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Ultrastructure of Pulmonary Granulomatosis Induced in Rats by Intravenous Complete Freund's Adjuvant

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Summary. Following the intravenous injection of complete Freund's adjuvant, changes in the rat lung were studied with the electron microscope. Interstitial granulomas were produced and whereas on light microscopy these appeared to consist mainly of epithelioid cells, electron microscopy showed that the granulomas were largely made up of macrophages.

Epithelioid cells were in fact few in number, atypical in appearance and limited to the periphery of some granulomas. Fenestrated capillaries were also found at the edge of the granulomas. The alveolar macrophages were increased in number and size but marked cytoplasmic vacuolation and a paucity of lysosomes are consistent with our previous suggestion that the phagocytic and migratory properties of these cells are weakened or inhibited. Alterations were found in both types of alveolar epithelial cell with the appearance of intermediate cell forms.

In various species, the intravenous injection of complete Freund's adjuvant (CFA) produces a widespread cellular reaction (Rist, 1938) involving particularly the lungs where the lesions are morphologically similar, in some evolutionary stages, to human granulomatoses such as sarcoidosis or extrinsic allergic alveolitis.

This very simple and efficient method provides an experimental model which should be helpful in understanding the pathogenesis of these histologically similar human conditions. We have studied the fine structural development of this model in the rat.

Material and Methods

Twenty nine female Wistar rats weighing 80 g were used: 9 were controls whilst the other 20 received 2 injections, separated by a 24 hr interval, of 0.05 ml CFA into the dorsal tail vein. Oral aureomycin was administered daily for 1 week.

Two animals were killed weekly, from 3 to 16 weeks after the 2nd injection of CFA. Under light intraperitoneal nembutal anesthesia, the lungs were fixed in situ by the intratracheal instillation of cold (4° C) 1.7% glutaraldehyde in cacodylate buffer (pH 7.4). The heart and lungs were then removed together and immersed in the fixative for 1 hr. Small pieces of the left superior lobe were taken for electron microscopy and placed in fresh fixative for 15 min, the other lobes being kept for light microscopy. After rinsing in cacodylate buffer, the tissues for electron microscopy were osmicated, dehydrated and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined in a Siemens Elmiskop 101 at 80 KV. Standard procedures were followed for light microscopy.

Results

Light Microscopy

The characteristic changes previously described (Basset *et al.*, 1972) are found in all animals treated with CFA, namely large vacuolated cells in the alveolar

walls or connective tissue septa, together with epithelioid or more rarely giant-cell follicular formations, composed of large cells with faintly acidophilic cytoplasm and oval or kidney-shaped, finely reticulated nuclei.

In the early stages (2 to 4 weeks) the alveolar walls are thickened by large numbers of vacuolated cells, a few lymphocytes and a few small epithelioid nodules chiefly in the subpleural region and distal parts of the lobes. Some alveoli show cuboidal metaplasia or contain numerous foamy macrophages with an eccentric nucleus. From 5 to 10 weeks numerous nodules and vacuolated cells are scattered throughout the lungs, particularly in a peribronchovascular and subpleural position. Their distribution is irregular from one lobe to another. In the later stages (10 to 15 weeks) the lesions regress irregularly and fibrosis develops around residual granulomas.

Electron Microscopy

Changes were observed in the perivascular interstitium, the alveolar walls and their epithelial lining, and in the air spaces.

1. Perivascular Interstitium. Corresponding to the granulomas, collections of large mononuclear cells are found. Their nuclei are usually elongated and kidney-shaped, composed of a thin band of peripheral hetero-chromatin, sometimes with very prominent nucleoli and 1 or 2 intranuclear bodies, type I of Bouteille *et al.* (1967) (Fig. 1b). Cell contours are irregular, sometimes exhibiting short cytoplasmic processes. Cytoplasmic organelles are generally numerous. The mitochondria are frequently elongated, the rough endoplasmic reticulum abundant and the Golgi complex always well developed. These cells also have phagocytic characteristics: lysosomes, phagolysosomes and numerous vacuoles containing lipidic material, sometimes extracted by the processing techniques (Fig. 1a). The phagolysosomes often contain partially digested CFA mycobacteria (Fig. 2). They frequently show a paracrystalline content and, on their margin, pentalaminar myelin-like coils of about 13 nm periodicity with the core denser and darker than the outer layers (Fig. 3a and b).

Although the granulomas appeared on light microscopy to consist largely of epithelioid cells, elements with the ultrastructural features of such cells, as described in human pathology (Basset *et al.*, 1967, 1969; Bernaudin *et al.*, 1974; Douglas, 1967; Hirsch *et al.*, 1967; Kalifat *et al.*, 1967; Kelemen, 1967; Wanstrup and Christensen, 1966; Wanstrup, 1967) are very rare and present only at the periphery of some granulomas. In contrast to the more central cells, they possess relatively few lysosomes and phagolysosomes, but contain some small membrane-lined vesicles with a finely granular content, similar to the characteristic "grey vesicles" of the human epithelioid cell. Their plasma membrane shows long thin digitations (Fig. 4a and b).

Around the granulomas there is a loose collection of collagen fibres, fibroblasts and inflammatory cells, mostly lymphocytes and plasma cells, rarely eosinophils. There are also capillaries which only rarely penetrate the granulomas. Some have a thin continous endothelium, others a thicker one with irregular contours (Fig. 5a), whilst others have membrane-lined fenestrations (Fig. 5b). The endothelial basement membrane is almost always modified, showing several

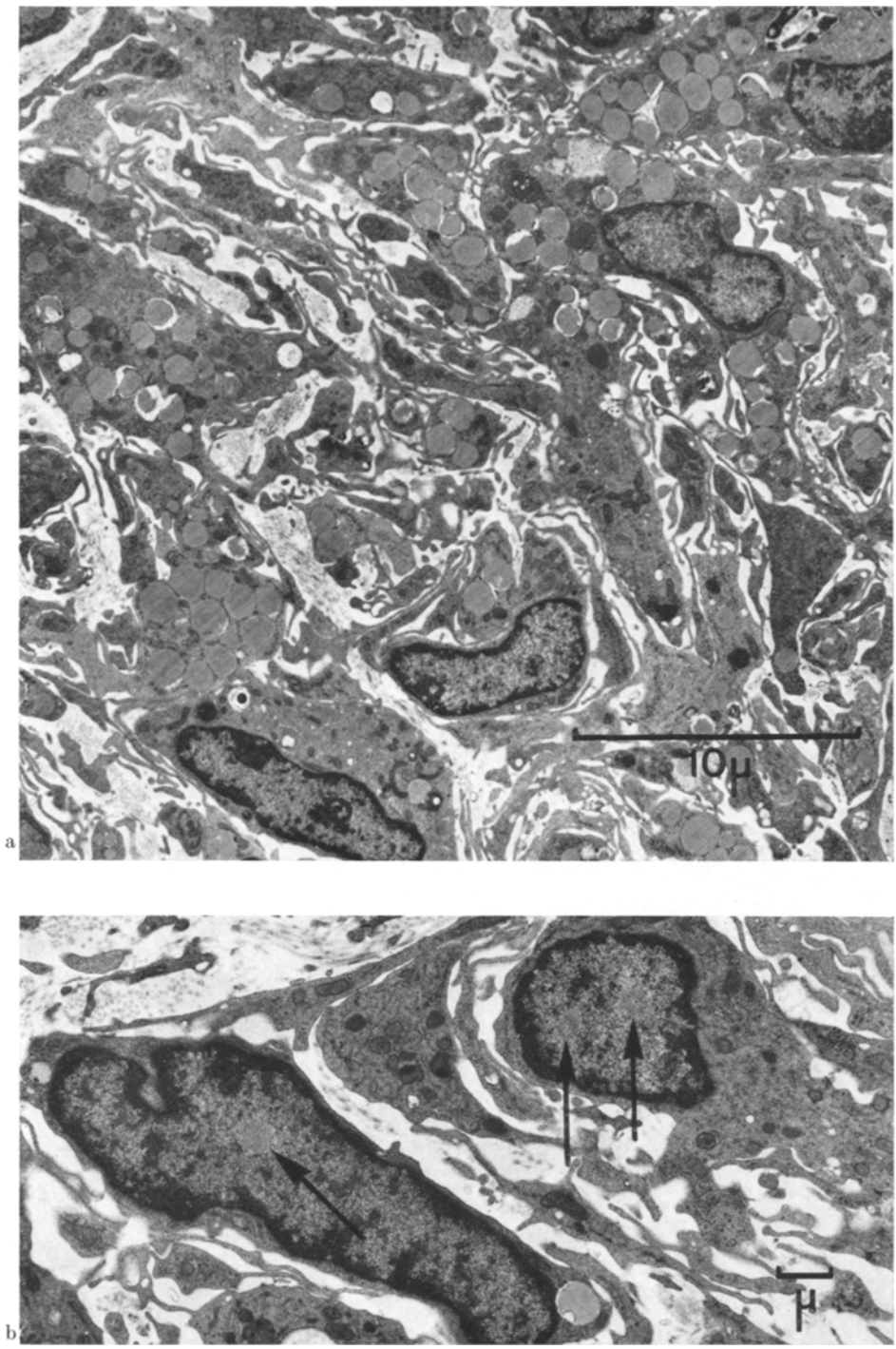


Fig. 1a and b. Eight weeks after the injection of CFA. (a) The granulomas are composed of large mononuclear cells presenting phagocytic characters: lysosomes, phagolysosomes and vacuoles containing lipidic material, sometimes extracted by processing techniques. ×4000. (b) Detail of some nuclei showing intranuclear bodies (arrows). ×7250

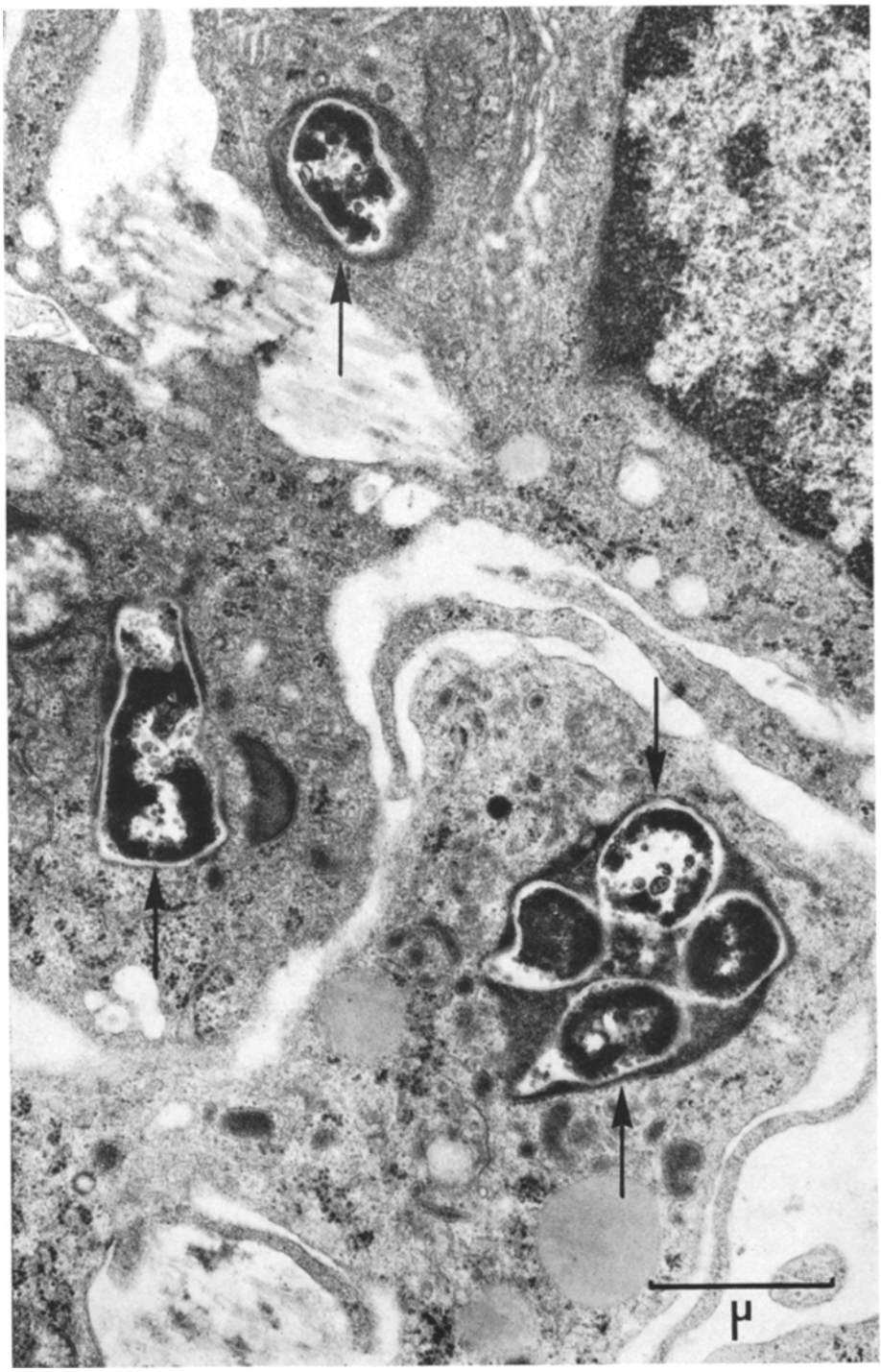
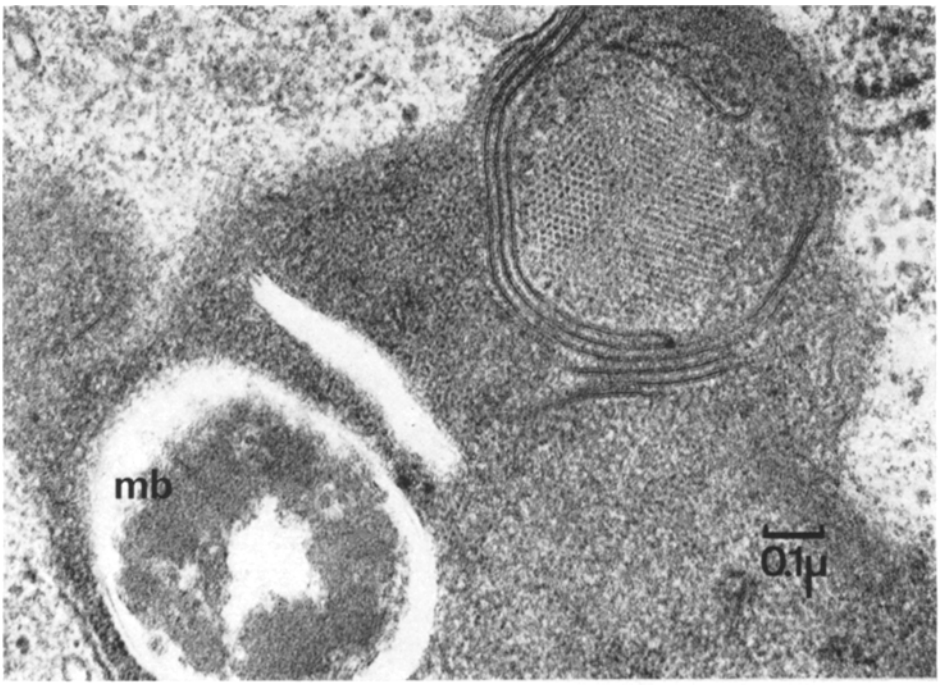


Fig. 2. Eight weeks after the injection of CFA. Cells of another granuloma showing the phagocytosed remains of CFA mycobacteria (arrows). ×25000



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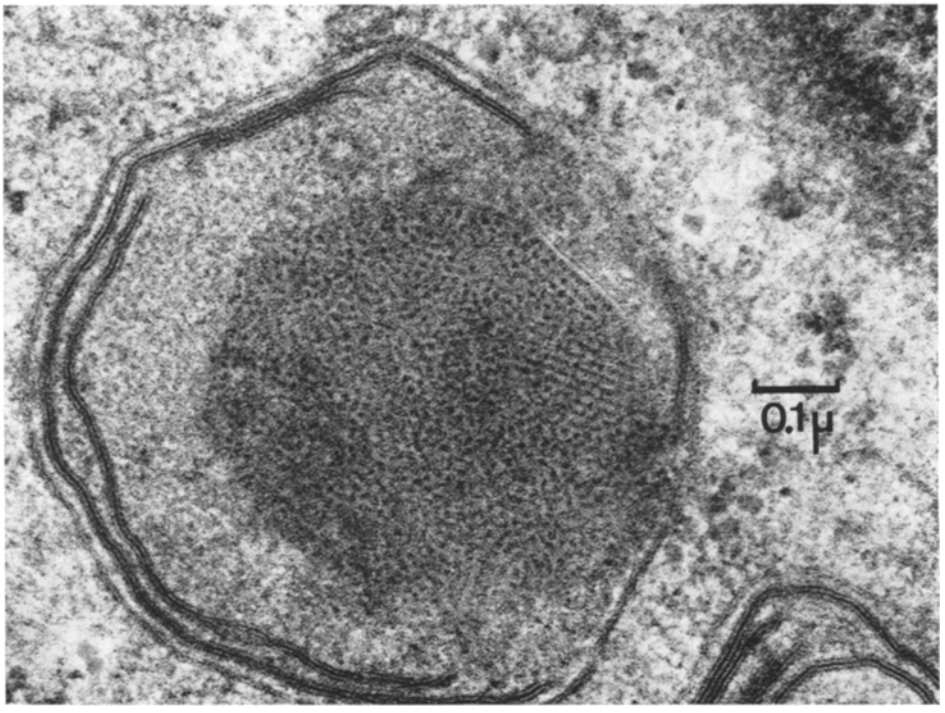

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Fig. 3a and b. Eight weeks after the injection of CFA. (a) Detail of phagolysosome showing a paracrystalline inclusion and pentalaminar myelin-like coils of about 13 nm periodicity, with the central band denser and darker than the lateral layers. *mb* ingested mycobacterium. ×75000. (b) Another similar phagolysosome. Detail of the myelin-like coils. ×112500

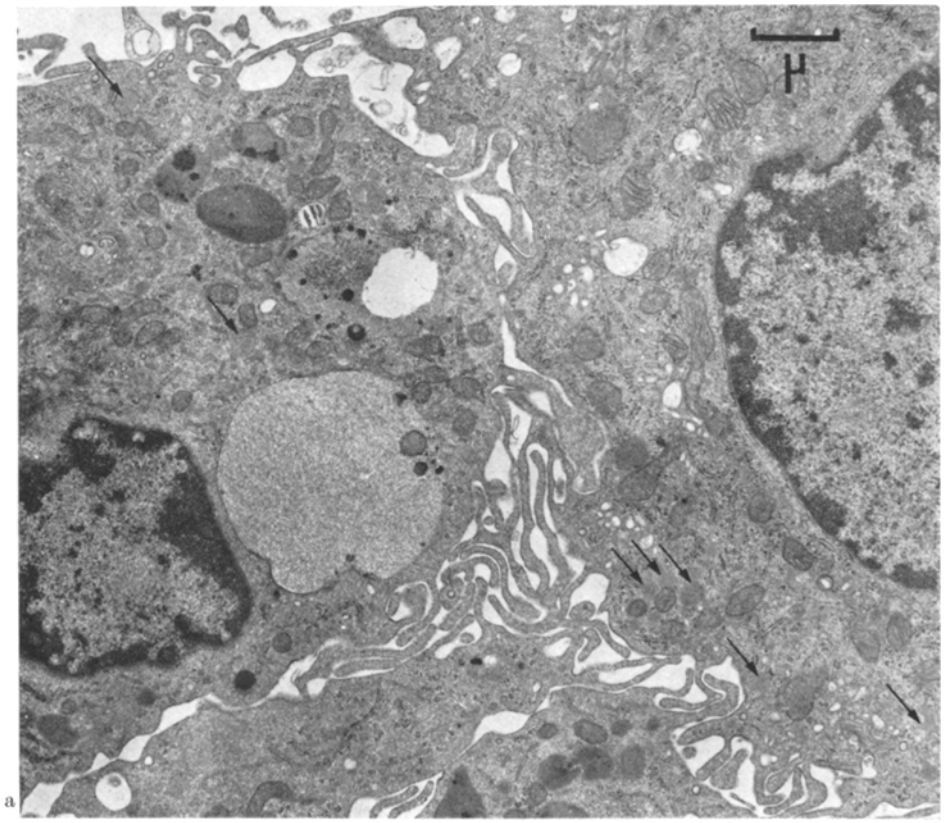


Fig. 4a and b

concentric layers of microfibrillar or granular material, often enclosing large portions of pericytes (Fig. 5a).

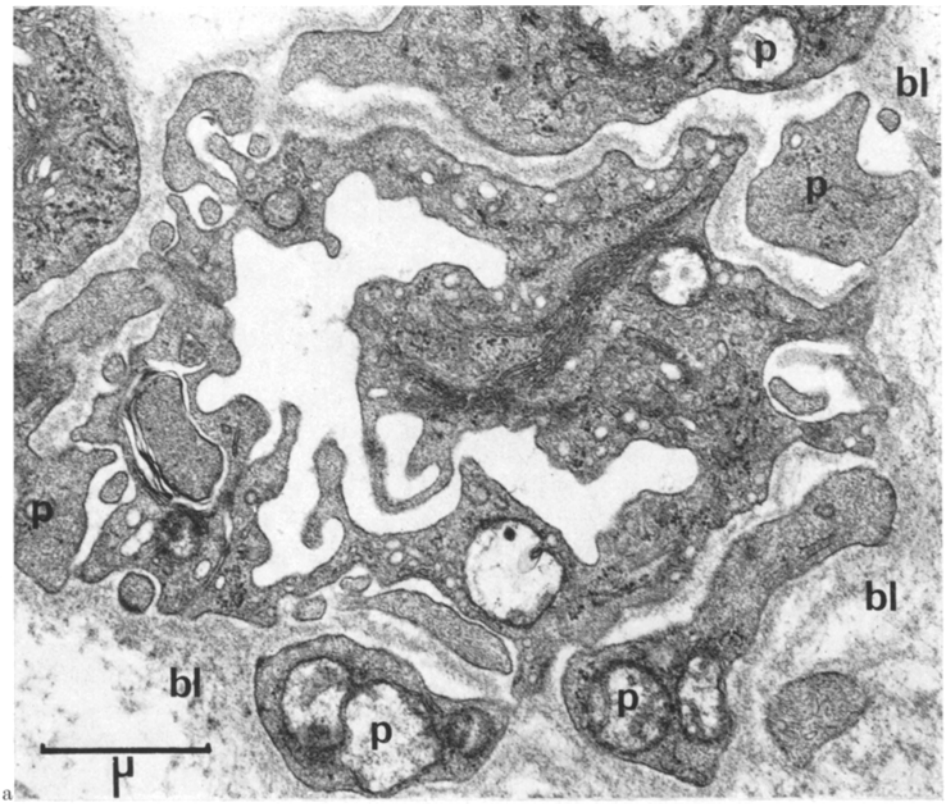
2. Alveolar Walls and Epithelium. The alveolar walls are infiltrated by numerous vacuolated cells and mononuclear inflammatory cells, and alterations are seen in both kinds of alveolar lining cell. The type II pneumocytes (granular pneumocytes) are increased in number and sometimes represent the sole component of the alveolar lining. Outside these areas of cuboidal metaplasia, there are frequently seen in the same alveolus 2 or 3 adjacent granular pneumocytes with junctions at their apical poles. These pneumocytes are atypical. Microvilli appear smaller and less numerous than normal. Lamellar bodies are sometimes larger than usual (Fig. 6a) and often occupy a basal position under the nucleus, near multivesicular bodies with a dense matrix, their possible precursor (Goldenberg *et al.*, 1969; Kikkawa and Spitzer, 1969; Sorokin, 1966) (Fig. 6b). Cytoplasmic slits corresponding to crystals of extracted fatty acids are occasionally seen. The type I pneumocytes (membranous pneumocytes) are also frequently altered, particularly near the granulomas. They exhibit small microvilli on their luminal aspect and their cytoplasm is thickened and sometimes contains multivesicular bodies and osmiophilic lamellar bodies identical to those of granular pneumocytes (Fig. 7a and b).

3. Air Spaces. Increased numbers of macrophages are clearly evident within the alveoli. They are generally large and round, devoid of cytoplasmic processes. They exhibit numerous intracytoplasmic vacuoles of irregular size. Many vacuoles appear empty whilst others contain small osmiophilic myelin figures. Where several vacuoles meet, the intervening cytoplasm is reduced to a few thin strands. Other cytoplasmic organelles, particularly primary lysosomes, are rare or absent (Fig. 8a). The nucleus is indented and often pushed towards the periphery by large vacuoles with an osmiophilic double lining, which occupy almost the whole cytoplasm (Fig. 8b).

Discussion

1. Epithelioid-type cells form the main component of the granulomas on light microscopy, but these cells differ in their fine structure from those described by other workers. On electron microscopy most appear to be macrophages. Their cytoplasm contains digestive vacuoles of varying size, some enclosing partly degraded mycobacteria, still recognizable despite the long interval since the last CFA injection. Some of these vacuoles, with a granular and sometimes paracrystalline content, exhibit on their periphery myelin-like whorls which could correspond to a particular component of the partly digested mycobacteria. Nevertheless one can also see at the periphery of follicular formations occasional cells resembling the epithelioid cells of human sarcoidosis or hypersensitivity

Fig. 4a and b. Eight weeks after the injection of CFA. (a) Portions of epithelioid cells located at the periphery of a granuloma. They possess relatively few lysosomes and phagolysosomes, but contain characteristic small "grey vesicles" (arrows). The plasma membrane shows long slim digitations. ×11250. (b) "Grey vesicles" (*v*) at a higher magnification. They show a finely granular content bounded by a single unit membrane. ×100000



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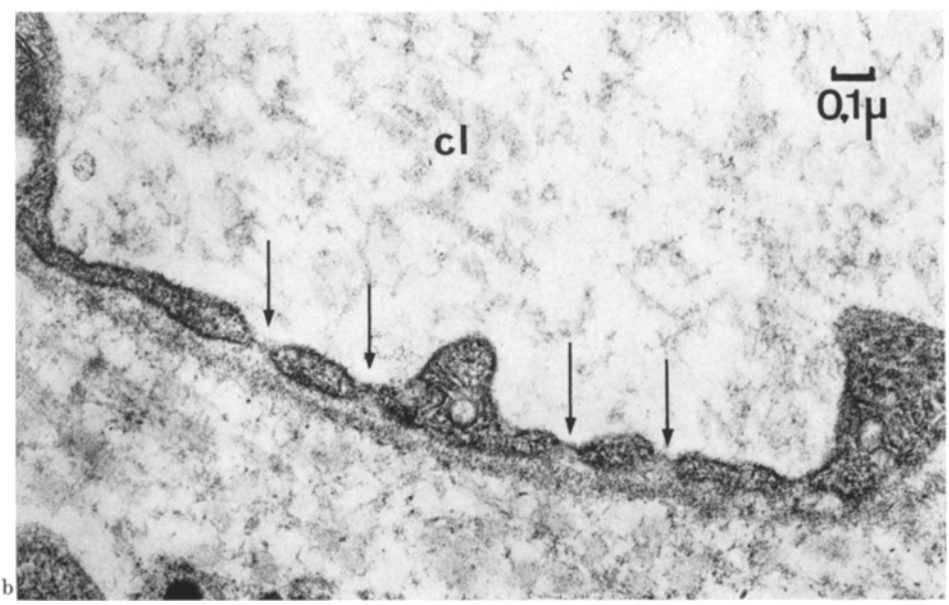


b

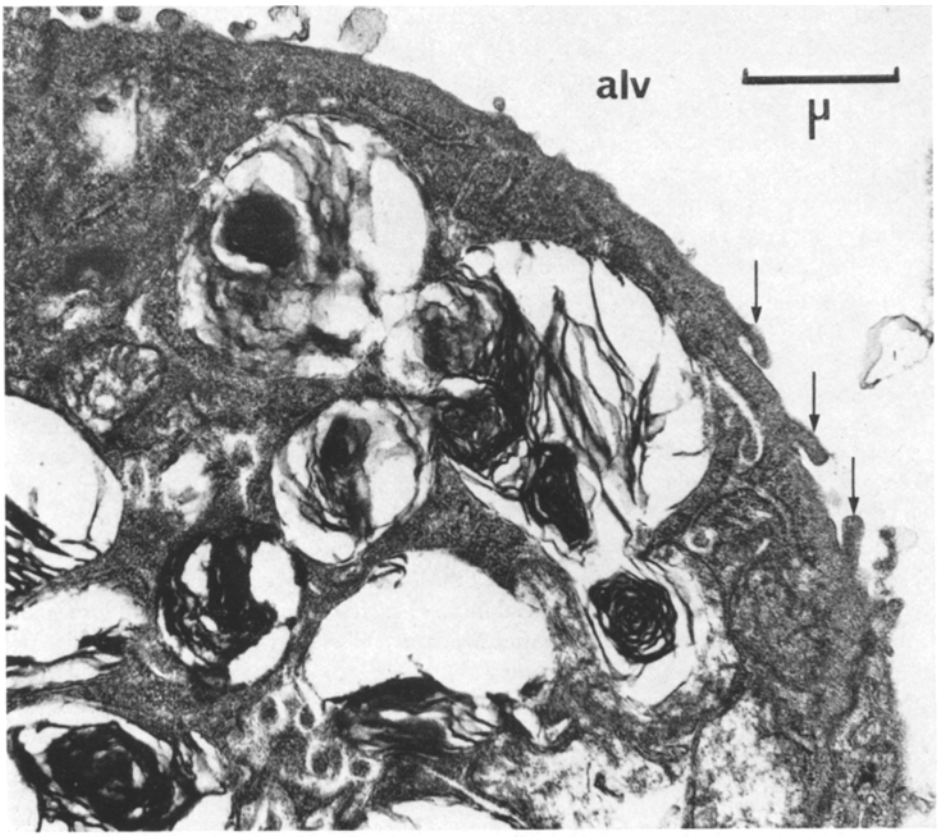
Fig. 5a and b

granulomas or more closely those described as epithelioid by Sutton and Weiss (1966) and Papadimitriou and Spector (1971) in the chicken and the mouse.

The exact nature and role of the epithelioid cell remain uncertain but several hypotheses have been advanced. James and Jones Williams (1974) believe that it may develop from a lymphocyte and that it is a secretory rather than phagocytic cell: its products would play a role in the formation and persistence of granulomas. Epstein (1967), on the other hand, believes that the epithelioid cell develops from a monocyte. It could be a "nonsense" cell devoid of function, or a stimulated monocyte specialized in the production of a protein mediator ("granuloma-inciting factor"). Papadimitriou and Spector (1971) suggest that epithelioid cells develop from macrophages mobilized at a site of inflammation. The macrophages turn into epithelioid cells only if there is little to ingest, or if the ingested material is thoroughly digested and reduced to completely soluble products. Uptake of indigestible, non-excretable material prevents macrophages from becoming epithelioid cells. Mycobacteria are not easily digested (Leake *et al.*, 1971; Papadimitriou and Spector, 1971) and it is therefore not surprising to find epithelioid cells limited to the periphery of BCG or CFA induced granulomas, between the mycobacteria-containing macrophages and the rest of the tissue. Here they could play a role in digesting, detoxifying or excreting toxic substances released from the granuloma. Our findings support the views of Papadimitriou and Spector (1971) and the hypotheses of Unanue and Benaceraff (1973), and Kasdon and Scholssman (1973), according to which the persisting presence of antigen is necessary for the formation of such granulomas. These views are not necessarily applicable to human lesions, such as sarcoidosis, however.

2. An increased number of alveolar macrophages has been previously noted following intravenous CFA (Bhagwat and Conen, 1969; Faulkner and Esterly, 1971; Laufer *et al.*, 1959; Moore and Schoenberg, 1963, 1964; Rupp *et al.*, 1960; Steiner *et al.*, 1960; Strauss *et al.*, 1970), probably reflecting the increase in interstitial macrophages which have been proposed as their immediate precursor (Vijeyaratnam and Corrin, 1972). In a previous experiment (Soler *et al.*, 1974) we reported that CFA did not cause a true stimulation of alveolar macrophages in spite of an increase in their numbers. Elimination graphs of inhaled marked hematite showed on the contrary a slowing or complete arrest of clearance. Pulmonary lavage yielded cells with a 40% decrease in their mobility in vitro. Our present ultrastructural evidence reflects these functional disorders of the cell. Pseudopodial processes and organelles are scanty and the cytoplasm is filled by numerous vacuoles instead of the characteristic lysosomes, supporting the suggestion that intracellular digestion and migration of such cells are weakened or inhibited.

Fig. 5a and b. Eight weeks after the injection of CFA. (a) Capillary located in the marginal zone of a granuloma. The endothelium is thickened with irregular contours. The basal lamina (*bl*) consists of several concentric layers of microfibrillar or granular material, enclosing numerous sections of pericytes (*p*). ×22500. (b) Detail of another type of capillary near a granuloma. The endothelium shows a few fenestrations, closed by a membrane (arrows). *cl* capillary lumen. ×50000



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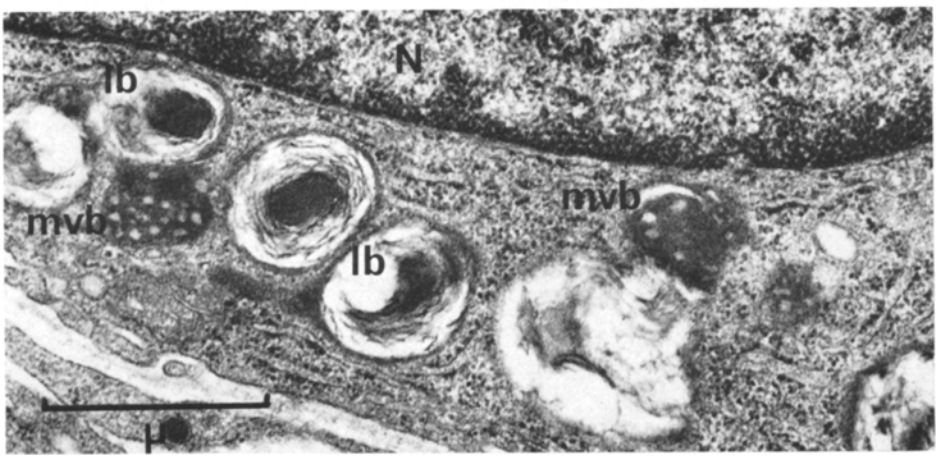

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Fig. 6. (a) Four weeks after the injection of CFA. Atypical granular pneumocyte. Microvilli are smaller and less numerous than normal (arrows). Lamellar bodies are larger than usual. *alv* alveolus. ×20000. (b) Eight weeks after the injection of CFA. Detail of another granular pneumocyte. Lamellar bodies (*lb*) are in a basal position under the nucleus (*N*), near multivesicular bodies with a dense matrix (*mvb*), their possible precursors. ×30000

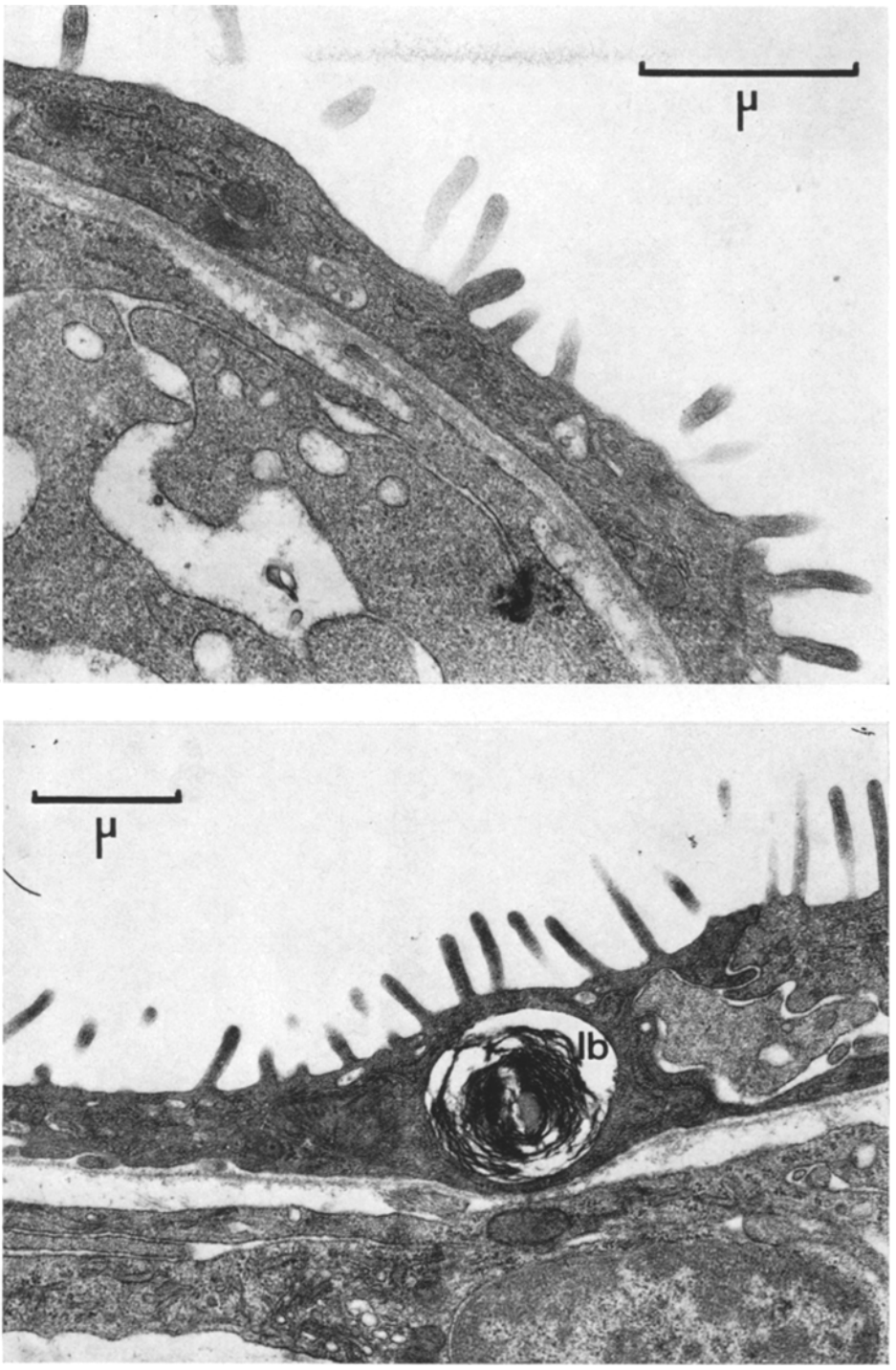

Fig. 7a and b. Eight weeks after the injection of CFA. (a) Membranous pneumocyte showing microvilli on its luminal surface. ×30000. (b) Another membranous pneumocyte possessing microvilli. The cytoplasm is thickened and contains an osmiophilic lamellar body (*lb*) identical to those of granular pneumocytes. ×20000

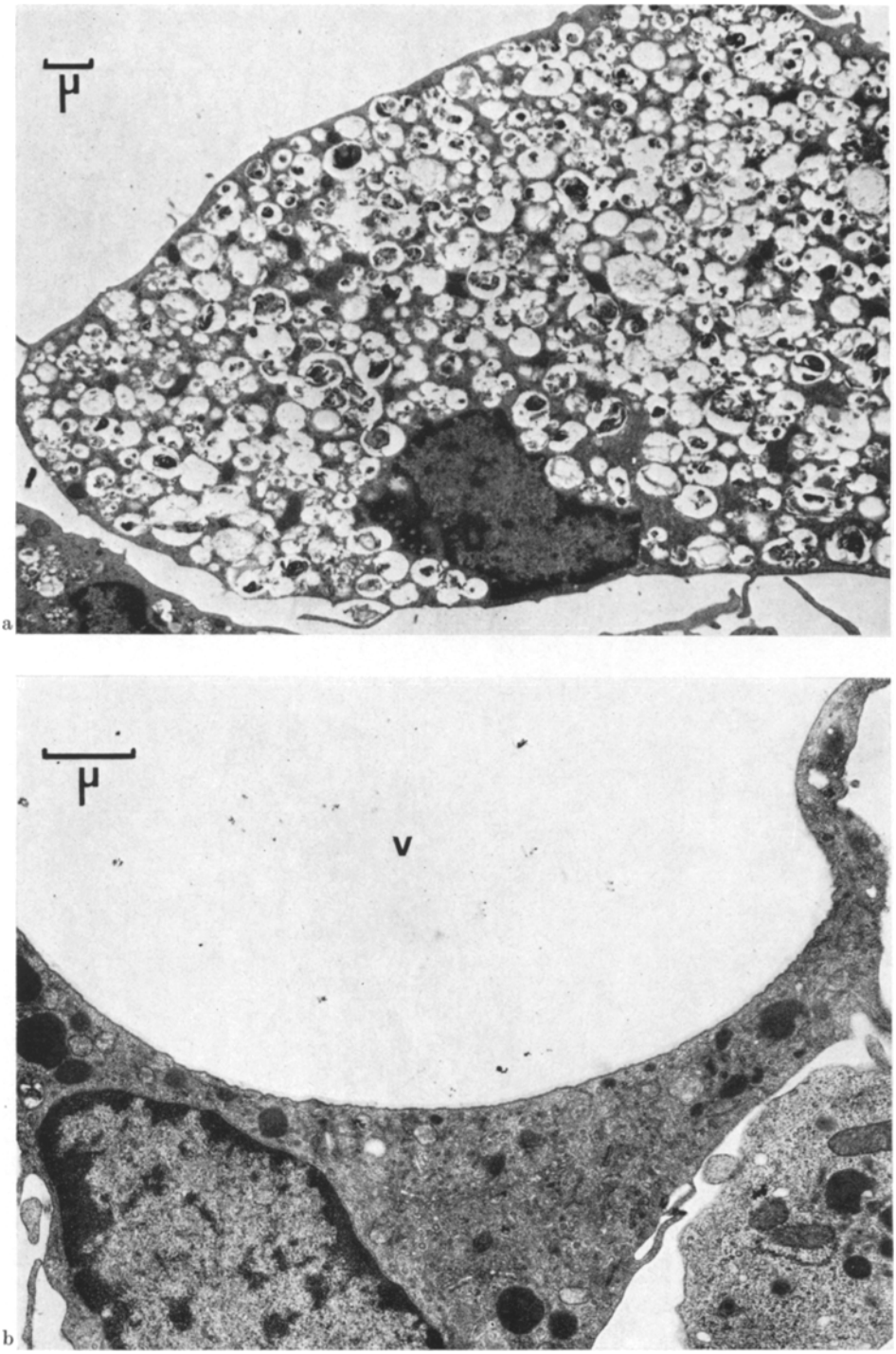

Fig. 8a and b. Five weeks after the injection of CFA. (a) Alveolar macrophage in a zone of alveolitis exhibiting numerous intracytoplasmic vacuoles of irregular size. Cytoplasmic pseudopodial processes and organelles, particularly lysosomes, are rare or absent. ×6250. (b) Extremely altered alveolar macrophage: the nucleus is pushed towards the periphery by a large vacuole (*v*) which occupies almost the whole cytoplasm and presents no visible content. ×12500

3. The various capillary alterations noted in this study have been described in several human (Basset *et al.*, 1969, 1970) and experimental conditions (Suzuki, 1969). The presence of fenestrated capillaries near CFA-induced granulomas raises the problem of their origin: are they modified pulmonary capillaries, as in hypersensitivity pneumopathies (Basset *et al.*, 1970), or are they the normally-fenestrated capillaries of the bronchial circulation? Our studies cannot answer this question which would require vascular injection techniques studied by electron microscopy. It will be noted however that many pathological processes in the lung (bronchiectasis, tuberculosis, neoplasm) have a bronchial blood supply (Cudkowicz, 1968; Liebow, 1949; Spencer, 1968).

4. The epithelial changes we noted have previously been described by Faulkner and Esterly (1971) in rats treated with CFA, and similar alterations have been reported in a great number of clinical and experimental disorders: neonatal respiratory distress (Kikkawa *et al.*, 1965; Balis *et al.*, 1966), cardiopulmonary by-pass (Sakashita *et al.*, 1968), desquamative interstitial pneumonia (Brewer *et al.*, 1969; Leroy, 1969; Shortland *et al.*, 1969), pulmonary alveolar proteinosis (Kuhn *et al.*, 1966), irradiation (Leroy *et al.*, 1966; Faulkner, 1971), CO_2 intoxication (Schaefer *et al.*, 1964), O_2 toxicity (Kapanci *et al.*, 1969; Rosenbaum *et al.*, 1969; Gould *et al.*, 1971), vagotomy (Goldenberg *et al.*, 1967), pilocarpine administration (Goldenberg *et al.*, 1969), hypocholesterolemic drugs (Esterly and Hruban, 1970), and the effect of nickel-carbonyl (Hackett and Sunderman, 1968). This similarity of reaction in such differing conditions supports the concept of a morphologically stereotyped pulmonary response to diverse injuries (Faulkner and Esterly, 1971; Kapanci *et al.*, 1969; Pariente *et al.*, 1973). The presence of cells intermediate in appearance between types I or II suggests the possibility of transformation of one cell type into the other and raises the problem of the direction of change (I to II or vice versa). Current views based on the autoradiographic evidence of Evans *et al.* (1973) and Adamson and Bowden (1974) propose that the type II cell is the precursor of the type I cell (Corrin, 1974), explaining that our intermediate cells are really type II cells differentiating into type I.

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