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Pulmonary Alveolar Proteinosis Developing from Desquamative Interstitial Pneumonia in Long Term Toxicity Studies of Iprindole* in the Rat

G. S. Vijeyaratnam and B. Corrin

Departments of Pathology, University of Ceylon, Colombo and St. Thomas's Hospital Medical School, London

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Summary. Rats fed on a diet containing 0.1% iprindole for up to 12 months were observed to develop pulmonary alveolar proteinosis, which evolved through a stage of desquamative interstitial pneumonia. The changes were studied sequentially by light and electron microscopy and histochemistry.

Degenerative changes in the alveolar capillary endothelium led to interstitial oedema, whilst there was marked lipidic degeneration of the type I alveolar epithelial cells. Myelin figures derived from the epithelial cells were initially taken up by alveolar macrophages which came to fill the air spaces. When these cells later broke down, compaction of the released lamellar lipid, originally derived from epithelial cells, resulted in the appearances of alveolar proteinosis.

In conjunction with previous reports of alveolar proteinosis developing in man and experimental animals following exposure to a wide variety of dusts, the present findings suggest that alveolar proteinosis represents a non-specific response to lung injury.

Introduction

We have previously shown that the anti-depressive drug iprindole adminstered orally in large doses to rats results in pulmonary histiocytosis which resembles human desquamative interstitial pneumonia (Vijeyaratnam and Corrin, 1972a). We have also described the primary changes in the alveolar wall which provoke this outpouring of alveolar macrophages: soon after iprindole administration is started degenerative changes appear in the alveolar epithelium and capillary endothelium, and interstitial oedema develops (Vijeyaratnam and Corrin, 1972b). It was anticipated that with continued iprindole administration these changes might progress to interstitial fibrosis, and long-term experiments were therefore undertaken. In these however, the mild alveolar wall thickening remained static and no fibrosis developed, buth the exudative changes gradually altered and from a desquamative interstitial pneumonia-like picture evolved the appearances of pulmonary alveolar proteinosis. The pathogenesis of both these conditions in man is obscure and their relationship poorly understood. A description of the experimental changes, which have been studied sequentially at both the light and electron microscopical level, should therefore be of value.

* Iprindole B.P., marketed as Prondol by John Wyeth and Brother Ltd., Maidenhead, Berkshire, England.

Materials and Methods

The animals used were 3-month-old specific pathogen-free Charles River strain male rats weighing 170 g. They were divided into two groups, 26 test animals and 14 controls, and each animal was weighed at the beginning and end of the experiment. All animals were fed a powdered diet ad libitum, to which iprindole (John Wyeth and Brother Ltd., Maidenhead, Berks.) was added in the case of the test rats in a concentration of 0.1% by weight. Four test rats and 2 controls were killed by exposure to pure nitrogen at the end of 3, 4, 6, 9, and 12 months. Iprindole was withdrawn from the diet of the surviving test rats at 12 months and all the remaining animals were killed 5 months later.

At death the thorax was opened and 2 ml of cold (4° C) 0.1 M cacodylate-buffered 4 per cent paraformaldehyde (pH 7.4) was gently instilled through the trachea into the lungs. The trachea was then tied off and the lungs immersed in the fixative for 10 mins. Tissue blocks, of sizes suitable for light and electron microscopy, were then cut and fixation continued at 4° C for 1–5 days. For light microscopy the tissues were processed to paraffin and 7 μm sections were stained with haematoxylin and eosin, periodic acid—Schiff reagents with and without diastase treatment and for iron. Frozen sections were stained for neutral fats with oil red 0 and Sudan Black and for phospholipids by the silver hydroxylamate method of Adams, Bayliss and Ibrahim (1963). The Schultz reaction and the digitonin method were employed for the detection of cholesterol and its esters. Oxidative and hydrolytic enzymes were localised by techniques used previously (Vijeyaratnam and Corrin, 1972a). For electron microscopy the tissues were post-fixed at 4° C for 1 hour in 1 per cent osmium tetroxide containing 0.12 per cent sucrose buffered to pH 7.4 with veronal acetate. Suitable thin Epon-embedded sections stained with uranyl acetate and lead citrate were examined in a Siemens Elmiskop I.

Results

The rats appeared to be in good health and showed no signs of respiratory distress. None died spontaneously during the experimental period, but the test rats did not gain weight as well as the controls. (Av. body weights after 12 months: test rats 510 g, control 600 g.) Post mortem, pale irregular areas were found on the lung surfaces of test rats, gradually increasing in number and size to measure 6–8 mms in diameter by 12 months.

Light Microscopy

The most striking lesion seen in the lungs after 3 months of medication is the presence of numerous cells within many alveolar spaces. The intra-alveolar cells are similar to those observed in the desquamative interstitial pneumonia-like condition present at 6 weeks (Vijeyaratnam and Corrin, 1972a), but show marked vacuolation and have an abundant foamy cytoplasm (Fig. 1). Multinucleated forms are numerous. Nuclei are pyknotic in some cells and are not seen in many others. The free intra-alveolar cells progressively increase in number and size but later appear to break down, filling the alveoli with pale eosinophilic granular material (Fig. 2). Thus by 6 to 9 months, several alveolar spaces are filled with this granular material, whilst others still contain numerous intact cells. Some alveoli contain both granular material and cells, many of which are in various stages of degeneration and disintegration. The pale granular eosinophilic material is weakly periodic acid-Schiff positive. After 1 year of drug treatment, the cellular breakdown products in some regions condense to form a more deeply eosinophilic mass (Fig. 3) which is strongly periodic acid-Schiff positive and diastase resistant. In these areas the appearances mimic those of human

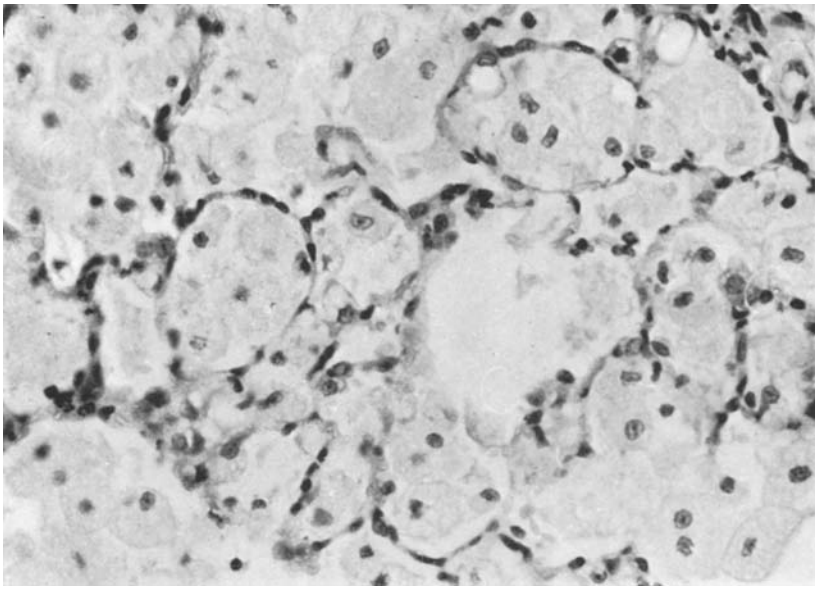

Fig. 1. After 3 months of iprindole administration the alveoli are filled by large foamy cells. Haematoxylin and eosin. × 340

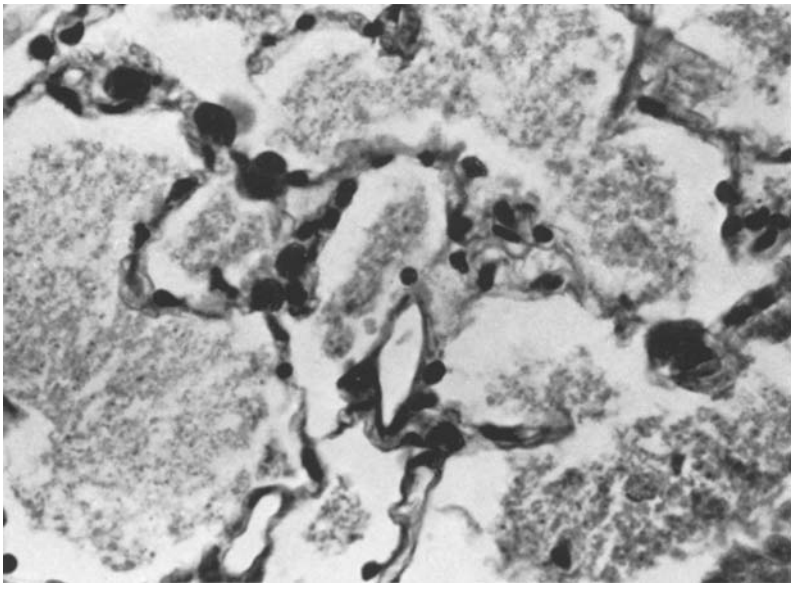

Fig. 2. At 9 months the alveoli are filled by a pale eosinophilic granular material. This gives a weakly positive periodic acid-Schiff reaction. Haematoxylin and eosin. ×560

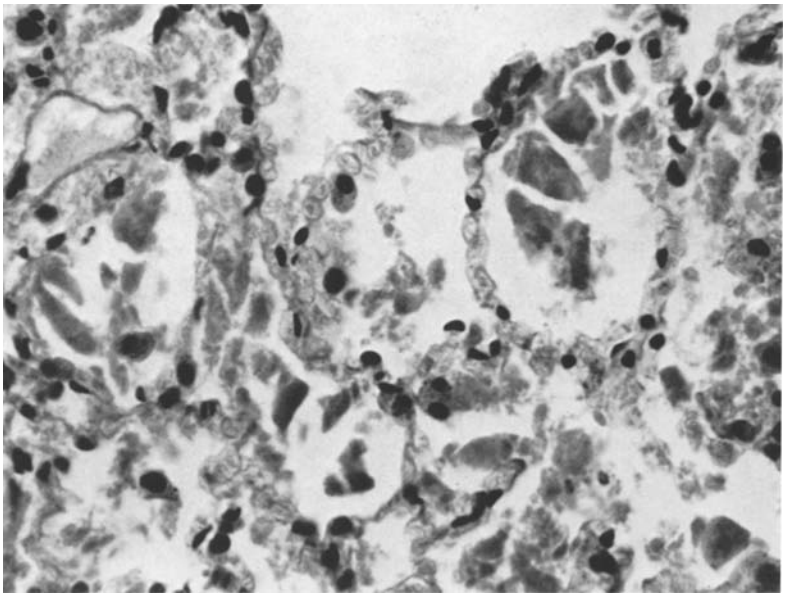

Fig. 3. At 12 months some alveoli are filled by compact densely eosinophilic material, an appearance simulating alveolar proteinosis. Haematoxylin and eosin. × 560

pulmonary alveolar proteinosis (Rosen *et al.*, 1958). Frozen sections treated with silver hydroxylamate show an intensely positive reaction indicating the presence of abundant phospholipid. The intra-alveolar material and free cells are both strongly stained. A few positive granules are seen in the lining epithelial cells. The bulk of intra-alveolar material and free cells are not stained with oil red 0 or Sudan Black, indicating a paucity of neutral fat. Stains for cholesterol and its esters are negative. Neither any cells nor acellular material with these staining reactions are found in the hilar lymph nodes.

The intra-alveolar cells initially show strong oxidative enzyme activity, but after 6 months the degenerate cells give only a weak reaction. The great majority of the intra-alveolar cells are strongly positive for acid phosphatase and β-glucuronidase, but here again the cells showing degenerative changes are less intensely stained. These enzymic characteristics, together with a marked avidity for particulate matter introduced into the lung via the trachea (Vijeyaratnam and Corrin, 1972a) identify the intra-alveolar cells as macrophages.

The alveolar walls are slightly thickened in some places by interstitial oedema and cellular infiltration. However, this change is not increased over the state seen in short term toxicity studies (up to 6 weeks) and remains fairly static throughout the duration of these experiments. Especially where the alveoli are filled with granular material, the alveolar walls remain quite thin and are sparsely populated by cells. A mild increase in fine argyrophilic reticulin is seen in some areas but there is no increase in collagen or alteration in elastic tissue.

Five months after withdrawal of the drug, the alveolar spaces are empty except for a few foci of foamy cells. The alveolar walls are not thickened and

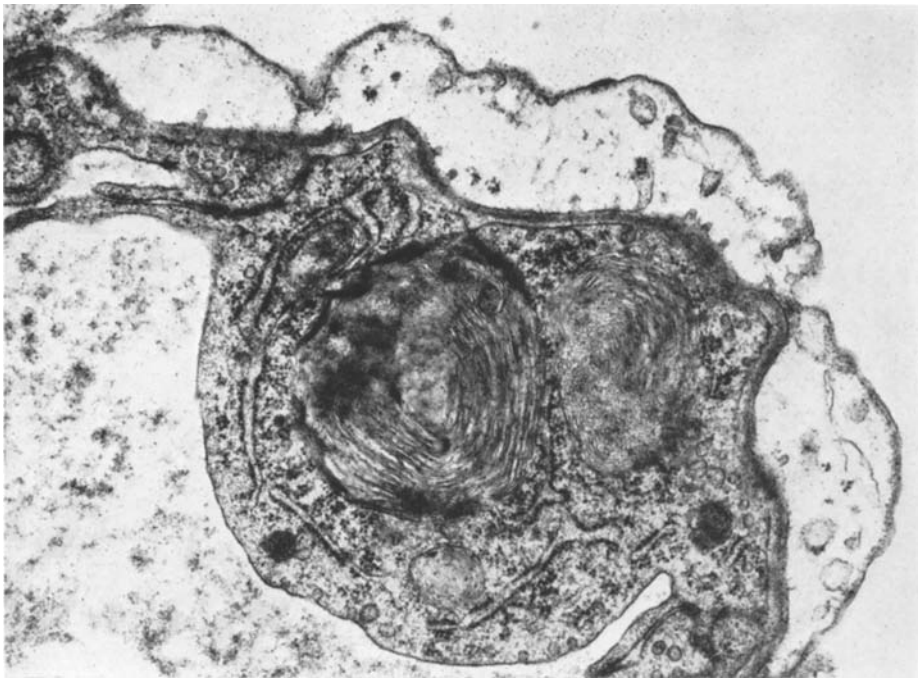

Fig. 4. A capillary endothelial cell contains myelin figures whilst the alveolar epithelium is swollen and oedematous. Lead and uranium. × 24000

there is no oedema. The changes induced by iprindole are therefore reversible. Control animals show no abnormalities throughout experiment.

Electron Microscopy

Interstitial oedema, which was a striking feature of short term toxicity studies, persists throughout these experiments but does not progress. In most areas it is associated with an increased number of pulmonary interstitial cells which are rich in mitochondria, free ribosomes and vacuoles of varying size. Fibroblasts are rarely seen and there is no increase in collagen.

The capillary endothelial cells exhibit pronounced changes. Some are thickened, with prominent pinocytosis and vacuolation of the cytoplasm whilst many contain myelin figures, abundant rough-surfaced endoplasmic reticulum and free ribosomes (Fig. 4). However, nowhere is the capillary endothelium destroyed.

The thin cytoplasmic processes of type I epithelial cells (squamous pneumocytes) are markedly swollen (Fig. 4). Numerous large myelin figures and other osmiophilic inclusions with a more homogeneous appearance are also seen (Fig. 5) and there are abundant free ribosomes. Sometimes the myelin figures appear identical to the specific lamellar inclusions of the type II cells, widely believed to represent surfactant secretion; but because similar myelin figures are observed in endothelial cells we have interpreted them as degenerative.

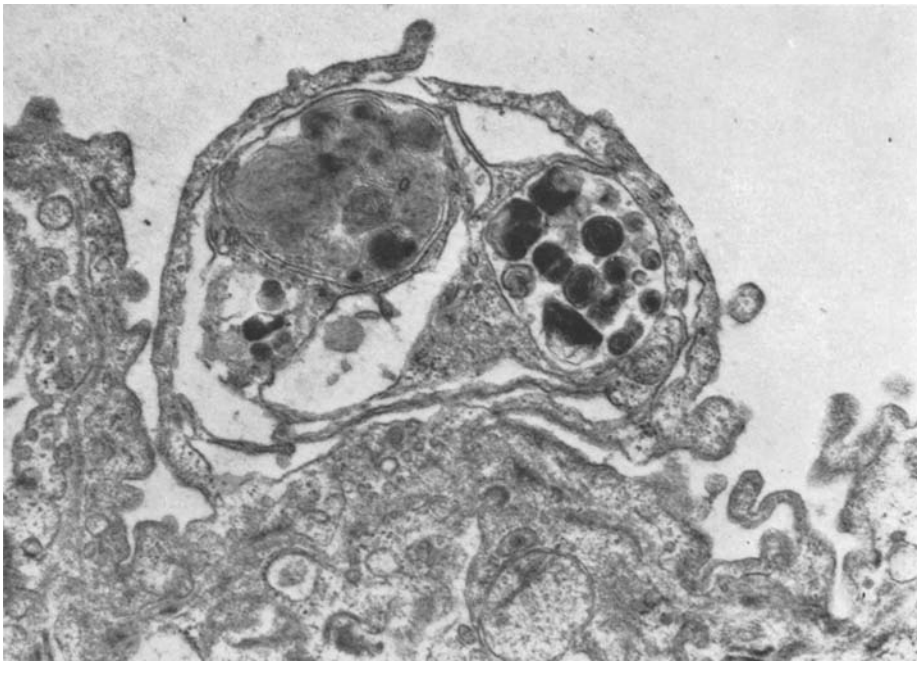

Fig. 5. Type I epithelial cell containing myelin figures and other lipidic inclusions. Lead and uranium. × 28000

The type II epithelial cells (granular pneumocytes) show increasingly severe dilatation of the vacuoles containing the specific lamellar inclusions, increased amounts of free ribosomes and abundant rough-surfaced endoplasmic reticulum. There is blunting of microvilli and the mitochondria are swollen with a few disrupted cristae. In addition, irregular cytoplasmic clefts are occasionally seen. After 9 months treatment, some of the type II cells contain pale cytoplasmic inclusions lacking the lamellar pattern of the specific osmiophilic inclusions. Some type II cells contain only very small amounts of osmiophilic material. The alveolar walls are lined in places by cells which largely resemble type II pneumocytes but differ in possessing thin lateral cytoplasmic processes more characteristic of type I cells. All these epithelial changes become progressively more pronounced with continued administration of the drug.

Many free cells are initially seen in the alveolar spaces. They measure up to 40 μm in diameter and their cytoplasm is filled with numerous inclusions, chiefly of the myelin figure type observed in the alveolar epithelial cells (Fig. 6). Later on they also show pale cytoplasmic inclusions, similar to those seen in some of the type II epithelial cells. Apart from these inclusions, the free alveolar cells contain few organelles. The majority of the free cells are rounded in shape with few cytoplasmic projections. These cells gradually break down liberating their cytoplasmic contents into the alveolar spaces. With continuing drug administration, many alveoli show increasing amounts of cellular debris and free myelin figures which become compressed together. Small amounts of membranous structures with

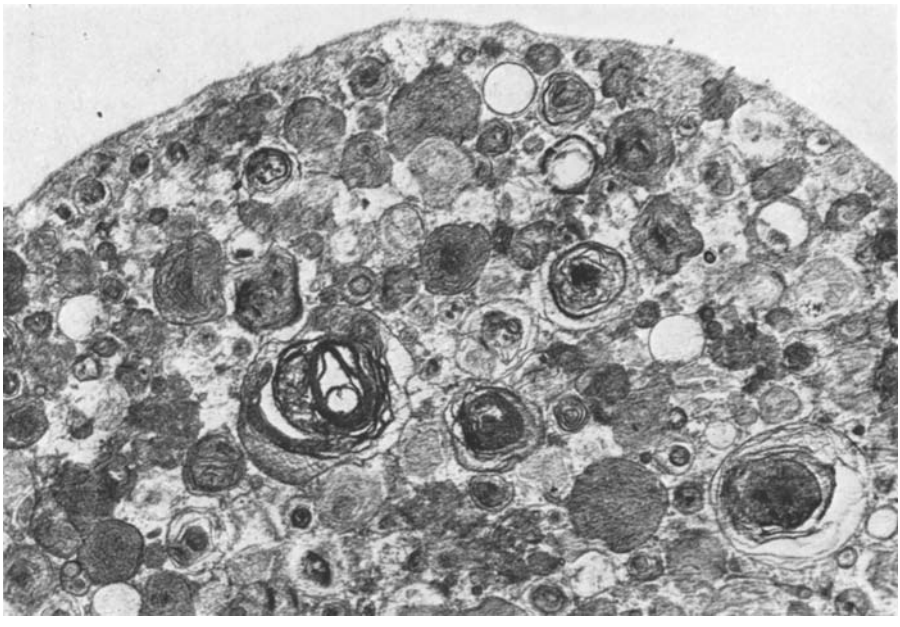

Fig. 6. Part of an alveolar macrophage distended by numerous myelin figures and rounded in shape. Lead and uranium. ×13000

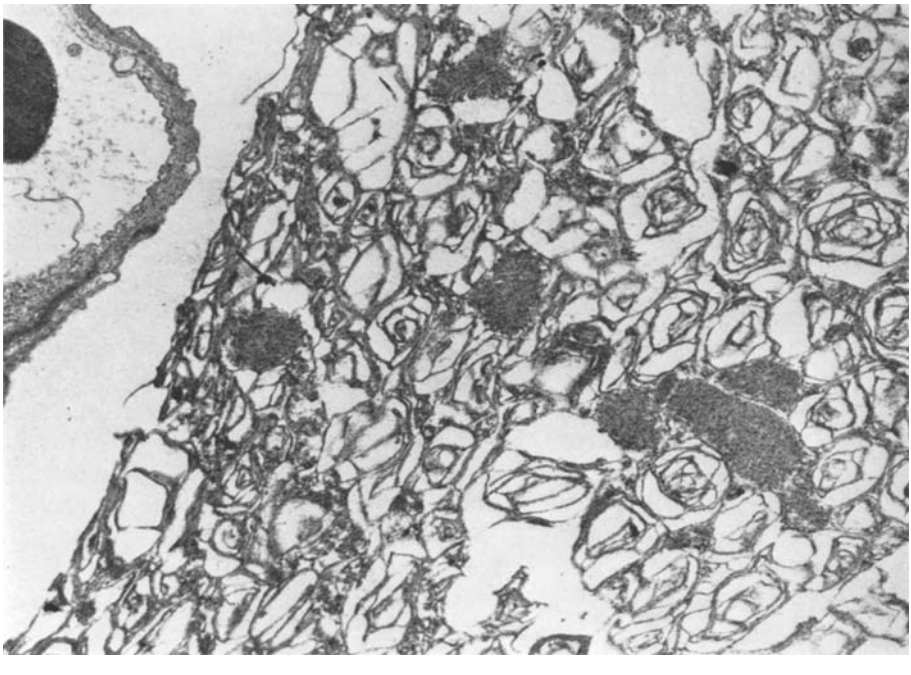

Fig. 7. Much of the alveolar lumen is filled by lamellar and "grid-iron" osmiophilic material. This corresponds to the stage of alveolar proteinosis seen on light microscopy. Lead and uranium. ×10500

a grid-iron pattern are also seen. After 1 year, some alveoli are filled with numerous osmiophilic laminated bodies (Fig. 7), which correspond to the densely eosinophilic masses observed in the alveolar spaces by light microscopy. The free lamellar bodies are similar to the inclusions in both the intra-alveolar and epithelial cells (Figs. 5–7).

Discussion

So far as the toxicity of iprindole is concerned it is reassuring that the early changes do not progress to fibrosis, and that even the late lesions are reversible. The development of alveolar proteinosis is disquieting but the amounts of iprindole used in these experiments greatly exceed the recommended dose. The drug is now widely used in clinical practice and so far there are no reports of it causing lung disease in man. Furthermore iprindole has no adverse effects when tested in other experimental species (Wyet and Brother, personal communication).

The interest of the present findings lies largely in their possible relevance to the spontaneous development of alveolar proteinosis in man. That they may be significant in this respect is suggested by a recent case-report describing the development of alveolar proteinosis from desquamative interstitial pneumonia in a child (Bhagwat *et al*, 1970a). Such a sequence had previously been described in experimental animals (Gross and deTreville, 1968; Corrin and King, 1970) but the cellular exudation was then termed exudative pneumonitis or endogeneous lipid pneumonia. Despite these reports a relationship between desquamative interstitial pneumonia and alveolar proteinosis is still not generally recognised, but should be considered in future studies of either condition.

In this paper attention is concentrated on the alveolar proteinosis-like changes, as we have already described the early lesions induced by iprindole and discussed their relationship to desquamative interstitial pneumonia (Vijeyaratnam and Corrin, 1972a, b). The late lesions observed in the present investigation appear to be identical to those of human alveolar proteinosis (Rosen *et al.*, 1958). In both conditions, the intra-alveolar material is deeply eosinophilic and strongly periodic acid-Schiff positive. Its basic lipidic nature, indicated in the original report of Rosen and his collagues, is confirmed in this study, whilst in its fine structure it contains lamellated structures of myelin figure type (Kuhn *et al.*, 1966; Divertie *et al.*, 1966).

The aetiology of alveolar proteinosis is man in poorly understood. There are similarities to Pneumocystis carinii infection but the organism cannot be demonstrated. That it could be a form of hypersensitivity reaction has been suggested following the experimental production of similar lesions in rabbits rendered hyperimmune (Powell and Gough, 1959) or given Freund's adjuvant (Bhagwat *et al.*, 1970b). Larson and Gordinier (1965) suggested that alveolar proteinosis may be the result of an inborn error of metabolism, but the predominant incidence in adults and the lack of familial occurrence argue against this possibility. Davidson and Macleod (1969), analysed all the recorded cases and found that nearly half had been exposed to a wide variety of dust including wood, silica, asbestos, cadmium, barium, broken fluorescent tubes, chlorinated resins, tin and molybdenum. They concluded that alveolar proteinosis may be a non-specific response to a variety of inhaled irritants. The production of similar lesions in experimental

animals by exposing them to silica and non-siliceous dusts lends support to this view (Gross and de Treville, 1968; Corrin and King, 1970; Heppleston *et al.*, 1970), whilst the present investigation shows that the responsible agent need not be inhaled.

The pathogenesis of alveolar proteinosis is also uncertain. Although there are structural similarities between the intra-alveolar material and pulmonary surfactant, Kuhn (1966) found that extracts of proteinotic tissue were not surface-active. Similarly Ramirez-R and Harlan (1969) have shown that lipid synthesis is not increased in alveolar proteinosis but that there is impaired removal or degradation of alveolar lipids. Evidence derived from animal experiments also indicates an impairment of the alveolar clearance mechanism (Gross and de Treville, 1968; Corrin and King, 1970). Rosen and his colleagues postulated that the intra-alveolar material is formed by a granular transformation of septal cells with a gradual accumulation of cellular debris until the alveoli are completely filled. This view is strongly supported by the present study. Ray and Salm (1962) however, proposed that the lipoproteinaceous material is merely derived from passive transudation of serum constituents; and Stanisfer and Bourgeois (1965) demonstrated that the chemical composition of the intra-alveolar material is similar to that of serum. The presence of capillary damage and fluid leakage in this study provides some support for this view but the fact that the oedema fluid is mainly confined to the alveolar walls makes it unlikely that all the alveolar material is derived from plasma.

In the present investigation, the bulk of the intra-alveolar material is derived from type I epithelial cells and represents degenerate portions of cytoplasm. Initially the degenerate fragments are taken up by alveolar macrophages which become distended by these inclusions. Such macrophages fail to reach the regional lymph nodes, suggesting that their mobility is impaired, probably because they are grossly overloaded by indigestible lipidic material. Lipid-laden macrophages persist in the alveoli for long periods but finally disintegrate, releasing their contents back into the lumen. Gradual compaction of this material then results in the characteristic appearance of pulmonary alveolar proteinosis. The evidence provided by this study therefore suggests that increased vascular permeability and lipidic epithelial degeneration may combine to overload the pulmonary macrophage clearance mechanism and so lead to alveolar proteinosis.

As the present experimental conditions are quite artificial it would appear that various unrelated factors may produce alveolar proteinosis, which would therefore represent a non-specific reaction to lung injury, as suggested by Davidson and Macleod (1969). There is evidence that desquamative interstitial pneumonia may similarly represent a non-specific pulmonary reaction (Corrin and Price, 1972).

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Dr. G. S. Vijeyaratnam
Dept. of Pathology
Faculty of Medicine
Kynsey Road
Colombo 8, Ceylon