deep submucosal glands. On the other hand, 80% oxygen may have been an irritative stimulus causing hypersecretion, especially from goblet cells of the surface epithelium. It will be important to ascertain whether hypersecretion occurs soon after introduction of HiO to infants with respiratory distress.

The reduction of secretion by explants after 3 days of exposure to 80% oxygen is surprising in view of the excellent submucosal gland preservation in explants exposed to this environment. It is difficult to ascribe this reduction of secretion to the loss of goblet cells, inasmuch as these cells of the surface epithelium comprise a total mass much smaller than the mass of mucus-secreting cells in submucosal glands [8]. The accumulation of large amounts of intracellular and intraluminal mucin seen in glands of explants exposed to 80% oxygen suggests that discharge of the accumulated secretory product was hindered. This suggestion is further supported by preliminary studies from our laboratory which show a four- to fivefold increase of nondialyzable $^{35}SO_4$ and tritium, and a two- to threefold increase of macromolecular fucose and sialic acid in the 20,000 × *g* supernatant fraction of homogenates of washed explants cultured in the presence of HiO for 7 days.

It appears that HiO concentrations in some way interfere with the discharge of mucin produced in submucosal glands. This interference is detected at the

same interval after exposure as is cessation of ciliary activity on the surface epithelium. We suggest that inhibition of ciliary motility in the ciliated portion of the submucosal gland duct may be the mechanism by

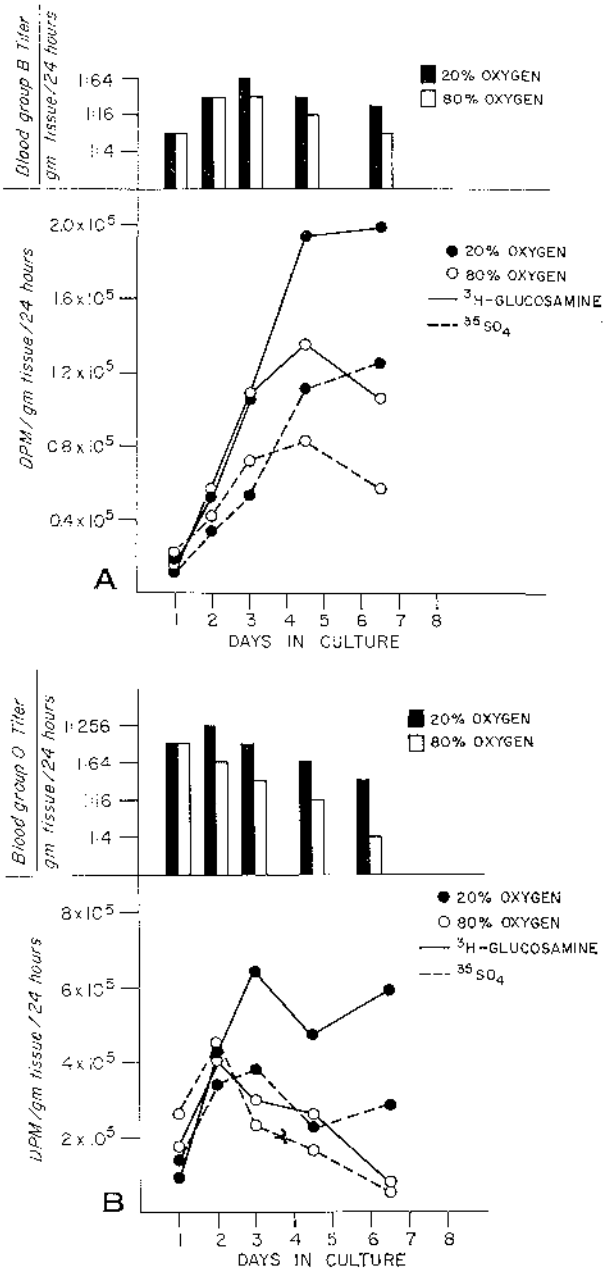

Fig. 6. Secretion of tritiated and sulfated macromolecules, and of blood group substance, in the presence of 20% and 80% oxygen by tracheal explants from neonates who died of hypoplastic left ventricle (*A*) and asphyxiating thoracic dystrophy (*B*). Media from *days 4* and *5* and *days 6* and *7* were pooled before analysis of samples from each of the oxygen exposure groups, but data from these analyses are calculated on a 24-hr basis.

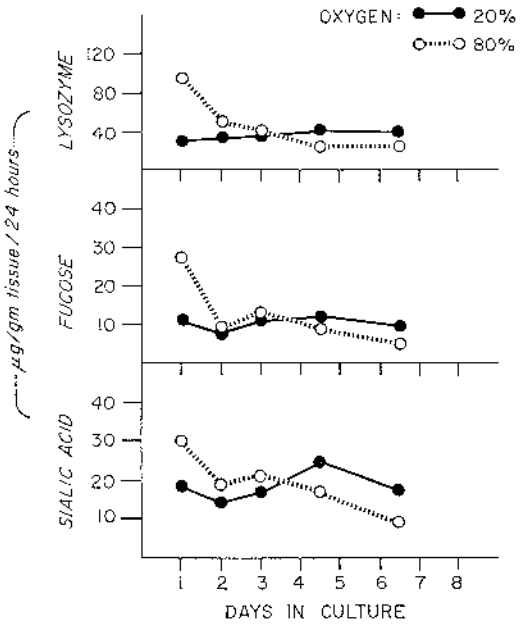

Fig. 7. Secretion of lysozyme and macromolecular fucose and sialic acid in the presence of 20% and 80% oxygen by tracheal explants from the infant who died of asphyxiating thoracic dystrophy.

which mucin discharge is prevented. Alternatively, HiO may interfere with the function of myoepithelial cells which presumably assist in the movement of secretions from the distal portions of the glands [10].

It is also possible that HiO oxidizes the sulfhydryl groups of mucin molecules, resulting in more intermolecular disulfide bonds [2], and subsequently in more viscous secretions which cannot be as easily expelled from the glands.

Summary

Human neonatal tracheal epithelium which has been cultured in the presence of 80% oxygen for at least 48 hr shows marked changes of ciliary function and mucus secretion. These changes, including cessation of ciliary movement, loss of ciliated surface epithelium, and initial hypersecretion followed by hyposecretion of mucin, were not observed during culture of explants from the same source in 20% oxygen for 7 days. The times of onset for changes induced by oxygen *in vitro* and the development of cytologic and roentgenographic evidence of bronchopulmonary dysplasia are strikingly similar and suggest that the alterations observed in cultured neonatal trachea also occur in the neonate exposed to high concentrations of oxygen.

References and Notes

1. Boat, T. F., Iyer, R. N., Macintyre, M. D., Carlson, D. M., and Matthews, L. W.: Evaluation of a culture method for study of human tracheobronchial secretion (Abstract). Amer. Rev. Resp. Dis., *103:* 915 (1971).
2. Boat, T. F., and Matthews, L. W.: Chemical composition of human tracheobronchial secretions. In: M. Dulfano: Sputum. (Charles C Thomas, Springfield, Ill., 1973).
3. Clements, J. A., and Fisher, H. K.: The oxygen dilemma. New Engl. J. Med., *282:* 976 (1970).
4. Davis, B. J.: Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci., *121:* 404 (1964).
5. Dische, Z., and Danilchenko, A.: Modifications of two color reactions of hexoses with cysteine and sulfuric acid. Anal. Biochem., *21:* 119, (1967).
6. Hoorn, B.: Organ cultures of ciliated epithelium for the study of respiratory viruses. Acta Pathol. Microbiol. Scand. Suppl., *183* (1966).
7. Kabat, E. A., and Mayer, M. M.: Experimental Immunochemistry, p. 127. (Charles C Thomas, Springfield, Ill., 1961).
8. Kilburn, K. H.: A hypothesis for pulmonary clearance and its implications. Amer. Rev. Resp. Dis., *98:* 449 (1968).
9. Laurenzi, G. A., Yin, S., and Guarneri, J. J.: Adverse effect of oxygen on tracheal mucus flow. New Engl. J. Med., *279:* 333 (1968).
10. Leeson, T. S., and Leeson, C. F.: Histology, p. 91. (W. B. Saunders Co., Philadelphia, Pa., 1970).
11. Marin, M. G., and Morrow, P. E.: Effect of changing inspired O_2 and CO_2 levels on tracheal mucociliary transport rate. J. Appl. Physiol., *27:* 385 (1969).
12. Mowry, R., and Winkler, C. H.: The coloration of acidic carbohydrates of bacteria and fungi in tissue sections with special reference to capsules of *Cryptococcus neoformans*, pneumococci, and staphylococci. Amer. J. Pathol., *32:* 628 (1956).
13. Northway, W. H., Rosan, R. C., and Porter, D. Y.: Pulmonary disease following respirator therapy of hyaline-membrane disease. New Engl. J. Med., *376:* 358 (1967).
14. Northway, W. H., and Rosan, R. C.: Oxygen therapy hazards in the neonate. Hosp. Practice, *4:* 59 (1969).
15. Osserman, E. F., and Lawlor, D. P.: Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J. Exp. Med., *124:* 921, 1967.
16. Warren, L.: The thiobarbituric acid assay of sialic acids. J. Biol. Chem., *234:* 1971 (1959).
17. Fungizone, Grand Island Biological, Grand Island, N. Y.
18. Mycostatin, Grand Island Biological, Grand Island, N. Y.
19. IMI Oxygen controller, B-D Electrodyne, Sharon, Mass.

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