Onderstepoort. J. vet. Res., 51, 1–13 (1984)

A SCANNING AND TRANSMISSION ELECTRON MICROSCOPY STUDY OF JAAGSIEKTE LESIONS

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ABSTRACT

PAYNE, ANNA-LISE & VERWOERD, D. W., 1984. A scanning and transmission electron microscopy study of jaagsiekte lesions. *Onderstepoort journal of Veterinary Research*, 51, 1-13 (1984).

A scanning electron microscopy (SEM) and transmission electron microscopy (TEM) study was made of lesions from acute, experimentally induced cases of jaagsiekte. In the SEM study tumour cells were easily identified by the abundant microvilli on their peripheral surface. The SEM study gave further insight into the development of lesions and the spatial relationship of cells involved in jaagsiekte. TEM revealed that the tumour cells were in a state of rapid protein synthesis and had many characteristics in common with other malignant cells.

INTRODUCTION

Jaagsiekte or ovine pulmonary adenomatosis is a transmissible, contagious tumour. Although jaagsiekte is classified histologically as a bronchiolo-alveolar adenocarcinoma, the disease is usually called "adenomatosis", because the incidence of metastases is rare (Stunzi, Head & Nielson, 1974). Such is not always the case, since in Israel metastases are common in Awassi sheep with jaagsiekte (Nobel, Neumann & Klopfer, 1969), and therefore "pulmonary carcinoma" was their suggested designation of the disease (Nobel & Perk, 1978). However, as the incidence of metastasis is rare in South Africa (Tustin, 1969), we prefer the former nomenclature. The difference in the incidence of metastases may be dependent on the breed of sheep (Nobel *et al.*, 1969) or it may indicate that there is a separate disease entity in Israel.

Recent experimental evidence has shown that the aetiological agent of jaagsiekte is a retrovirus (Verwoerd, Williamson & De Villiers, 1980b; Verwoerd & Williamson, 1982; Herring, Sharp, Scott & Angus, 1983). Jaagsiekte retrovirus (JSRV) replicates in the tumour cells and buds into the alveolar lumen (Payne, Verwoerd & Garnett, 1983). The virus differs morphologically as well as serologically from other retroviruses (Payne *et al.*, 1983; Verwoerd, Payne, York & Myer, 1983). In natural cases of jaagsiekte the disease is characterized by a long incubation time (Tustin, 1969). It has been possible to reduce the lag time to a few weeks by inoculating large amounts of JSRV intratracheally into neonatal lambs (Verwoerd, *et al.*, 1980b). All previous transmission electron microscopy (TEM) studies were performed on material from natural cases of jaagsiekte (Perk, Hod & Nobel, 1971; Wandera & Krauss, 1971; Nisbet, Mackay, Smith & Gray, 1971; Hod, Herz & Zimber, 1977). In this study, lesions from experimentally induced, acute jaagsiekte cases were examined, using both TEM and scanning electron microscopy (SEM). The unique ability of SEM to survey broad expanses of tissue allows for easier evaluation of the respiratory cell types involved in disease (Andrews, 1979). SEM also yields insight into the three-dimensional structure and the spatial relationships between cells.

MATERIALS AND METHODS

Lung samples

Samples of solid lung tumour and macroscopically normal lung were taken from 50 sheep with advanced, experimentally induced jaagsiekte (Verwoerd *et al.*, 1980b) within 40 min of slaughter. Most lungs were thoroughly rinsed with cold minimal essential medium (MEM) before processing. Samples from 10 normal sheep lungs were also taken. Only 4 of the normal lungs were rinsed with MEM.

Scanning electron microscopy (SEM)

Lung samples from 15 jaagsiekte sheep and 5 normal sheep were cut with a sharp blade into strips, 2–5 mm thick, and fixed in GA fixative (2,5 % glutaraldehyde in 0,1 M cacodylate buffer containing 4 % sucrose, pH 7,2) for a minimum of 2 hours at 4 °C (adapted from Arborgh, Bell, Brunk & Collins, 1976). This was followed by 2 ten-minute washes in buffer (0,1 M cacodylate buffer containing 4 % sucrose, pH 7,2), and post-fixation for 1 hour in osmium tetroxide (1 % osmium tetroxide in 0,1 M sodium cacodylate buffer containing 4 % glucose, pH 7,2). After 2 ten-minute washes the samples were dehydrated in acetone. The samples were then transferred to baskets and treated with 3 ten-minute changes of fluorocarbon (Freon 113, Dupont) and then placed into the bomb of an Hitachi HCP-1 critical point drier (Boyde, Franc & Maconnachie, 1981). The specimens were kept "wet" throughout processing to prevent air drying artifacts. After critical point drying the specimens were mounted with double-sided adhesive tape on aluminium stubs and coated with 25–35 mm of gold in a Balzer's SCO 020 sputter-coating unit. A touch of silver paint was applied to the edge of the specimen to ground it and help prevent charging in the microscope. The specimens were viewed on an International Scientific Instruments 100 scanning electron microscope operating at 20 to 30 KV.

Transmission Electron Microscopy (TEM)

Lung samples from 50 jaagsiekte sheep and 10 normal sheep were cut into 1 mm cubes with a sharp blade to reduce tissue trauma. The samples were fixed in GA fixative at 4 °C for at least 1 hour. They were post-fixed in osmium tetroxide, washed in buffer and dehydrated in a graded acetone series. After being cleared in propylene oxide, the specimens were infiltrated with epoxy resin. Resin polymerization was at 65 °C overnight. Sections were stained with 2 % uranyl acetate followed by lead citrate (Reynolds, 1963). The sections were viewed with a Siemens Elmiskope 102 transmission electron microscope operating at 80 KV.

RESULTS

Normal sheep lungs

As the alveoli and bronchioles are the areas affected by jaagsiekte (Nisbet *et al.*, 1971), the ultrastructure of these areas was examined in normal lung samples for comparative purposes. The ultrastructure of sheep alveoli and bronchioles corresponded to that previously described (Kikkawa & Spitzer, 1969; Tyler, De Lorimier, Manus & Nowell, 1971; Plopper, Mariassay & Hill, 1980.)

Received 19 September 1983—Editor

Jaagsiekte lungs

Surface of tumour cells

When lungs that had not been rinsed prior to fixation were examined using SEM techniques, the epithelial cells were covered with exudate, making it impossible to elucidate any surface structure. The alveoli were also in a collapsed state, making viewing and interpretation difficult. In SEM studies of rinsed lungs the free surface of all tumour cells was covered with abundant microvilli. The length and number of microvilli varied from tumour to tumour. However, there were always more microvilli on the tumour cells than on normal pneumocytes, which usually had a smooth appearance (Fig. 1 & 4).

The older tumour cells appeared flatter and had fewer microvilli than the younger cells. TEM studies revealed that in some lesions the alveolar lumen was almost entirely occluded and the microvilli were interlocked.

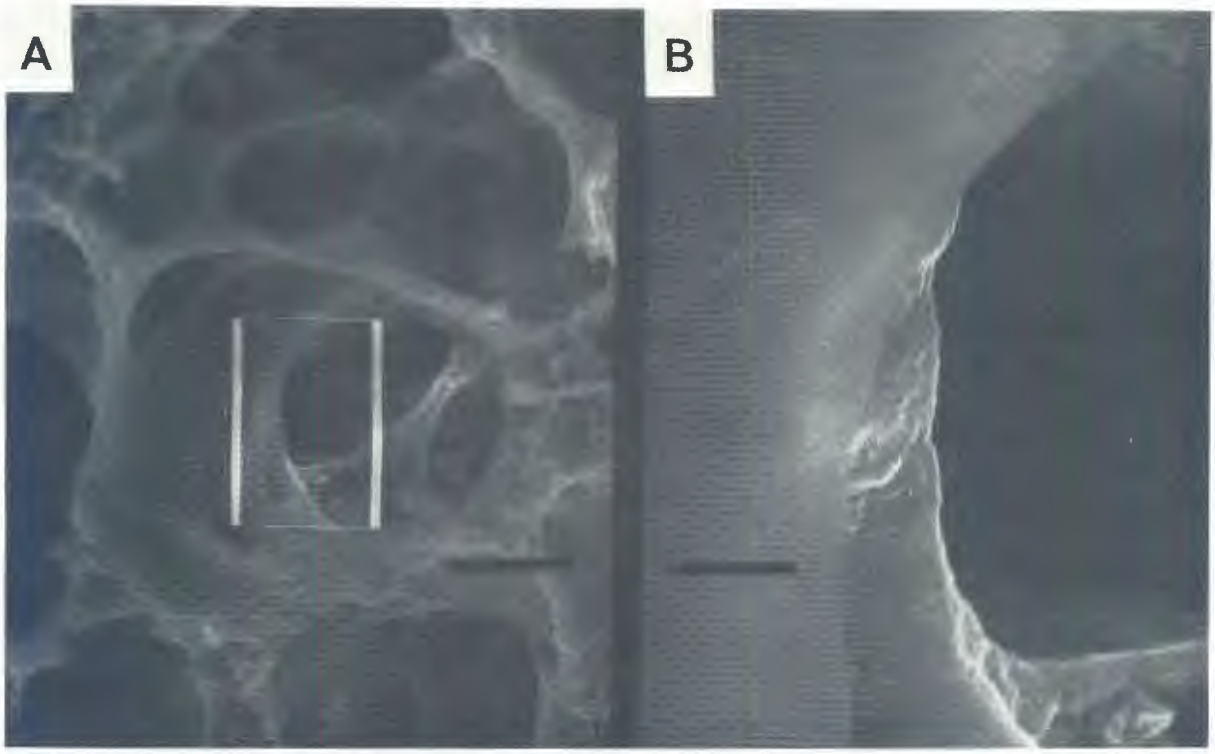

FIG. 1a SEM of normal sheep lung alveoli. Bar = 20 μm
1b Higher magnification of the smooth surface of normal sheep lung epithelial cells lining the alveolus. Bar = 4 μm

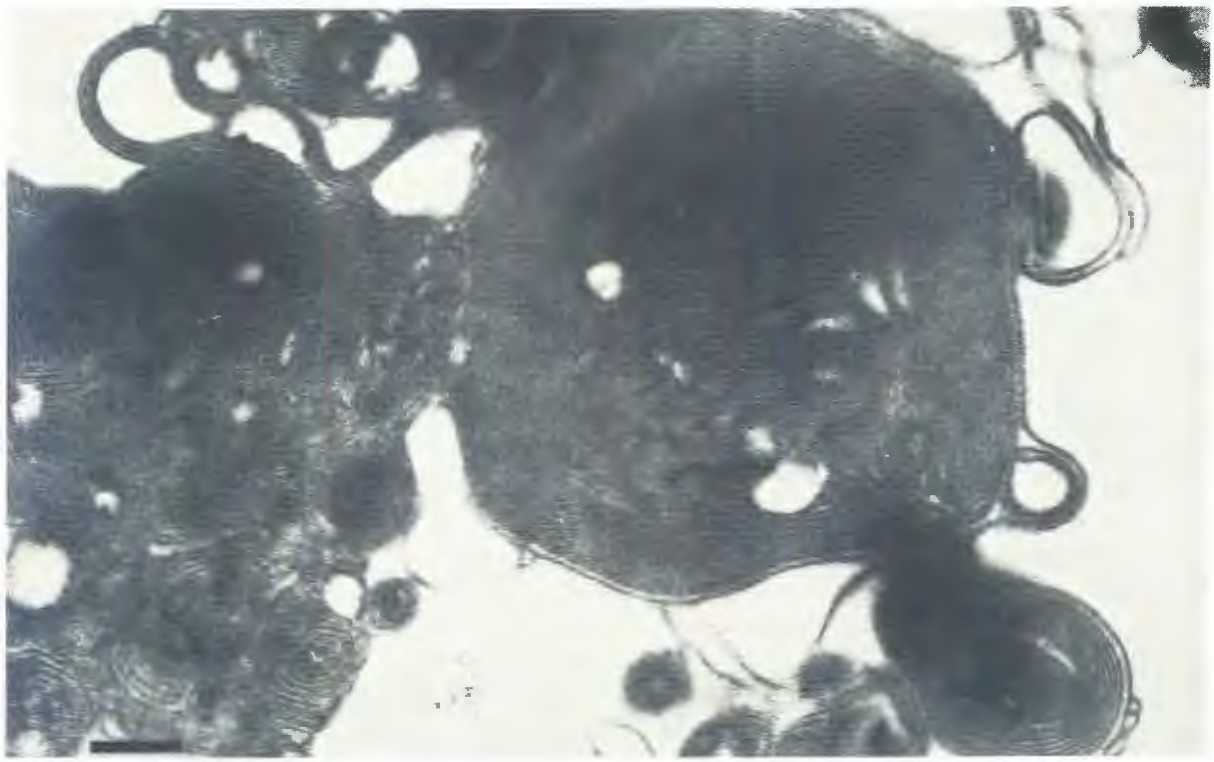

FIG. 2 Tubular myelin in the alveolar lumen of a jaagsiekte lung. Bar = 2 μm

An electron dense layer was visible on the surface of the tumour cells. This corresponds to the layer of surfactant described by Kikkawa, Motoyama & Cook (1965). Tubular myelin was often seen in the alveolar lumen in close proximity to tumour cells (Fig. 2). This is considered to be a storage form of pulmonary surfactant (Hassett, Engelman & Kuhn, 1980).

Arrangement of tumour cells

In the earliest lesions[1] pairs of tumour cells could be found in the corners of alveoli (Fig. 3). Various degrees of complexity of tumour cell arrangement were observed in the more advanced lesions. In some instances a complete layer of columnar or cuboidal cells was observed (Fig. 4 & 5). More advanced lesions consisted of papilliform projections and grape-like clusters (Fig. 6). SEM showed clearly that the most advanced lesions were made up of large proliferations of cells filling the alveolar lumen and spreading to adjacent alveoli (Fig. 7). TEM revealed sheets of cells, often in acinar arrangement (Fig. 8). The shapes of the cells, the majority of which were in the alveoli, varied according to the amount of packing. The cells on the periphery of the tumour were usually better differentiated than those within the tumour.

Tumour cells were occasionally observed with SEM in the respiratory bronchioles. They were distinguished from normal Clara cells by means of their abundant microvilli. In some cases, the tumour cells had a cobblestone appearance, with the cilia from the bronchiolar epithelial cells protruding between them (Fig. 9). Bunches of tumour cells were also seen in some bronchioles. The tumour cells in respiratory bronchioles were often confluent with those in adjacent alveoli (Fig. 9). No bronchiolar lesions could be identified with TEM.

Intracellular relationships

Desmosomes connected adjacent tumour cells on their lateral surfaces. In some tumours interdigitation was observed between tumour cells. In SEM of tumour cells the intercellular junctions appeared as distinct grooves making it possible to identify the surface of individual cells (Fig. 4).

Cytoplasmic characteristics of tumour cells

Most of the tumour cells on the periphery of the lesion contained secretory granules which varied in appearance in different lesions. In some the granules were very small and few in number, whereas in others the granules were very prominent and filled the apical portion of the tumour cells. There was also a large variation in the content of secretory granules. In the granular pneumocytes of normal sheep the secretory granules contained very little osmiophilic, electron dense material (Kikkawa & Spitzer, 1969). In jaagsiekte tumour cells the secretory granules varied from electron lucent to electron dense to granules filled with myelinoid whorls (Fig. 10). Some tumour cells were in the process of releasing the contents of their secretory granules. This is probably the source of the lung exudate that is pathognomonic for jaagsiekte.

The tumour cells contained a fair amount of rough endoplasmic reticulum and a large number of free polysomes. The Golgi apparatus was usually well developed and associated with many membrane-bound vesicles. Hypertrophic and degenerating mitochondria were observed as well as elongated mitochondria and mitochondria containing membrane whorls. Glycogen granules were only observed on rare occasions, but were not a characteristic of all tumour cells. In one tumour cell a cytoplasmic inclusion of electron dense material was seen (Fig. 11).

Most tumour cells had large numbers of filaments, sometimes in bundles, running through the cytoplasm (Fig. 12). Centrioles found in some tumour cells indicated a state of mitosis. An unusual finding was a single centriole that resembled early cilium formation at the plasma membrane (Fig. 13). This phenomenon was observed on 4 occasions in 3 different lung samples. It was not seen in lung samples of normal sheep. Wandera & Krauss (1971) observed ciliated cells in 1 of 8 jaagsiekte lung tumours examined electron microscopically. However, they did not state whether these ciliated cells had tumour cell characteristics. Cutlip & Young (1982) observed ciliated cells amongst tumour cells, but regarded them as remnants of the original pulmonary epithelium.

Cytoplasmic clefts were observed in some tumour cells. These may have represented a preparation artifact, as they were also present in some granular pneumocytes of the normal lung.

Tumour cell nuclei

In the columnar tumour cells the nucleus was always situated towards the base of the cell. In cuboidal tumour cells the nuclei were large and centrally located with prominent nucleoli and peripherally located chromatin (Fig. 8). Some nuclei were convoluted, whereas others showed no abnormalities. Margination of the nucleoli was often observed (Fig. 5 & 8).

The endothelium and the interstitial space

In some lungs there was an increase in the amount of collagen in the interstitium as a result of progressive fibroplasia (Tustin, 1969). This was not pronounced in most of the lungs examined, because the disease was acute in most of the experimental cases. Fibroblasts as well as various cells of the immune systems (see below) were observed in the interstitial space. The endothelium did not seem to be affected by the disease.

Cells of the immune system

An important characteristic of jaagsiekte lesions is the large number of macrophages present in the alveolar lumen, despite the lungs having been washed before fixation. The macrophages are usually found in normal and adenomatous areas of jaagsiekte lungs. Some macrophages, however, appeared to be attached to the surface of transformed or normal cells (Fig. 14), while the majority formed large clusters in the alveolar lumen (Fig. 15).

The large size of the macrophages as well as their highly ruffled surface indicated that they were in an activated state. TEM of macrophages revealed that the cytoplasm contained many lysosomes, phagolysosomes, abundant organelles and myelin bodies (Fig. 16). On rare occasions JSRV particles were observed being phagocytosed by macrophages (Fig. 17). Bacteria and mycoplasma-like organisms were also observed being phagocytosed by macrophages.

Plasma cells were often observed in the interstitial space in close association with lymphocytes. In cases where there was an inflammatory reaction, neutrophils were seen in the alveolar lumen, between tumour cells, and in the interstitial space. Although monocytes were observed in the lesions, they were comparatively rare compared with the other types of immune cells.

[1] It should be noted that all lesions came from animals in terminal stages of jaagsiekte and that the terms "early" and "advanced" refer to the size of the lesion and not the disease status of the animal

JSRV in jaagsiekte lungs

JSRV was observed in only 11 of the lungs examined, despite the fact that virus could be isolated from all the lung washes (Verwoerd *et al.*, 1983). Intracellular particles were found within the tumour cells in 7 of these lungs. These particles were round and electron dense (Fig. 18) and sometimes found near centrioles. Extracellular virus particles were also found in 7 jaagsiekte lungs (Fig. 19). This figure is not a true reflection of the number of lungs containing extracellular particles, as most of the lungs had been rinsed with MEM. A detailed description of the morphology and morphogenesis of JSRV in jaagsiekte lungs is given elsewhere (Payne *et al.*, 1983).

Discussion

Jaagsiekte can be transmitted by means of the transplantation of whole cells (Verwoerd, De Villiers & Tustin, 1980a) or by infection with jaagsiekte retrovirus (Verwoerd *et al.*, 1980b; Verwoerd & Williamson, 1982; Herring *et al.*, 1983). In the present study it was possible to locate pairs of transformed cells in very early lesions. These cells appeared to proliferate first, forming a single layer lining the alveolar lumen and developing into grape-like clusters. These clusters proliferated further to fill the alveolar space and spread to other parts of the lung. The SEM study of jaagsiekte lesions clearly showed that tumour cells proliferated and spread from one alveolus to the other. No matter how small the alveolar lumen, there always appeared to be a free tumour cell surface covered in microvilli.

The tumour cells on the periphery of the lesions appeared to be well differentiated and usually contained secretory granules resembling those found in normal sheep granular pneumocytes. However, there was much more osmiophilic substance in the tumour granules, and some contained myelinoid bodies that were not present in granular pneumocytes of normal sheep. The content of the secretory granules may be related to the rate of surfactant production. The myelin whorls observed within secretory granules and the extracellular tubular myelin are considered to be surfactant reserves in the lungs (Massaro, 1981). The surfactant reserves are much lower in lungs of normal sheep than observed in most jaagsiekte tumours. This is probably due to the great increase in the number of cells producing surfactant in the diseased sheep, and also explains the large amount of lung exudate that is pathognomonic of the disease. It is interesting to note that the granular pneumocytes in foetal lamb lungs contain more osmiophilic substance than mature granular pneumocytes (Kikkawa *et al.*, 1965). This implies that the tumour cells resemble the embryonal stages of the granular pneumocyte, which is an indication of malignancy (Ghadially, 1980).

The high incidence of free polysomes and nucleolar margination seen in the tumour cells indicate that they were in a state of rapid protein synthesis and cell division (Ghadially, 1980). The presence of centrioles in some tumour cells was another indication of mitosis. Intracytoplasmic JSRV was often found in close association with centrioles, indicating that the virus replicates under conditions of cell division (Payne *et al.*, 1983). As in other malignant cells, the cells had abnormal nuclei and the mitochondria were abnormal and fragile. Perk *et al.* (1971) a well as Wandera & Krauss (1971) reported abnormal mitochondria, while Nisbet *et al.* (1971) reported no change.

The majority of the tumour cells were found in alveoli, indicating that the granular pneumocyte is the major site of transformation in experimentally induced jaagsiekte. However, some bronchiolar proliferations were observed by means of SEM. In most lesions the bronchiolar tumour cells seemed to be spreading to or from the adjacent alveoli. It is not clear, however, if the tumour cells originated in the alveolus or the bronchioles.

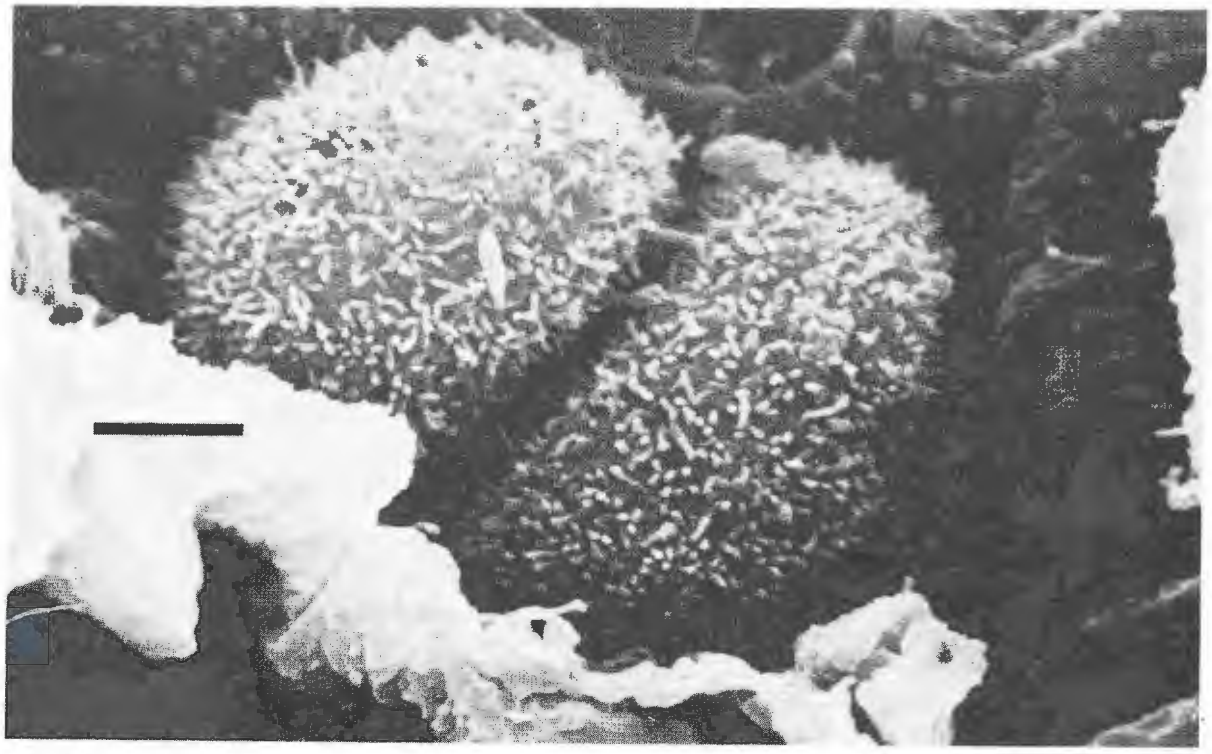

FIG. 3 A couple of tumour cells in a corner of an alveolus. Bar = 1 μm

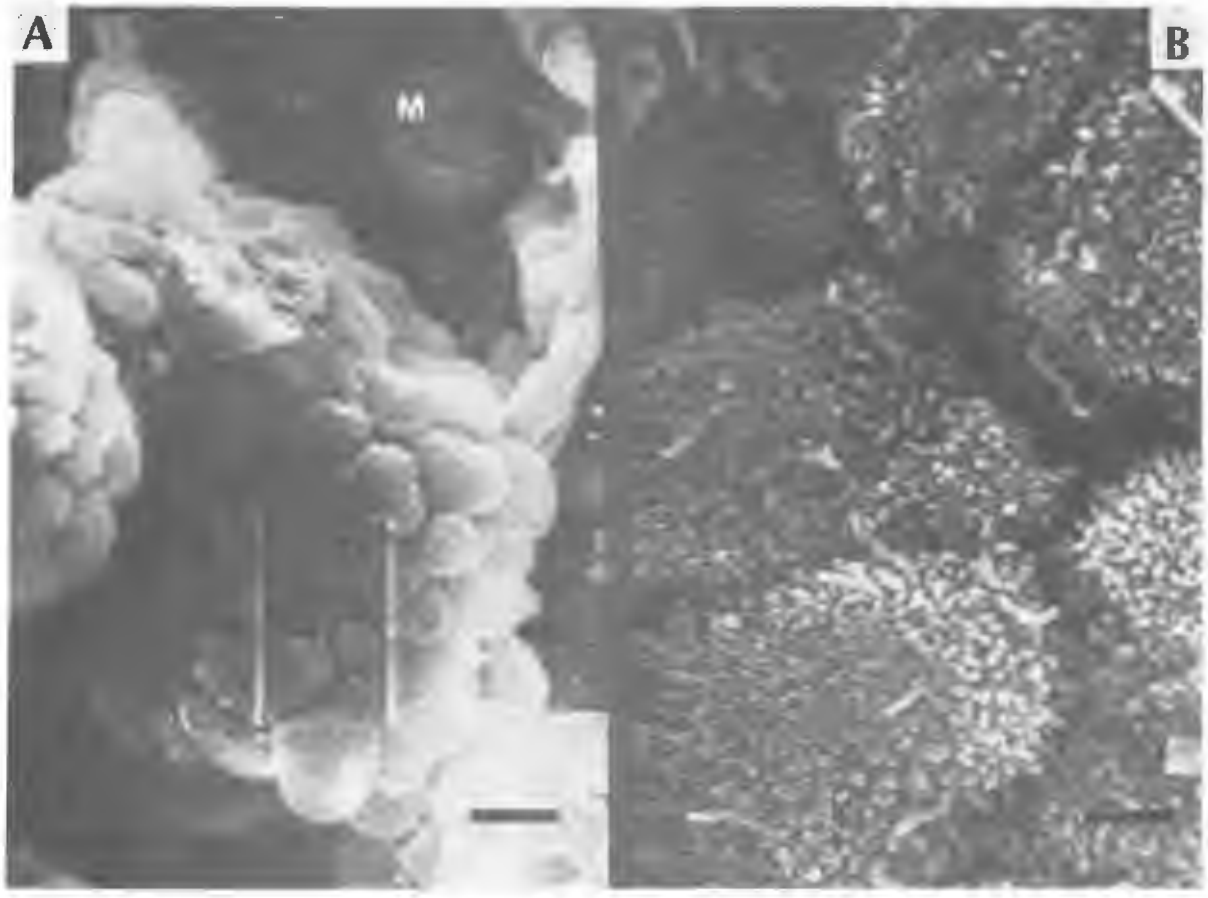

FIG. 4a Tumour cells lining an alveolus. Note the macrophage (M) in the adjacent alveolus. Bar = 7,5 μm
4b Higher magnification to show the abundant microvilli on the surface of the tumour cells. Bar = 1,5 μm

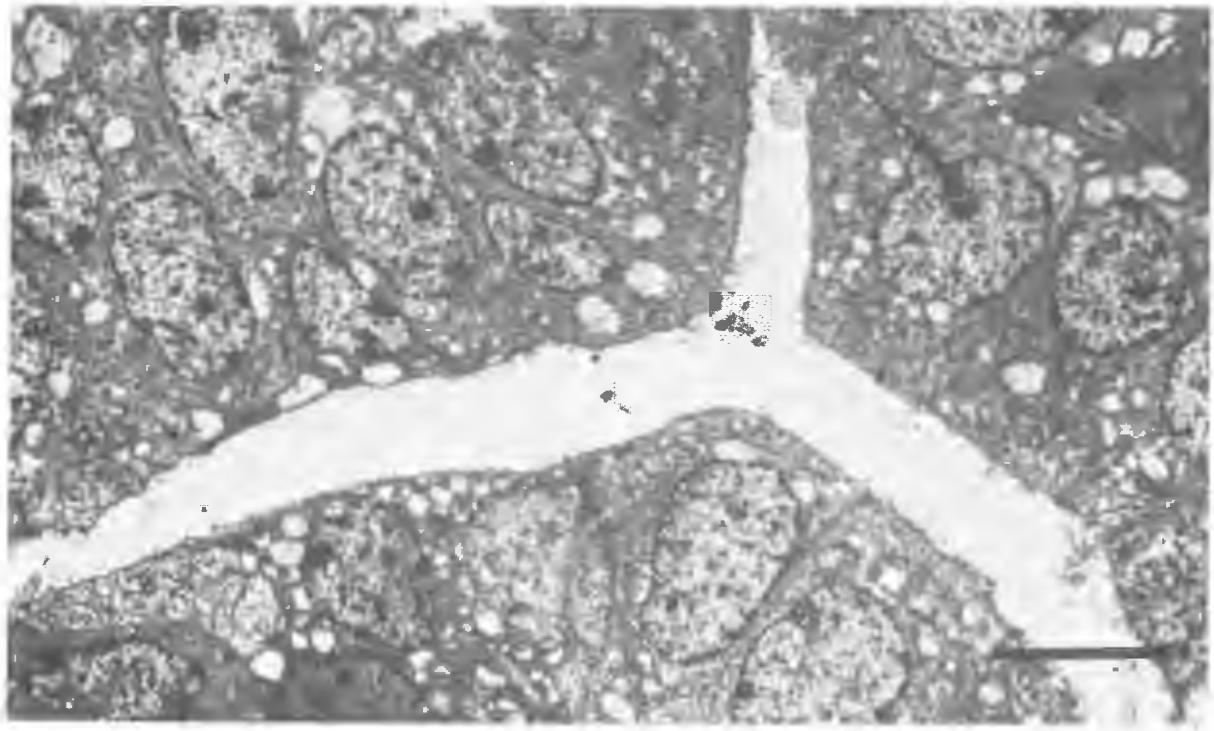

FIG. 5 Tumour cells lining the alveolar lumen. Note the marginated nucleoli (arrow). Bar = 5 μm

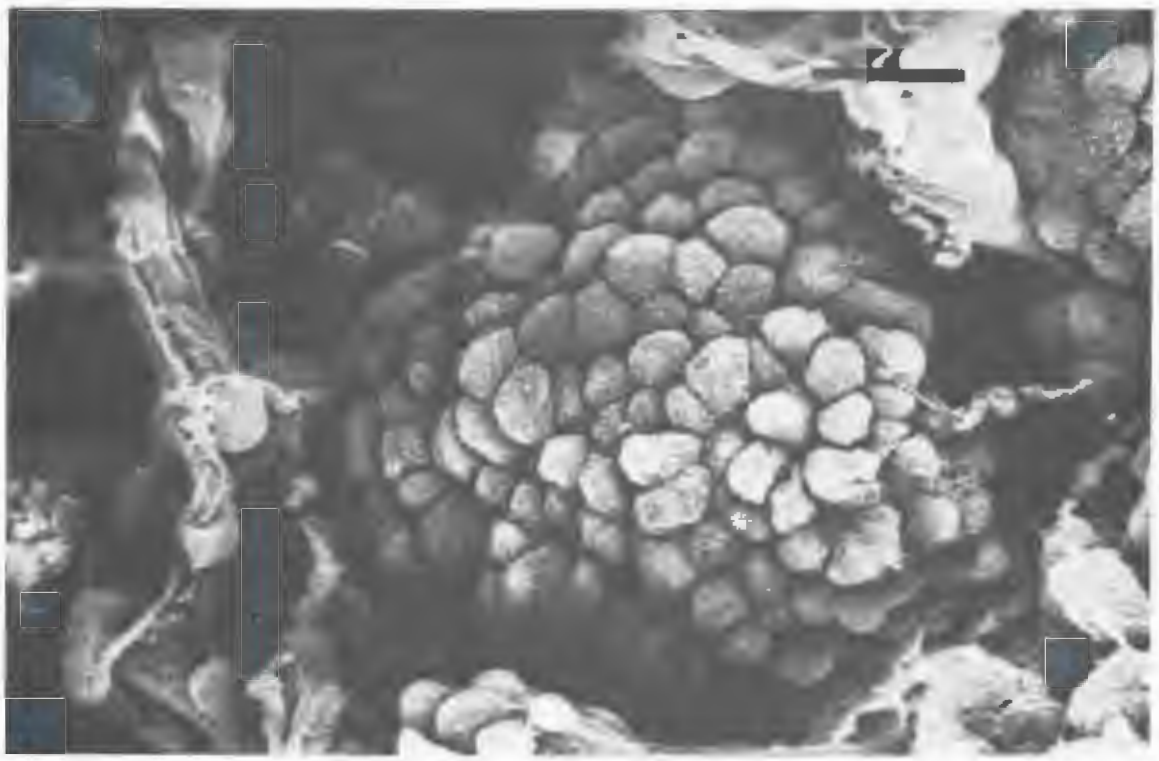

FIG. 6 A large cluster of tumour cells. Bar = 15 μm

FIG. 7 An alveolus filled with tumour cells. Bar = 6 μm

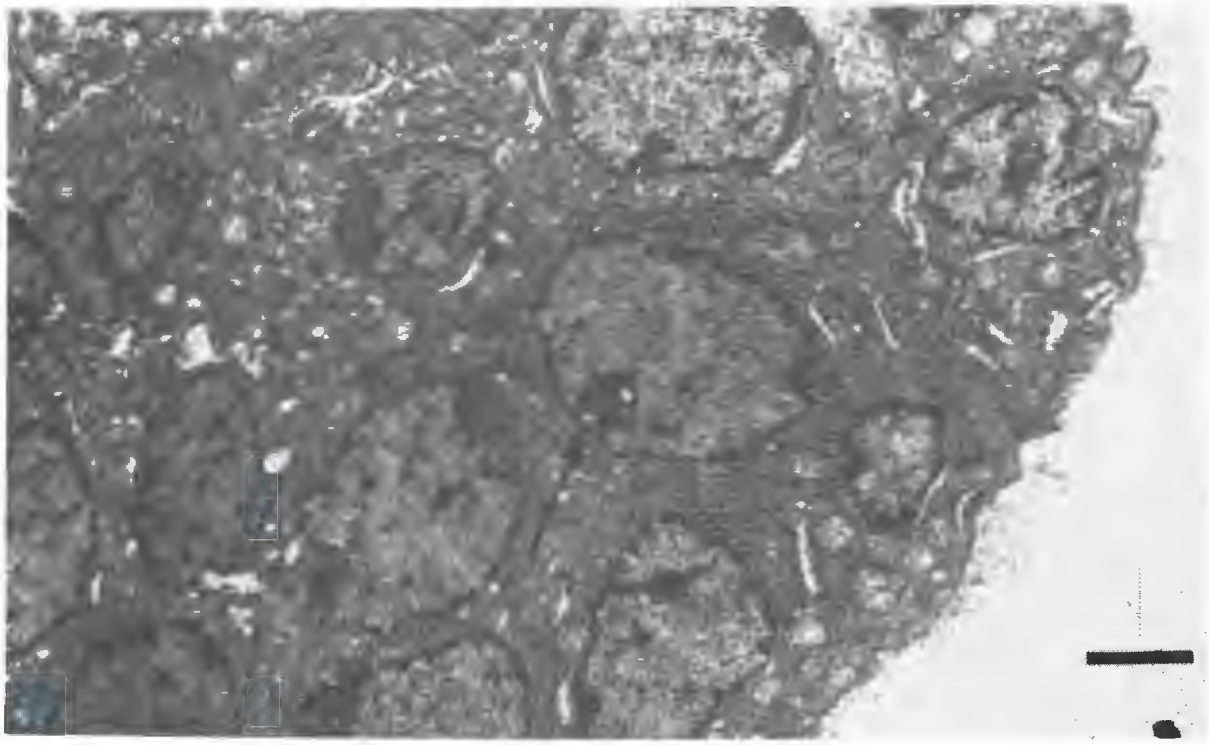

FIG. 8 Sheet of jaagsiekte tumour cells. Note the marginated nucleoli (arrow). Bar = 2 μm

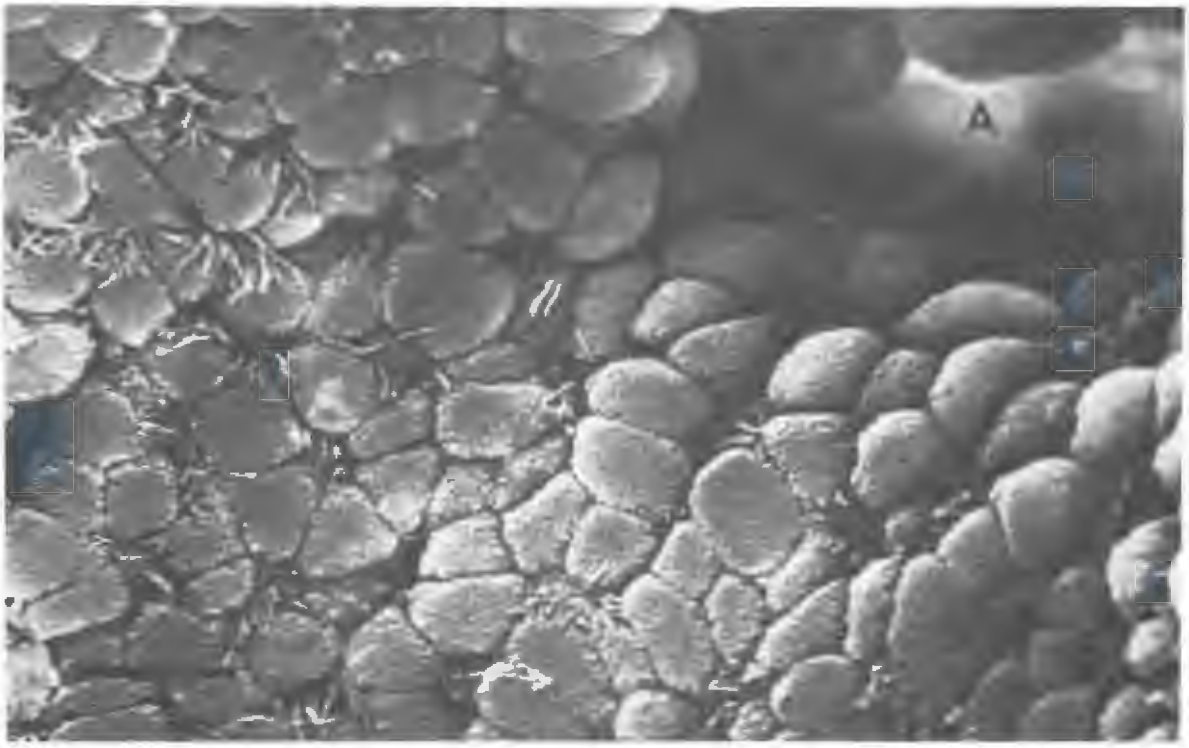

FIG. 9 Tumour cells in a respiratory bronchiolus. Note the adjacent alveolus (A) and the cilia (arrow) from the underlying ciliated epithelial cells. Bar = 5 μm

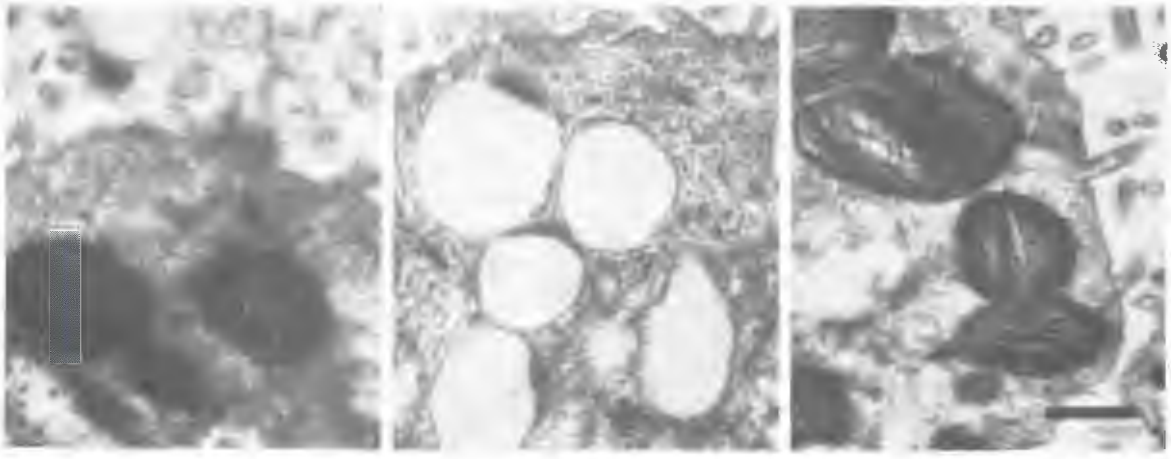

FIG. 10 A comparison of the different forms of secretory granules found in jaagsiekte tumour cells. Bar = 1 μm

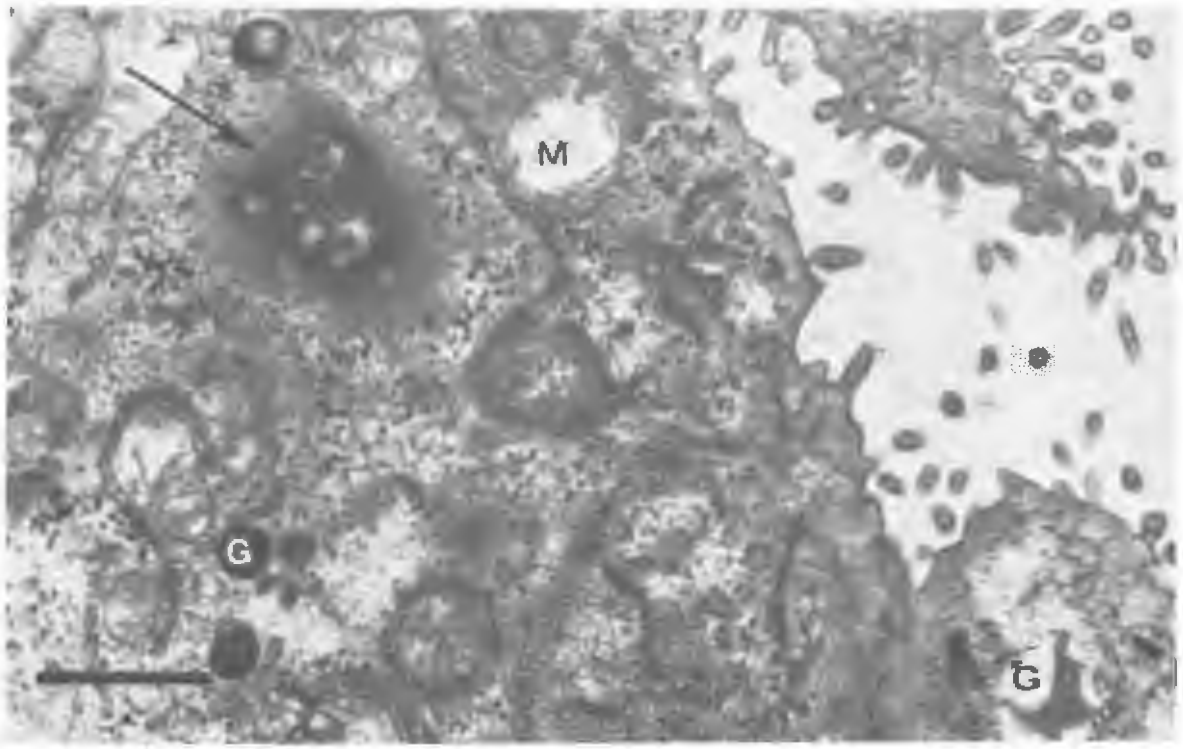

FIG. 11 Cytoplasmic inclusion (arrow) in a jaagsiekte tumour cell. Note the degenerating mitochondria (M) and the secretory granules (G). Bar = 1 μm

FIG. 12 Filaments and bundle of filaments which are characteristic of jaagsiekte tumour cells. Note the desmosome (arrow). Bar = 200 nm

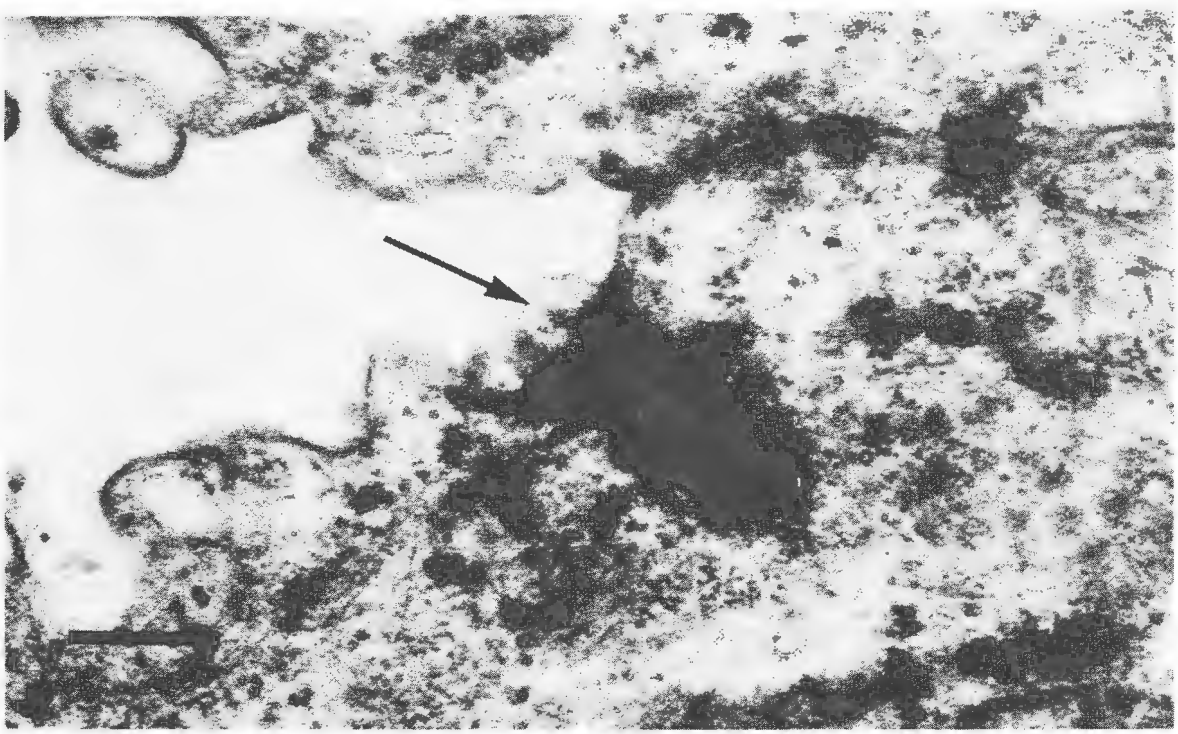

FIG. 13 Attempted cilium formation from a tumour cell (arrow). Bar = 300 nm

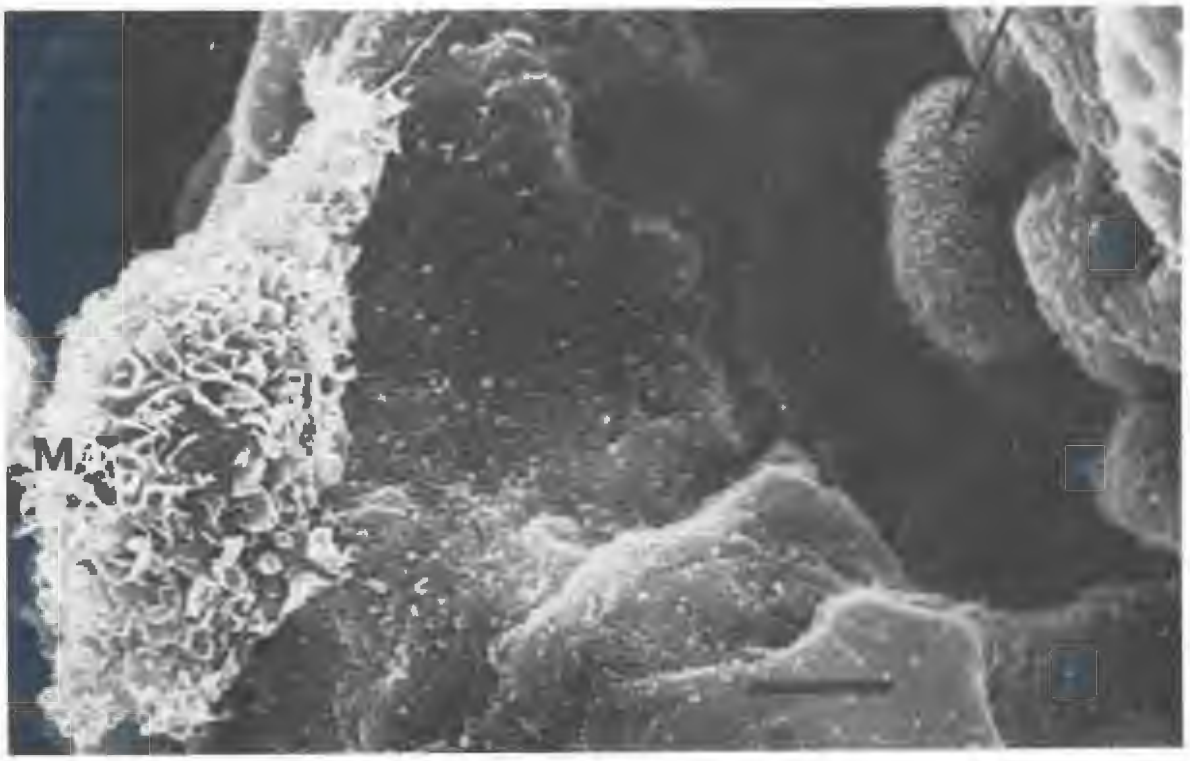

FIG. 14 Large ruffled macrophage (M) in close proximity to some tumour cells (arrow). Bar = 2,5 μm

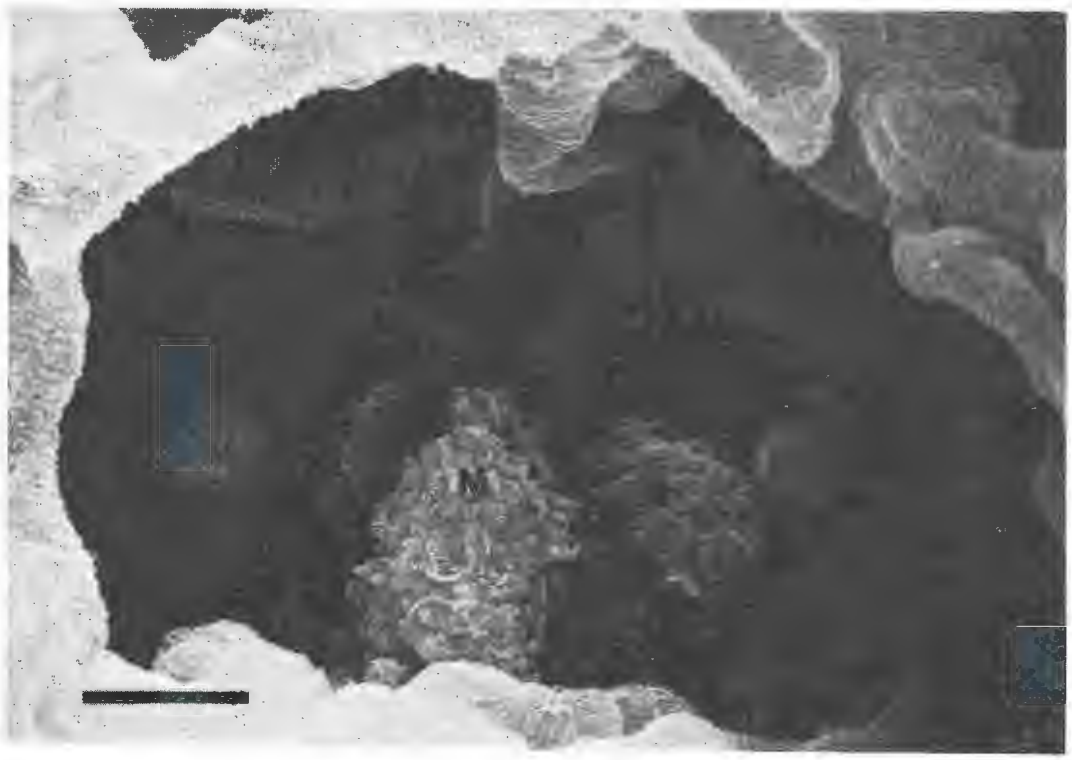

FIG. 15 Cluster of macrophages (M) in a jaagsiekte lung. Bar = 5 μm

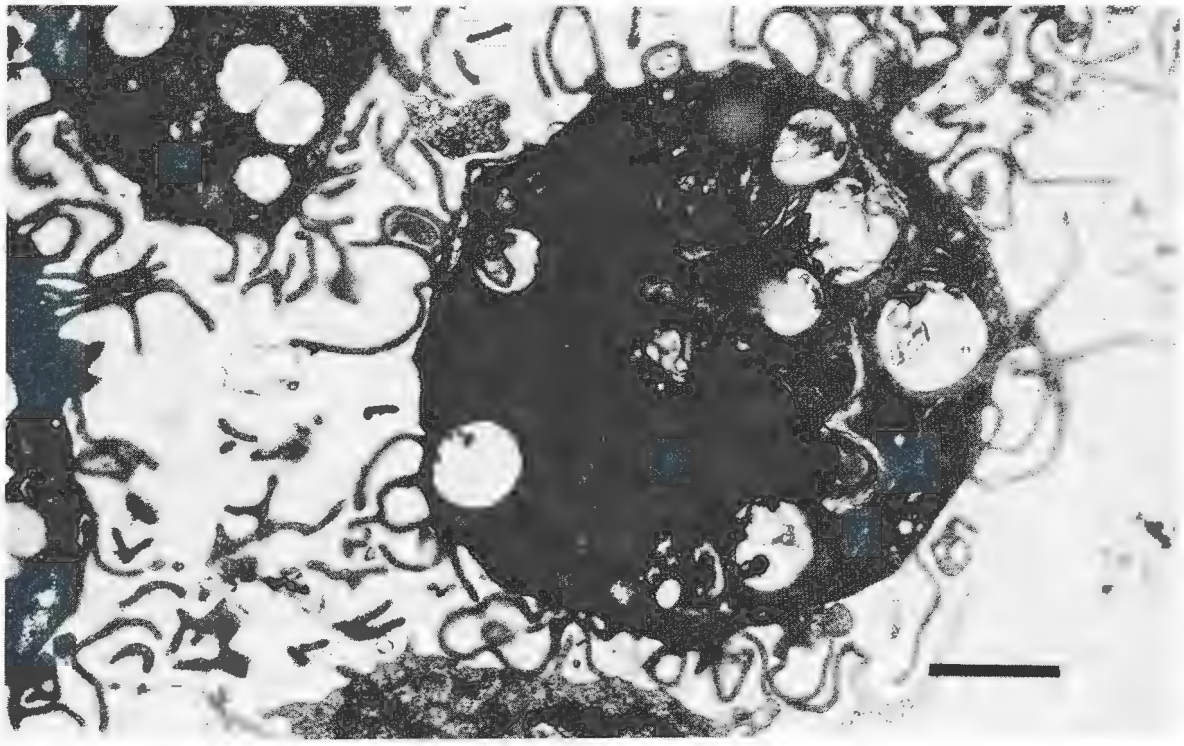

FIG. 16 Macrophage from a jaagsiekte lung. Bar = 2 μm

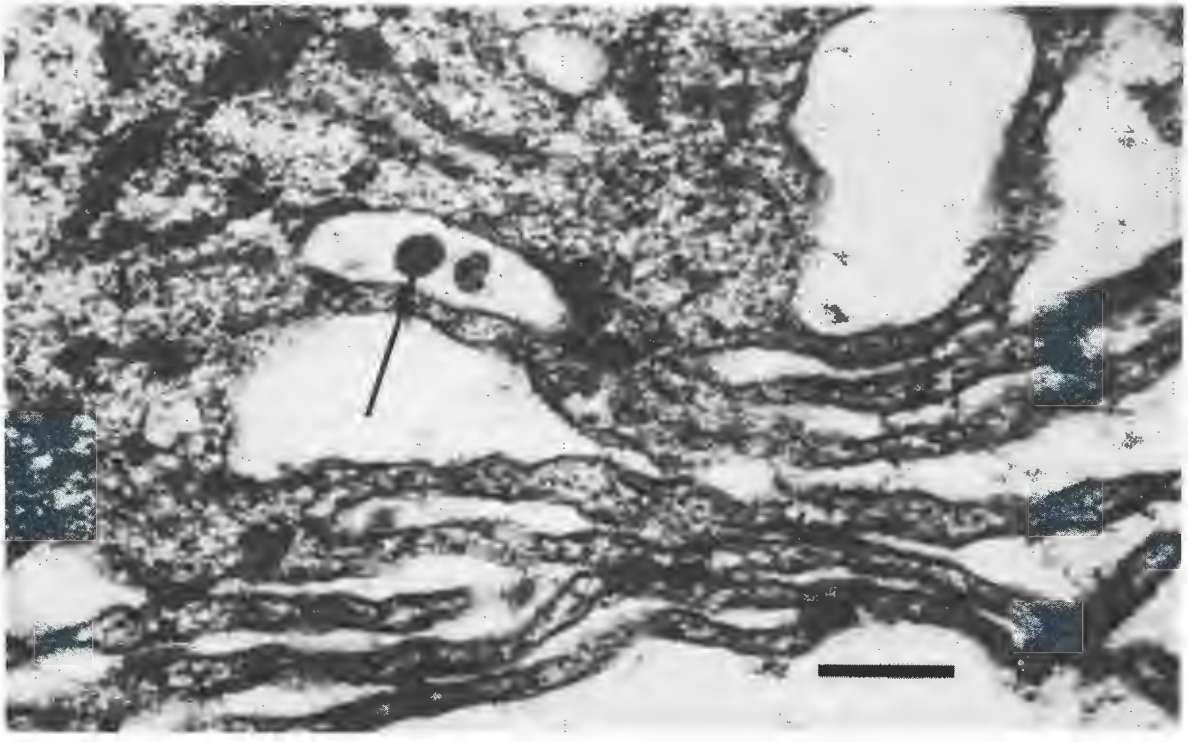

FIG. 17 JSRV (arrow) being phagocytosed by a macrophage. Bar = 300 nm

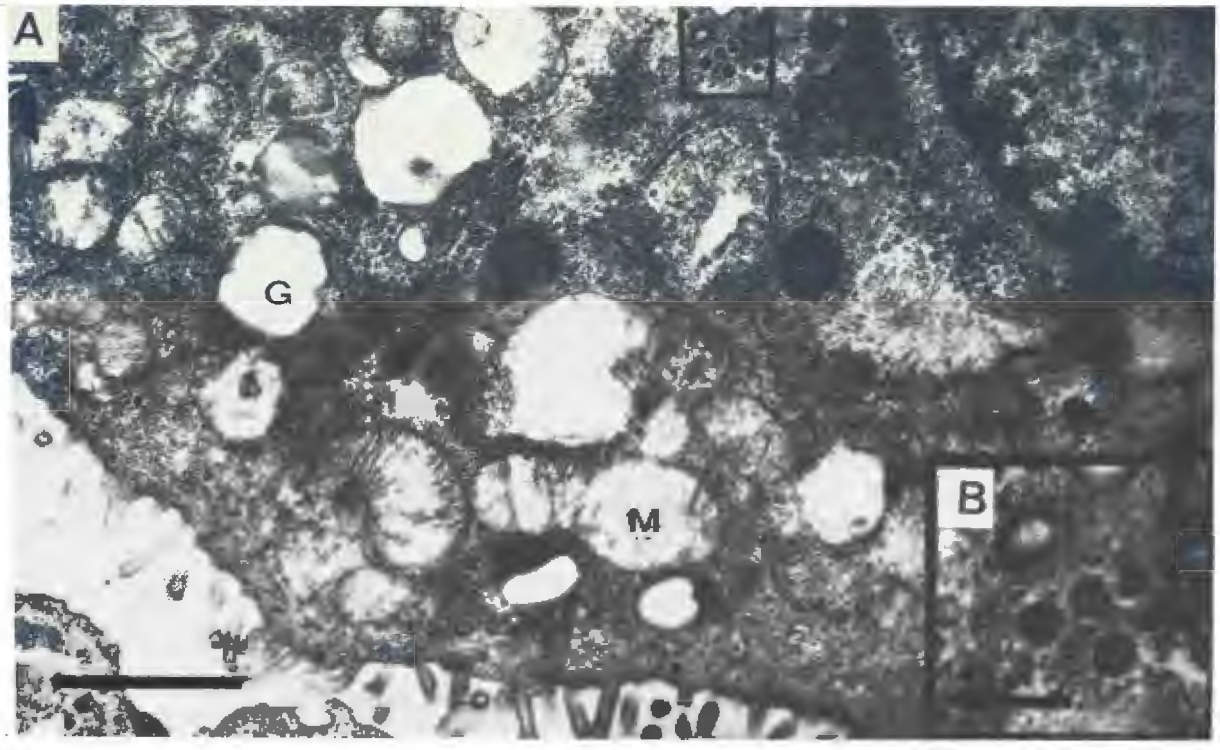

FIG. 18a Intracytoplasmic JSRV (box) in jaagsiekte tumour cells. Note the degenerating mitochondria (M) and the secretory granules (G). Bar = 1 μm
18b Higher magnification of the intracytoplasmic JSRV particles. Bar = 200 nm

FIG. 19 Extracellular JSRV particles in jaagsiekte lung alveolus. Bar = 200 nm

Nisbet *et al.* (1971) concluded that bronchiolar proliferations in jaagsiekte were derived from Clara cells, but little evidence was provided to support their conclusion. In seems reasonable that transformed Clara cells and transformed granular pneumocytes should be morphologically alike, as they have a common stem cell (Nagaishi, Okada, Daido, Genka, Ikeda & Kitano, 1965). These authors postulated that, in the process of carcinogenesis, young undifferentiated cells proliferate neoplastically and that totipotent primitive epithelial cells may produce tumour cells which take the form of any of the epithelial cells of the bronchiolo-alveolar system. This suggestion may explain the attempted cilium formation by non-ciliated cells: Attempted cilium formation was also reported in human bronchiolo-alveolar carcinomas (Greenberg, Smith & Spjut, 1975). As jaagsiekte proliferations are found to a greater extent in the alveoli (Tustin, 1969), it seems probable that the principal cell of origin is the granular pneumocyte. However, without following the development of the few bronchiolar proliferations, it will remain impossible to assess whether these lesions arise from transformed Clara cells or from a metastasis of transformed granular pneumocytes.

The most prevalent cell of the immune system found in jaagsiekte lungs was the alveolar macrophage. The role of the macrophage in the disease is uncertain, except for their role in removing micro-organisms, including JSRV, from the alveoli. In normal lungs, alveolar macrophages have a major role in the clearance of surfactant material (Hocking & Golde, 1979). It is therefore also possible that the increase in macrophages may be

due to the excess of surfactant in jaagsiekte lungs. The possibility that macrophages may be cytotoxic to tumour cells cannot be ruled out, as Hocking & Golde (1979) reported that macrophages are an important host defence mechanism against neoplasms that arise in the lower respiratory tract. Nathan, Murray & Cohn (1980) found that the macrophage content of a tumour tends to correlate inversely with their metastatic potential, which may explain the low incidence of metastasis in jaagsiekte.

Plasma cells found in the interstitial spaces of jaagsiekte lungs probably play an important role in the production of local immunoglobulins against JSRV. Verwoerd *et al.* (1983) reported that JSRV is mainly found in the form of immune complexes in the lung, but the site of immunoglobulin production against the virus has not yet been elucidated.

The ultrastructure of lesions from acute cases of experimentally induced jaagsiekte was similar to, but not identical with that described in previous studies of natural jaagsiekte cases (Perk *et al.*, 1971; Wandera & Krauss. 1971; Nisbet *et al.*, 1971; Hod *et al.*, 1977; Cutlip & Young, 1982). One of the differences was in the amount of glycogen observed in the tumour cells. Nisbet *et al.* (1971) also found that only a small proportion of the tumour cells contained glycogen. However, Perk *et al.* (1971), Wandera & Krauss (1971) and Cutlip & Young (1982) reported that many of their tumour cells contained large amounts of glycogen. Furthermore, we observed less fibroplasia in the acute experimental lesions than in natural cases which are more chronic in nature. Finally, the Israeli researchers observed virus particles in a number of natural jaagsiekte cases (Hod, Perk, Nobel & Klopfer 1972; Perk *et al.*, 1971). These particles differed morphologically from those particles observed in experimentally induced jaagsiekte cases in South Africa (Payne *et al.*, 1983).

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