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Early bronchopulmonary lesions in rat lung after normobaric 100% oxygen exposure and their evolution

A light and electron microscopic study

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Summary. In order to clarify the early phenomena involved in the lung reaction to hyperoxia, twenty adult male rats were exposed to 100% oxygen at 1 ATA. Morphological pulmonary lesions were detectable after only 24 h hyperoxia, and included vasoconstriction and perivascular oedema, bronchiolar constriction, and pericyte reaction. The lesions were irregularly scattered within the lung parenchyma and occurred preferentially in areas centred on bronchiolo-vascular stems. Even at the latest stages, pulmonary heterogeneity was obvious, from the coexistence of areas damaged at different times. Neuro-epithelial-bodies were found under the bronchiolar epithelium; the morphological aspect of the neuro-endocrine cells observed was consistent with hyperoxia-induced modulation of their secretory activity. Taken together, our findings show the speed of development of hyperoxia-induced pulmonary changes and raise some pathogenic considerations.

Key words: Hyperoxia – Lung broncho-vascular reaction – Electron microscopy – Rat

Introduction

Oxygen (O_2) is necessary for life, but is also a drug detrimental when used at high concentrations, on account of its toxicity. In numerous anatomical, physiological, biochemical studies, this toxic effect on pulmonary parenchyma has been reported and examined (Pratt 1958; Nash et al. 1967; Kistler et al. 1967; Kapanci et al. 1969; Crystal 1974; Crapo et al. 1974; Kimball et al. 1976; Halliwell 1978; Balentine 1982; Ainsworth et al. 1986). Hydrogen peroxide, superoxide radical, hydroxyl radical and singlet O_2 have all been implicated as aggressive factors in the pathogenesis of O_2 induced lesions. The pathways of damage, the physiological protective mechanisms and how they are overwhelmed and the therapeutic possibilities continue to be explored, since the problem of the mechanism of O_2 toxicity is not yet clearly understood.

This is partly because of our incomplete knowledge of the different functions of the lung. In recent years, numerous reports have focused on metabolic, immunological, endocrine functions of the lung (Widdicombe 1981; Said 1982; Ryan JW 1982; Davis et al. 1983; Jamieson et al. 1986; Lauweryns et al. 1986; Polak et al. 1986; Ryan US 1986; Shepro et al. 1986).

The aetiology of human pulmonary fibrosis is known to be varied and includes O_2 therapy (Porte et al. 1978). We therefore thought it might be of interest to investigate O_2 pulmonary toxicity experimentally, since an histological study of very early O_2 pulmonary damage may help to clarify the phenomenon and perhaps improve the treatment of patients needing high concentration of inspired O_2. In preliminary experiments, we first established that rats exposed to 100% normobaric (at 1 ATA) O_2 were a reliable model for the study of human diffuse interstitial fibrosis. The purpose of the present study was to observe, in this model, the early elementary pulmonary lesions after 24 and 48 h exposure to 100% normobaric O_2 and to set out the chronology and the evolution of the changes.

Materials and methods

Two groups of ten adult male Wistar rats, mean weight 300 g, were exposed to 100% O_2 at 1 ATA in an air-tight transparent caisson. A 100% O_2 exposure was chosen because in an earlier experiment on rats, using 80% O_2, lesions had progressed more

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slowly. The 100% O_2 concentration in the caisson was checked by continuous oxymetry. When the O_2 concentration was found to oscillate between 95 and 98%, the animals were not examined. The animals had not been exposed to O_2 previously, ruling out any risk of acquired resistance (Davis et al. 1983). For all the animals, food and water were continuously available. Rats exposed to 100% O_2 are known to survive only for a limited time: 3 out of 20 rats survived more than five days; we killed them after 18, 28 and 32 days. The lungs from 4 animals found dead in the caisson were removed for histopathological analysis and examined but were excluded from this study.

We found fixation by perfusion, whether intra-cardiac, intra-tracheal, or both, gave rise to artefacts in pulmonary airways and vessels (the sites that we wanted to study) so this method was abandoned.

Animals were killed by decapitation: after 24 h ($n=8$), 48 h ($n=4$), 72 h ($n=1$), 18 days ($n=1$), 28 days ($n=1$), and after 32 days ($n=1$). Control animals (room air) ($n=6$) were killed in the same way.

Specimens of lung parenchyma were taken from different parts of both lungs; some brain, liver and kidney specimens were taken after 48 h. They were immersed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and embedded in an Araldite-Epon mixture. Some samples were post-fixed with 1% osmium tetroxide in the same buffer, and then embedded in an Araldite-Epon mixture. Ten to fifteen samples from different parts of the lungs of each rat were examined on semi-thin sections (1 μ) stained for light microscopic examination either directly with toluidine blue (TB) in 5% sodium borate, or, after removal of the embedding medium with sodium methoxide, with the usual methods: haematoxylin eosin (HE), periodic acid Schiff (PAS), phospho tungstic acid haematoxylin (PTAH).

Thin sections for electron microscopy were contrasted with uranyl acetate and lead citrate, and examined under a Jeol 100 CX electron microscope.

In the two rats surviving after 18 and 32 days' exposure, lung specimens were also fixed in formalin, and paraffin embedded for routine histopathological examination.

Results

In controls (room air), the pulmonary structure remained normal as was observed in both light and electron microscopy.

In the hyperoxic group (100% O_2 at 1 ATA) asthenia, polypnoea, bristling of the hair were seen. The weight of the rats tended to increase (by 10 to 15% after 48 h exposure). When the chest and abdomen were opened, visceral oedema was observed and fluid flowed out of the cavities. However, after several weeks' exposure, weight loss became evident.

After 24 h exposure, light microscopy showed that the lung parenchyma was hardly modified. However, in all animals examined, we found some alveolar collapse and a definite vascular congestion in several irregularly scattered areas. In such areas, intense bronchiolar and vascular constriction, obviously different from the post-mortem constriction which could be found in the control animals, was observed. Alveolar septa were folded and squeezed together, resulting in increase in local tissue density alongside overdistension caused by air, as is usually observed in ventilation disorders (Fig. 1). Arteriolar adventitia was distended by oedema. The interalveolar septa were thickened. On PTAH stained sections, discrete fibrin deposits were found in some arteriolar walls and intervalveolar septa. In the strongly constricted bronchioles, the apices of epithelial cells were unusually pale. Subpleural mast-cells were crammed with granules.

On electron microscopy, early modifications mainly affected vascular structures. Arteriolar changes, such as the occasional vacuolisation of endothelial cells (Fig. 2), infiltration of the elastic layers by fibrin and distension of the tunica adventitia by oedema were observed in restricted areas. In the capillaries, some endothelial cells were partly and discretly separated from their basement membrane (Fig. 3). In some capillaries, a pericyte reaction was observed. The alveolar epithelium remained almost unchanged: in type II pneumocytes apical villosities and lamellar bodies seemed slightly more numerous. Thus, after 24 h exposure, obvious modifications of the lung were observed, affecting some broncho-vascular units and sparing others.

After 48 h exposure, appearances were not such as to explain the frequent death of animals at this early stage. Injured and normal areas were either juxtaposed or mixed in the same section. In the injured territories, we observed bronchiolar, vascular and interstitial changes. Intense broncho-constriction greatly reduced the lumen of terminal bronchioles. The apices of bronchial epithelial cells were paler than usual. Blood vessels were congested and lympathic vessels highly distended (Fig. 4). Arteriolar constriction was particularly marked. Moreover arterioles were surrounded by muff-like oedema which greatly distended their adventitia (Fig. 5). Fibrin deposits were revealed within the vessel walls by PTAH staining. The vessel walls were thinner than usual with elastic and muscular layers dissociated by oedema. The interstitium, especially around the bronchiolo-vascular stem, was oedematous and dividing cells were seen (Fig. 4). The interalveolar septa were thickened. In the most modified zones, the alveoli were invaded by an exsudate which was very poorly stained with TB or PAS. Thin fibrin deposits were revealed by PTAH staining in some alveolar septa, in connective tissue lining bronchiolar epithelium and also in alveoli. The alveolar epithelium seemed to be scarcely affected, even inside the zones of

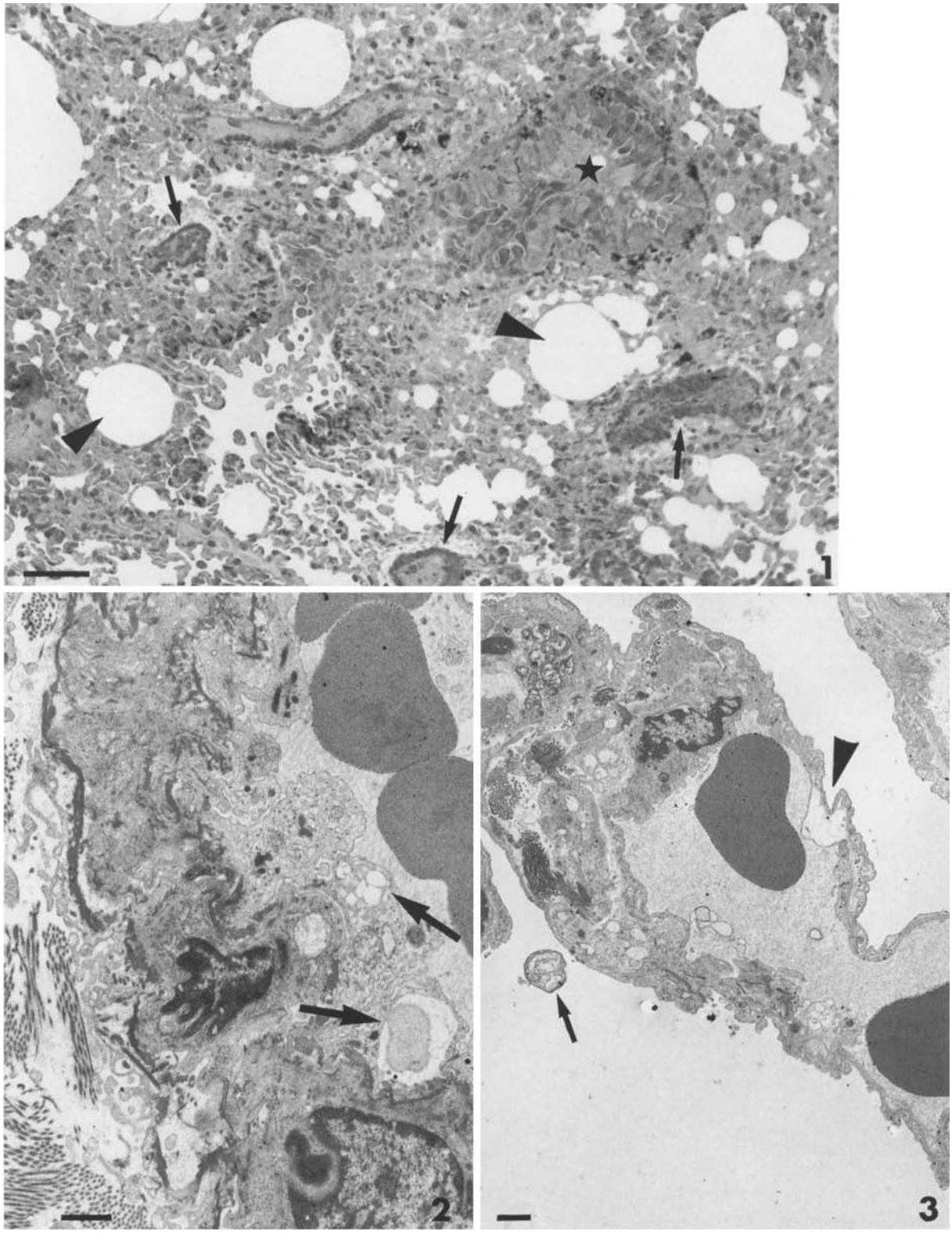

Fig. 1. 24 h hyperoxia. A strongly constricted bronchiole (*asterisk*), different degrees of atelectasis and microscopical emphysema (*arrow-heads*) are seen. Oedema is observed around constricted arterioles (*arrows*). Semi-thin section, PTAH, ×230. *Bar*: 50 μm

Fig. 2. 24 h hyperoxia. Arteriolar wall showing vacuolization of endothelial cells (*arrows*). ×8200. *Bar*: 1 μm

Fig. 3. 24 h hyperoxia. Localised detachment of a capillary endothelial cell (*arrow-head*) in an otherwise normal alveolar septum. Note the lamellar body in the lower alveolus (*arrow*). ×5500. *Bar*: 1 μm

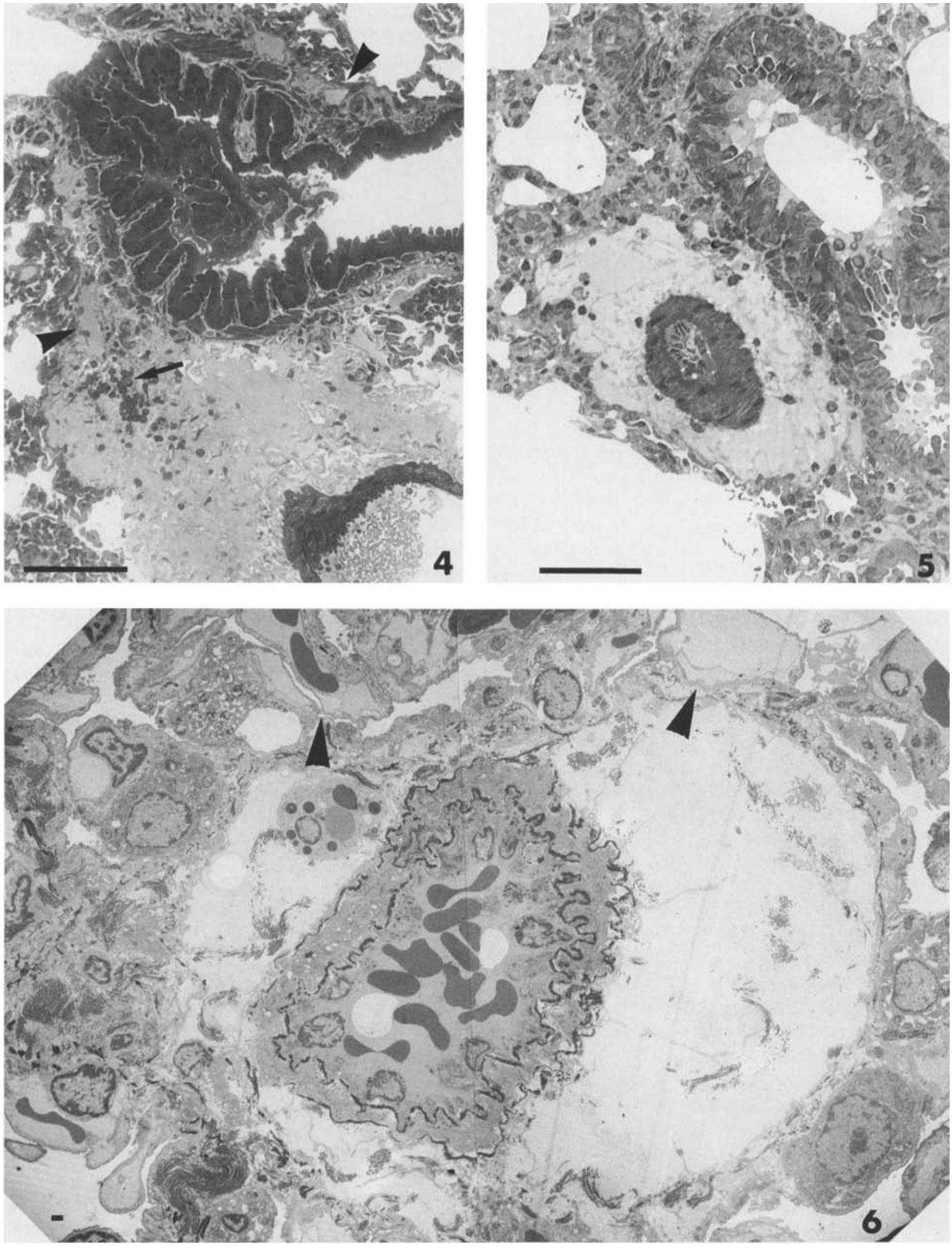

Fig. 4. 48 h hyperoxia. Broncho-arteriolar stem: peri-arteriolar and peri-bronchiolar oedema. Note a dividing cell (*arrow*) and, in the peri-bronchiolar space, distended lymphatic vessels (*arrow-heads*). Semi-thin section, TB, × 360. *Bar*: 50 μm

Fig. 5. 48 h hyperoxia. Transverse section of a constricted bronchiolo-alveolar stem showing a peri-arteriolar adventitia distended by oedema. Semi-thin section, TB, × 360. *Bar*: 50 μm

pulmonary distension or collapsus. Subpleural mast-cells were less obvious than at 24 h and were apparently degranulating.

On electron microscopy, the oedematous periarteriolar tissue (Fig. 6) was filled with electron lucent material containing scattered collagen bundles, fibroblasts, macrophages, plasmocytes, polymorphonuclear neutrophils and mast-cells, some of the latter obviously releasing their granules. Adventitial oedema seemed more extensive where the lumen of the vessel was electron dense. The elastic and muscular layers were infiltrated by oedema; the elastic layer was discontinuous and the electron density of the elastin suggested fibrin infiltration (Fig. 6). Endothelial damage was found in some places, taking the form of vacuolisation, swollen mitochondria, or plasma membrane detachment from the basement membrane. In most capillaries, endothelial cells were irregularly lifted off their basement membrane (Fig. 6) thus increasing the air/blood distance. Alveolar septa were thickened by interstitial oedema. Lesions were especially marked around the bronchiolovascular stem. Some capillaries or post-capillaries had their lumen blocked by thrombus, and pericyte formation could be observed there. The alveolar epithelium was only slightly modified: in some places, vacuolised pneumocytes had assumed irregular forms and were releasing their lamellar bodies. Neither macrophages nor polymorphonuclear neutrophils appeared to be increased in number. In one sample we found a neuro-epithelial-body under the bronchial epithelium, next to the basal lamina, in a zone where perivascular oedema was particularly noteworthy. It was formed by a homogeneous population of cells characterised by an accumulation in their cytoplasm of dense-cored vesicles, roughly 130 nm in diameter (Fig. 7).

In the other organs (liver, kidney), a generalized vasoconstriction, with congestion of the hepatic sinuses and of the renal capillaries, was observed. In light and electron microscopy, arteriolar and capillary walls did not seem to exhibit any obvious damage. Some renal glomeruli did, however, show a swelling of the podocytes and occasional capillary thrombosis. This preliminary examination of the kidney did not reveal any other obvious modifications.

After 72 h exposure, the disposition of lesions remained heterogeneous, with severely damaged and almost intact parenchyma areas coexisting side by side. In injured territories, pulmonary lesions were more marked: exsudative and proliferative changes were obvious at this stage. Oedema, located around the bronchiolovascular axes at 48 h, was more diffuse. Arterial walls exhibited dissociation and fragmentation of elastic and muscular layers, localized decreases in thickness and, in some areas, the endothelium was missing. The muff-like periarterial oedema had broadened, and blood cells, macrophages, mast-cells, fibroblasts were identified therein. Fibroblastic proliferation was obvious around vessels. Interalveolar septa were thickened. In some places, the alveoli were filled with strongly chromophilic fluid, enclosing abundant fibrin precipitates. This change was the most regularly observed in subpleural areas, previously almost undamaged. At this stage macrophages and polymorphonuclear neutrophils were present in alveoli, but we found no massive epithelial necrosis, nor typical hyaline membrane. Subpleural mast-cells seemed scarce.

After 18 and 32 days'exposure only three animals survived. No significant difference was observed between the animals'lungs at 18 and 32 days. In light microscopy, the most striking finding at this stage was once again the heterogeneity of structure observed in paraffin-embedded sections of pulmonary lobules: damaged territories adjoined normal ones and all the intermediate stages of the lesions were found (Fig. 10). In some sites, dense fibrosis consisting in an intense proliferation of fibroblasts and collagen deposits was observed: air and blood pathways had practically disappeared from a pulmonary parenchyma which was no longer recognizable. In other sites, we found intermediate stages : a looser fibrosis texture spared a few alveoli and vascular structures; macrophages were obvious; pericyte proliferation had narrowed the lumen of arterioles. Electron microscopy showed a pericyte reaction (Fig. 11) and numerous fibroblasts with morphological signs of intense secretory activity. In the alveoli, squeezed by fibrosis, pneumocytes had become cuboidal (Fig. 12). In yet other sites, the appearance was similar to that observed in the early stages: congestion, oedema, interstitial and intra alveolar fibrin were obvious.

Fig. 6. 48 h hyperoxia. Around a contracted arteriole, the adventitia is largely distended by oedema containing scattered collagen fibrils and a macrophage. Note the high electron density of elastic arteriolar layers related to fibrin infiltration. The endothelium is more or less detached from the basement membrane of some alveolar capillaries (*arrow-heads*). × 2000. *Bar*: 1 μm

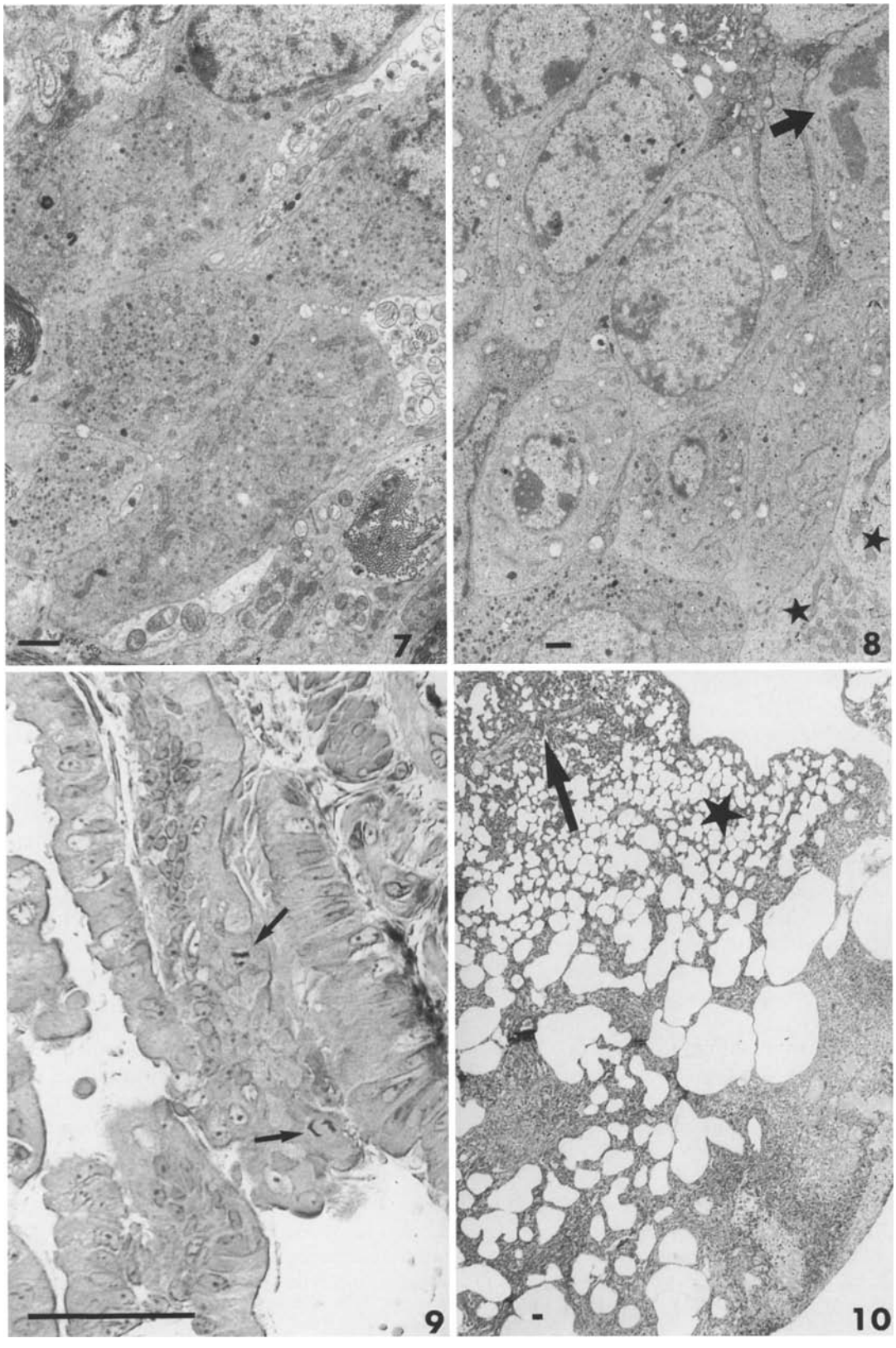

Fig. 7. 48 h hyperoxia. Neuro-epithelial-body. Endocrine cells are filled with dense-cored secretory vesicles. ×6750. *Bar*: 1 μm

Fig. 8. 18 days' hyperoxia. Neuro-epithelial-body observed under bronchiolar epithelium (*asterisks*). Most endocrine cells are nearly empty of secretory vesicles and contain well-developed r.E.R.; a dividing endocrine cell (*arrow*) can be seen at the top right. ×4200. *Bar*: 1 μm

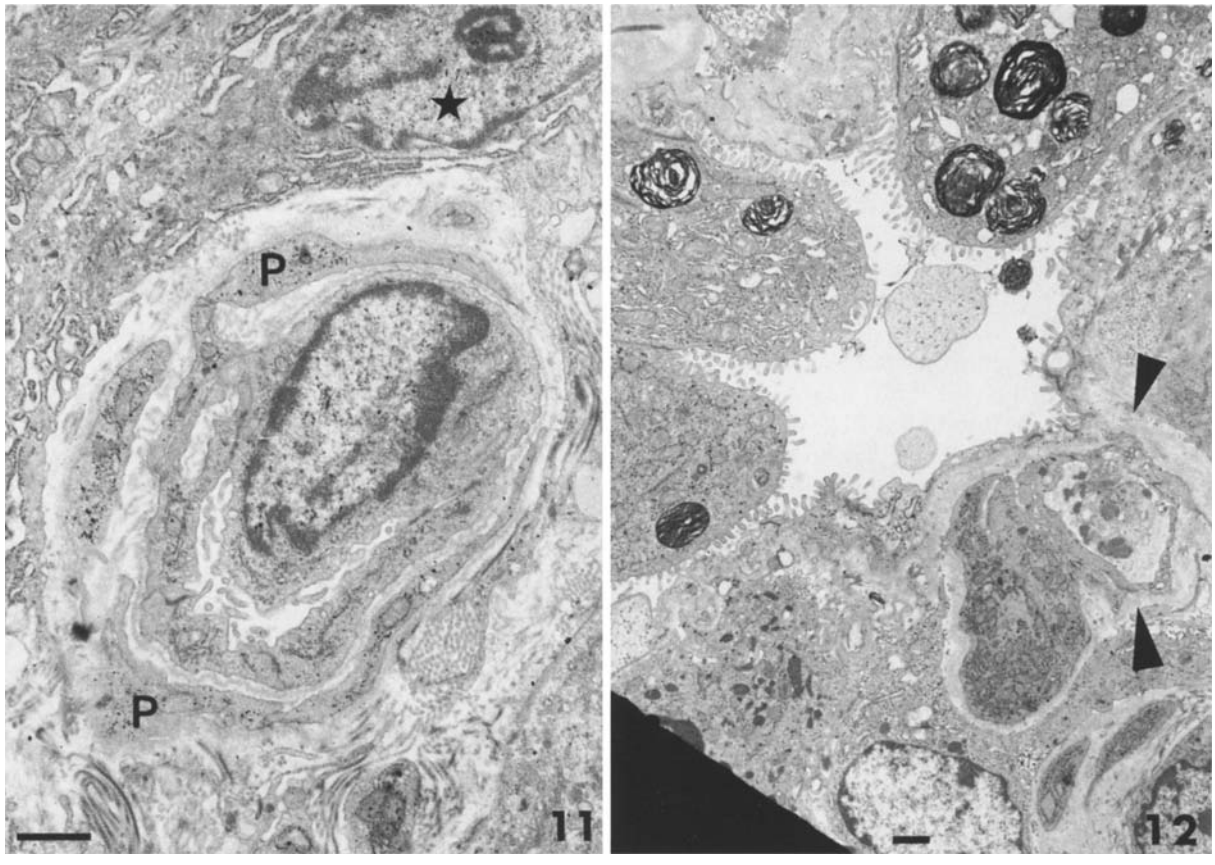

Fig. 11. 18 days' hyperoxia. Pericytes (P) in a vessel wall; at the top, a fibroblast (*asterisk*) with signs of intense secretory activity. ×9700. *Bar*: 1 μm

Fig. 12. 18 days' hyperoxia. In an alveolus squeezed by fibrosis, pneumocytes have become cuboïdal. Note the amorphous substance in the interstitial spaces in contact with the capillary basement membrane (*arrow-heads*). ×4800. *Bar*: 1 μm

Bronchiolar epithelium reactions were far more striking than alveolar epithelium ones. After 18 days' O_2 exposure, a decrease in number of the ciliae of epithelial bronchiolar cells and hyperplasia with dividing cells were observed by light microscopy (Fig. 9). In electron microscopy, a neuroepithelial-body was detected at the bottom of the bronchiolar epithelium (Fig. 8). Compared with a similar formation found at the 48 h stage (Fig. 7) the endocrine cells contained far fewer secretory vesicles, more developed Golgi apparatus and r.E.R. cisternae. These features and the presence of a dividing cell indicated secretory stimulation at the 18 days stage.

Discussion

This study shows that early bronchiolar and vascular pulmonary histopathological changes occur in rats exposed to 100% O_2 at 1 ATA. The remarkable speed of development of the pulmonary modifications, detectable after 24 h exposure is thus confirmed. Davis et al. (1983), using broncho-alveolar lavage in their study on normal human

Fig. 9. 18 days' hyperoxia. Papillary hyperplasia of bronchiolar epithelium with two dividing cells (*arrows*). Semi-thin section, TB, ×540. *Bar*: 50 μm

Fig. 10. 32 days' hyperoxia. Heterogeneous pulmonary parenchyma with dense fibrotic area, atelectasis and emphysema (*bottom half*), coexisting with incipient parenchymal densification around a bronchiolo-vascular stem at the top (*arrow*), and also with apparently normal alveoli (*asterisk*). Paraffin-embedded section, HE, ×27. *Bar*: 30 μm

adults exposed to hyperoxia, suggested these early changes. They were also observed by Ainsworth et al. (1986) in infant rhesus monkeys and recognized in cultured endothelial cells by others (Block et al. 1981; Housset et al. 1983). Thus, as Davis et al. (1983) stated, the notion that exposure to 100% O_2 for at least 24 h is safe can no longer be admitted.

According to our observations, early changes after O_2 exposure include a vascular reaction (vasoconstriction and perivascular oedema), a bronchiolar reaction (bronchoconstriction, changes in the bronchiolar epithelium and neuroendocrine cells), and the onset of interstitial fibrosis (pericyte reaction and active fibroblast proliferation). A striking feature of these associated lesions is their irregular topographic distribution within the lung parenchyma and their early preferential disposition around a bronchiolovascular axis. Lesions progress in each damaged area until they reach the stage of dense fibrosis – provided, of course, that the rat survives. At this stage, the juxtaposition of more or less anciently damaged and subnormal territories confers a pronounced heterogeneity on the lung parenchyma.

The vascular reaction was obvious in light microscopy after the first 24 h: the arterioles were strongly constricted and their tunica adventitia was distended by fluid. Electron microscopic examination confirmed a vacuolisation of arteriolar endothelial cells, likely to increase the permeability of the vessel, and the lifting of the capillary endothelial cells from their basement membrane. However we did not observe the formation of typical hyaline membranes although the fibrinous exsudate flooded the alveoli in injured territories. Typical hyaline membrane, usually observed in acute human pulmonary fibrosis, probably results from the simultaneous action of infectious, nervous, immunological and toxic factors, including oxygen therapy (Porte et al. 1978).

The bronchiolar reaction, detected in the present study as early as the vascular one, seems to have been underestimated. O_2-induced modifications in airways have seldom been investigated and generally only with regard to superficial epithelial changes (Harrison et al. 1970; Obara et al. 1979). The intense bronchiolar constriction, observed after the first 24 hours, was seen associated with atelectasis and emphysema, a typical morphological pattern of ventilation troubles. Early atelectasis and emphysema could thus be explained by this bronchiolar constriction rather than by an altered surfactant production. The present findings about the exocytosis of lamellar surfactant bodies from pneumocytes II, occurring at 48 h onwards are in agreement with the observations of Ainsworth et al. (1986) who, after exposing infant monkeys to 98% O_2, did not detect any significant change in the number of lamellar bodies of pneumocytes II within the first 24 h of exposure. The bronchiolar epithelium had developed severe papillary hyperplasia after 18 days' exposure. The histogenesis of this alteration and the role of the neuro-epithelial-bodies located under the bronchiolar epithelium need further investigation. The involvement of the neuro-endocrine cells in the pathogenesis of O_2 induced lesions will be discussed later.

The onset of a rapidly developing interstitial fibrosis within the first 24 h was indicated by the proliferation of pericytes and hyperactivity of fibroblasts. This observation is in agreement with that of Davis et al. (1983) who evaluated, by broncho-alveolar lavage, 14 normal subjects exposed to hyperoxia for 17 h. These authors found that in hyperoxia, alveolar macrophages released increased amounts of fibronectin and alveolar-macrophage-derived fibroblast growth factor, both of which are thought to modulate fibroblast recruitment and proliferation in the alveolar wall, while the release of neutrophil chemotactic factor had not yet occured. Our observations confirm that interstitial fibrosis develops before any leukocytes migration into the lung has occured. The process of fibrosis observed in the present study around vessels and in alveolar septa, is seen to intensify in the surviving animals. The mechanism underlying the onset and the maintenance of fibroblast proliferation, the upset of epithelium/stroma balance are not clearly understood. Several factors have been shown to be involved in this process: the release of mediators either stimulating mesenchymal cell proliferation and collagen production (Crystal et al. 1984; Bitterman et al. 1986; Claman 1987; Martinet et al. 1987), or keeping mesenchymal production in check (Ko et al. 1977; Hill et al. 1986), and the variety of receptors in the fibroblasts (James et al. 1984; Heldin et asl. 1984).

The heterogeneity of the pulmonary lesions may result in underestimation or even non recognition of change if the number of samples examined is insufficient. It has been shown in this study that, from the very first hours, broncho-vascular lesions and reactions are scattered throughout the pulmonary parenchyma. These modifications, preferentially centred on a bronchiolo vascular axis, strike new sites successively so that, when the rats survive several weeks in 100% O_2, several stages of evolution coexist in the same section. The causes of this heterogeneous distribution of pulmonary lesions

remain to be established: a possible difference of vulnerability between pulmonary areas, or perhaps the physiological alternance in functional pulmonary units are to be taken into account. Heterogeneity of lung lesions has been already reported in the Adult Respiratory Distress Syndrome (ADRS) (Porte et al. 1978). Computerized tomography studies in ADRS have lead Gattinoni et al. (1987) to challenge the patho-physiological signification for the whole lung of the appearances of one biopsy, or the results of some lung function tests.

The morphologic data reported in this study may help to resolve some pathogenic problems. The high mortality rate of animals after 24 or 48 h exposure to 100% O_2 at 1 ATA cannot be accounted for by the alterations observed in the lung. Generalised vasoconstriction due to hyperoxia is well known and, in the present study, has been observed in the liver and the kidney as well as in the lung. Brain disorders after hyperoxia have been reported (Hammond et al. 1984) and may contribute to the early deaths of rats exposed to hyperoxia. It has also been noted that some organs appear to react specifically to hyperoxia: the eye in the premature infant, perhaps the kidney glomeruli (Ward 1986), and the lung.

The reaction of the lung is a particularly complex one and it is generally admitted that it results partly from the direct cytotoxic effects of O_2 and partly from the stimulation by O_2 of endogenous pulmonary structures. O_2 and its metabolites (Hess et al. 1984; Pryor 1986) have a direct toxic effect on the cells of lung parenchyma that antioxydant enzymes cannot protect. Endothelial cells, which form an important metabolic organ in the lung (Ryan US 1982, 1986) are known to be highly susceptible to O_2 cyto-toxicity and showed signs of injury after 24 h exposure in this study. Indirect oxygen toxicity works through the stimulation of pulmonary elements or structures: macrophages, platelets, polymorphonuclear neutrophils, mast-cells, or neuro-epithelial bodies, or axonal endings, each of which can release one or more factors. We have seen that pulmonary macrophages, polymorphonuclear neutrophils and platelets increase in number, but not before 48 h exposure. The same observation applies for mast-cells. We found them overloaded with granules after 24 h exposure. The morphometric measurement of Ainsworth et al. (1986), in the infant monkey exposed to 98% O_2, did not show any decrease in number after 24 h. But, later on at 48 h, we saw them degranulating while the perivascular oedema increased and cellular migration appeared. These effects could be related to the possible liberation of leukotrienes among other products by mast-cells (Garcia et al. 1982; Gutner et al. 1987). Moreover degranulating mast-cells are suspected to participate in the activation of fibroblasts (Claman 1987).

The modification observed in neuro-epithelial-bodies (NEBs) indicate that they are affected by hyperoxia. In infant monkeys exposed to 98% O_2, Ainsworth et al. (1986) found a significant decrease in the number of detectable NEBs, related to the massive exocytosis of secretory vesicles. After a 48 h O_2 exposure, NEB endocrine cells were heavily loaded with secretory granules. At 18 days, granules had almost completely disappeared, signs of secretory stimulation were obvious and dividing cells were noted. So these structures (Jeffery et al. 1972; Will 1982; Lauweryns et al. 1983; Pack et al. 1984) may be suspected to play a part in pulmonary reaction to O_2, but further studies are needed to elucidate this problem.

Last but not least, the role of tachykinins in the lung has to be considered. The precocity and intensity of the reaction observed in the bronchiolovascular stem suggest that it could result from a nervous reflex response to O_2 inhalation. As substance P, neurokinin A and probably neuropeptide K are present in sensory capsaicin-sensitive axons innervating airways in mammals (Wharton et al. 1979; Lundberg et al. 1984; Hua et al. 1985; D'Orleans-Juste et al. 1985; Castairs et al. 1986; Saria et al. 1987). These neuropeptides by their effects on smooth muscle constriction and vascular permeability may play a part in the early changes we found after a 24 h exposure.

In conclusion, the toxic effect of 100% O_2 at 1 ATA upon lung is an early and complex one. The rapidity of onset and the intensity of initial vaso and bronchoconstriction suggest a reflex action with a local or central origin. Several actions, both direct and indirect, successive or cumulative, induce early vascular permeability changes, and the early onset of severe fibrosis in the heterogeneously injured lung. Further experimental investigations are still needed to improve the knowledge of respective mechanisms and the evaluation of their relative importance.

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