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THE HISTOPATHOLOGY AND ULTRASTRUCTURE OF PLEURAL MESOTHELIOMAS PRODUCED IN THE RAT BY INJECTIONS OF CROCIDOLITE ASBESTOS

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Summary.—Primary tumours of the pleural cavity were produced in rats by the intrapleural injection of crocidolite asbestos. Their histological structure as seen with both light and electron microscopy was very variable and tumours frequently contained elements of both connective-tissue and epithelial type. In some instances the connective-tissue elements predominated from the start and the earliest tumour nodules consisted mainly of pleomorphic connective-tissue cells with only a few layers of cells more nearly epithelial in type on the surface. This pattern was largely retained when tumour nodules increased in size and coalesced, but in the deeper layers of advanced tumours the pleomorphic connective-tissue pattern was often replaced by a more uniform spindle-cell form. Other tumours were more predominantly epithelial in type, showing either a papillary pattern with rounded epithelial cells growing in solid columns, or a vesicular form in which large tissue spaces, often intracellular, were lined by very thin layers of extended cell cytoplasm. Whereas early tumours showed only one histological pattern, the more advanced stages often exhibited areas of all 3, so that there seemed to be some degree of histological mutability. The spindle-cell areas of advanced tumours frequently showed evidence of direct invasion of the surrounding tissue but this was never seen with the epithelial forms of rat mesothelioma.

MESOTHELIOMAS of both the pleural and peritoneal cavities have been associated with exposure to asbestos dust since Wagner's report from South Africa in 1960 (Wagner, Sleggs and Marchand, 1960). Wagner's cases came from the mining population in areas where blue asbestos or crocidolite was produced, but cases have now been reported from the asbestos-processing industries of many industrial nations, although the tumours remain extremely rare. It has proved difficult to isolate working populations exposed to only one type of asbestos dust during their working lives, but evidence is now accumulating that, in addition to crocidolite, both amosite and chrysotile can be responsible for the development of mesotheliomas (Selikoff, Hammond and Seidman, 1973; McDonald, 1973).

In animal experimental studies using the artificial technique of intrapleural or intraperitoneal injection, all asbestos types will produce a high percentage of mesotheliomas (Wagner and Berry, 1969; Wagner, Berry and Timbrell, 1973) but in inhalation experiments only occasional mesotheliomas have been reported (Gross and de Treville, 1967; Wagner *et al.*, 1974). However, mesothelial tumours have now been shown to develop in experimental animals following inhalation with all the main asbestos types. Mesotheliomas have been characterized by many workers as having a very variable histological structure, sometimes epithelial, sometimes of connective-tissue type and sometimes both (Hourihane, 1964; Churg, Rosen and Moolten, 1965). This variation led some authorities to conclude that most reported

human cases are in fact secondary deposits of unrecognised primary carcinomas (Robertson, 1924; Willis, 1967).

Since animal mesotheliomas show the same variation in structure as their human counterparts, it was considered that electron microscope examination of the early stages of tumour development might help to explain the histological differences. The structure of peritoneal mesotheliomas produced in rats was the subject of an earlier publication (Davis, 1974). The structure and development of the more variable pleural mesotheliomas forms the material for the present report.

MATERIALS AND METHODS

Forty-eight white Han rats were given an intrapleural injection of 25 mg of crocidolite asbestos suspended in sterile saline. The crocidolite was that distributed by UICC (Timbrell, 1969). At intervals from 12 to 20 months after dust injection the animals were killed and both definite tumours and possible early lesions from the pleural cavity were fixed for detailed examination. For light microscope studies, tissues were fixed in formol saline and sections stained with haematoxylin and eosin, van Gieson's method for collagen or Gordon Sweet's stain for reticulin. For electron microscope examination specimens were fixed in buffered osmium tetroxide, embedded in Araldite, and thin sections were stained with both lead citrate and uranyl acetate.

RESULTS

Tumours, including very early growths confirmed only after histological sectioning, developed in 16 animals (33%) of those injected. The earliest microscopic signs of development of pleural mesotheliomas in the rats examined consisted of multiple white nodules 0·25–0·5 mm in diameter, scattered over both visceral and parietal surfaces. Some were situated close to and even in contact with the original asbestos granulomas but, once they were visible with the naked eye at least, there was no obvious tumour dissemination from granulomatous areas. In more advanced stages, some tumour nodules increased in size up to 3–5 mm in diameter and some nodule fusion occurred to produce sheets of solid tumour. This appeared to occur more frequently on the parietal pleural surface than the visceral. In 3 cases the tumour was confined to a single nodule which reached 7–11 mm in diameter.

The multiple mesotheliomas exhibited 3 distinct histological patterns and these were discernible in the earliest lesions. In one variety the early nodules consisted of several layers of rounded epithelial cells on the surface, with spindle-shaped cells of connective type in the centre (Fig. 1). This pattern remained constant as the size of the nodules increased but with the largest nodules the central regions often became fibrosed and acellular. When this type of nodule fused to form a sheet of tumour growth, the surface layers remained epithelial while those below were of spindle-cell type. In the second type, the tumour pattern was papillary with cells of rounded epithelial type growing in solid columns. Very early papillomatous growth on the visceral pleural surface is shown in Fig. 2 and the more advanced pattern in Fig. 4. The third histological variety took the form of a vesicular growth in which multiple tissue spaces were lined with greatly extended and flattened epithelial cells supported by the minimum of connective tissue. Initially this type of growth appeared as a variety of the papillary pattern but with large tissue spaces visible from the first (Fig. 3). In more advanced cases, however, solid masses of tumour were produced showing only the vesicular pattern (Fig. 5).

All small tumour nodules examined, below 1 mm in diameter, showed only one histological variety but larger nodules and areas of confluent tumour frequently showed more than one pattern in close connection. The 3 solitary tumours were an exception: these all consisted entirely of spindle cells and appeared very similar to benign fibromas (Fig. 6). The epithelial varieties of mesotheliomas examined during this study showed no tendency to metastasize but, with the exception of the 3 solitary tumours, areas of spindle-cell growth frequently invaded the muscle

FIG. 1.—An early tumour nodule between folds in the visceral pleural surface of the lung of a rat injected with crocidolite asbestos. The nodule consists mainly of irregularly shaped connective-tissue cells. × 230.

FIG. 2.—A very small papillomatous growth developing on the visceral pleural surface of a rat injected with crocidolite asbestos. The cells are of rounded epithelial type and appear to have developed as modifications of the normal flattened mesothelial surface layer. × 375.

FIG. 3.—A group of small papillomatous growths found on the visceral pleural surface of a crocidolite-treated rat. Within the columns of rounded epithelial cells, large tissue spaces are present. × 230.

FIG. 4.—A papillomatous growth from the pleural cavity of a rat with an advanced mesothelioma. The cell columns consist of cells of rounded epithelial type. × 85.

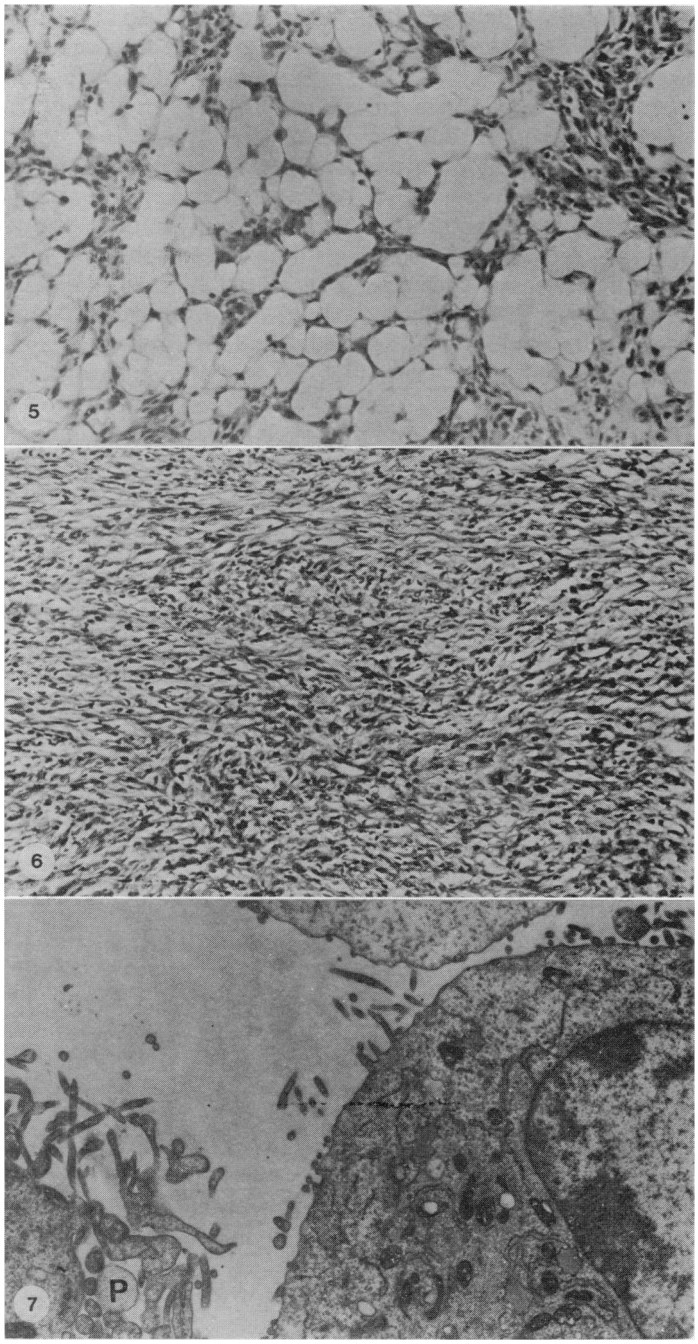

FIG. 5.—Part of a solid tumour mass from the pleural cavity of a rat treated with crocidolite asbestos. Many large tissue spaces are present surrounded by thin layers of extended cell cytoplasm. × 90.

FIG. 6.—An area of spindle-shaped cells from a solitary tumour nodule found within the pleural cavity of an asbestos-treated rat. × 90.

FIG. 7.—Electron micrograph of parts of rounded cells from the surface of a very early tumour nodule. Elongated surface processes are present on all cells, many cylindrical but others globular and pseudopodium-like (P). × 9000.

coating of the body wall. However, no sign of direct invasion of lung tissue was found in any of these cases.

The first histological pattern appeared identical to that already described for rat peritoneal mesothelioma (Davis, 1974) and this was confirmed by electron microscopy. The cells on the surface of tumour nodules or sheets were large, rounded and loosely arranged. There were very few desmosomes on the cell-surface membrane which were covered with elongated irregular projections (Fig. 7). Sometimes these projections were like microvilli but on other occasions they were globular and similar to very small pseudopodia. Within the cell cytoplasm lipid droplets were usually present and granulated endoplasmic reticulum was present in large quantities although usually in very short lengths. In the deeper layers of the earliest nodules cells were irregular in shape and were arranged in a loose network of reticulin fibres. The surface membrane showed only a few processes but those present were often as much as 10 μm in length. Lipid droplets were still commonly found within the cytoplasm and the granular endoplasmic reticulum was very well developed with elongated and branching sacs, frequently dilated and filled with amorphous material (Fig. 8).

In the central regions of more advanced nodules and in areas of invasion, the cells were more closely packed and spindle-shaped. However, the cell cytoplasm still contained large amounts of dilated and branching endoplasmic reticulum (Fig. 9). Surface processes were much less common at this stage of tumour development but occasional elongated processes were still present, usually compressed against the cell-surface membrane.

In mesotheliomas of the papillary type, the cells growing in columns were large and rounded but usually firmly attached to each other by desmosomes. The free cell surfaces were covered with microvilli but these were much more regular in structure than those seen on the surface cells of the first tumour pattern described. They could vary from 0·5 to 2·0 μm in length but almost always appeared cylindrical, with a diameter of approximately 1,000 Å (Fig. 10). Where membranes were not held closely together with desmosomes, some microvilli could persist between the cells although they were usually compressed. Although the papillary cell columns were usually solid, at some points occasional tissue spaces occurred. Often these were intercellular, although some cells had become flattened and extended to form a lining. In other instances, however, spaces were intracellular although they could be as much as 10–15 μm in diameter. The cell membrane lining both intercellular and intracellular cell spaces was surfaced with numerous microvilli. Within the cell cytoplasm of all cells making up the papillomatous type of growth, the endoplasmic reticulum was well developed, but was more commonly present as short lengths than as large branching sacs and was rarely seen in a distended condition. The Golgi apparatus was distinct and often several were present within each cell surrounded by large numbers of small, smooth, spherical membranous vesicles (Fig. 11).

In the vesicular tumours, the size of the tumour spaces and thickness of their epithelium linings varied greatly. In some cases the spaces were small enough to be contained within the normal diameter of a single cell and the thick cytoplasmic layer surrounding the space could exhibit all the normal cytoplasmic organelles, which were similar in type, size and distribution to those of the epithelial cells from papillary tumours (Fig. 12). In these cases the plasma membrane adjacent to the space was usually covered with elongated microvilli. In many cases, however, tissue spaces were as much as 50 μm in diameter and spaces of this size were lined with extremely thin layers of extended cytoplasm often only 1000 Å thick. These extremely thin layers contained no cell organelles and frequently there were no microvilli so that the spaces had smooth surface lining over large areas.

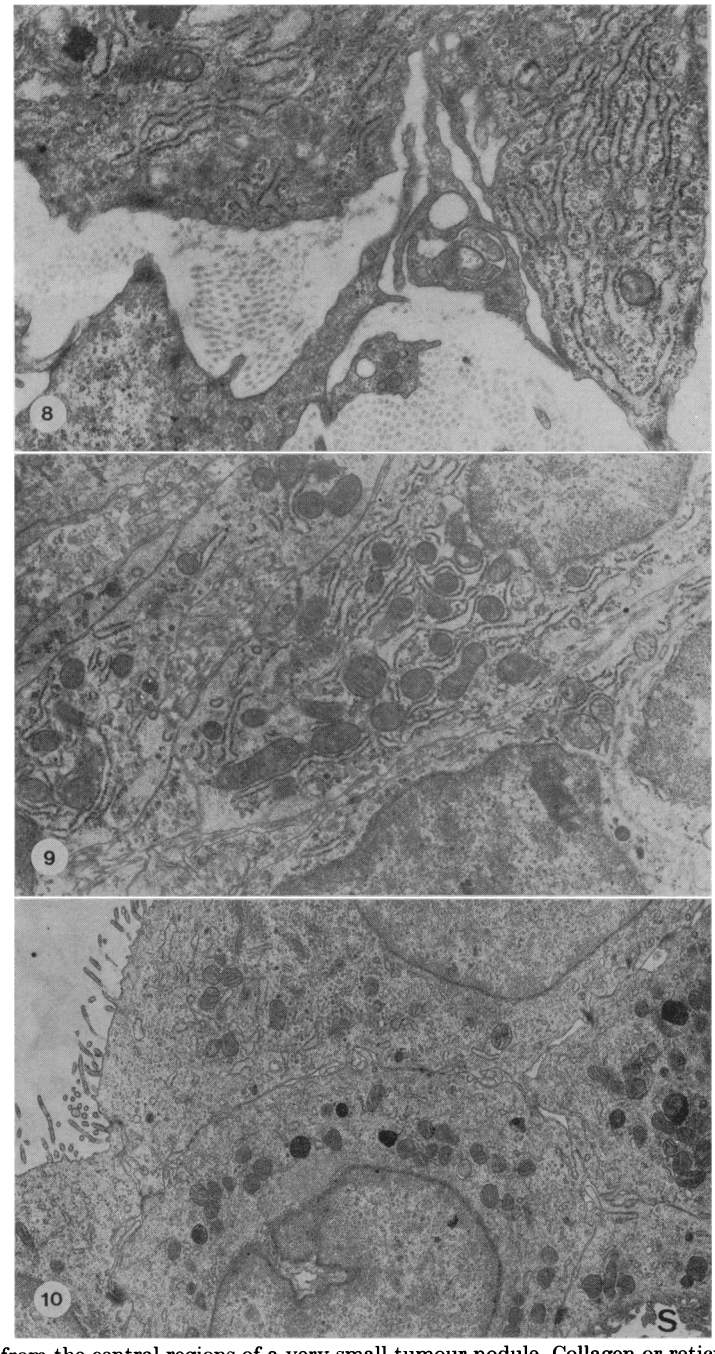

FIG. 8.—Cells from the central regions of a very small tumour nodule. Collagen or reticulin is present between the cells, the surface membranes of which are modified to form long irregular processes. × 22,800.

FIG. 9.—A group of closely packed spindle-shaped cells. No surface processes are present on the cell membranes, although the cells are separated in some places by small amounts of collagen or reticulin fibres. × 21,000.

FIG. 10.—A group of rounded cells from a papillomatous mesothelioma. A few microvilli are present between the closely packed cells, but these processes are numerous on free cell surfaces or lining intracellular spaces (S). × 7200.

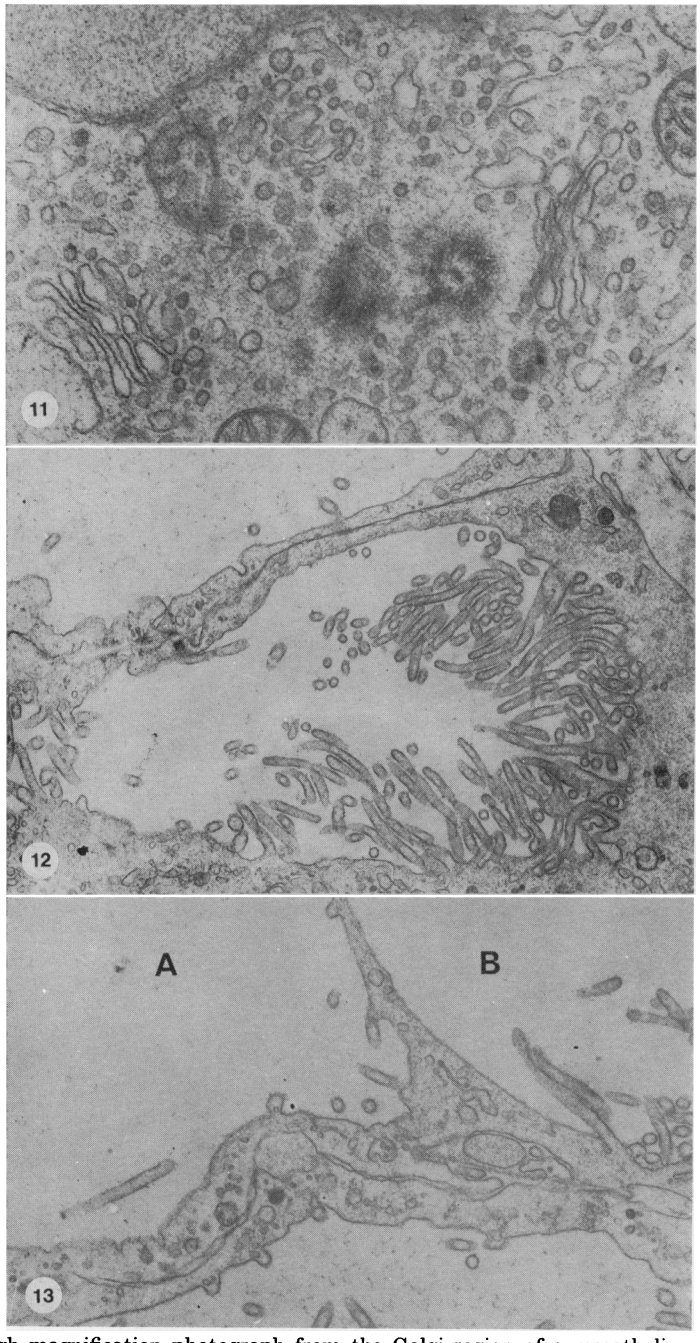

FIG. 11.—A high magnification photograph from the Golgi region of a mesothelioma cell. Several groups of Golgi membranes are present as well as large numbers of smooth-membraned vesicles. × 39,000.

FIG. 12.—Intracellular spaces within the cytoplasm of 2 mesothelioma cells. In some areas the cytoplasmic lining of the spaces is thick and contains normal cell organelles. In other areas it forms a layer with a depth of only about 0·2 μm. × 21,000.

FIG. 13.—Intracellular spaces within the cytoplasm of mesothelioma cells. Spaces labelled A and B are within the same cell and separated by a cytoplasmic layer only 0·1 μm thick. × 25,200.

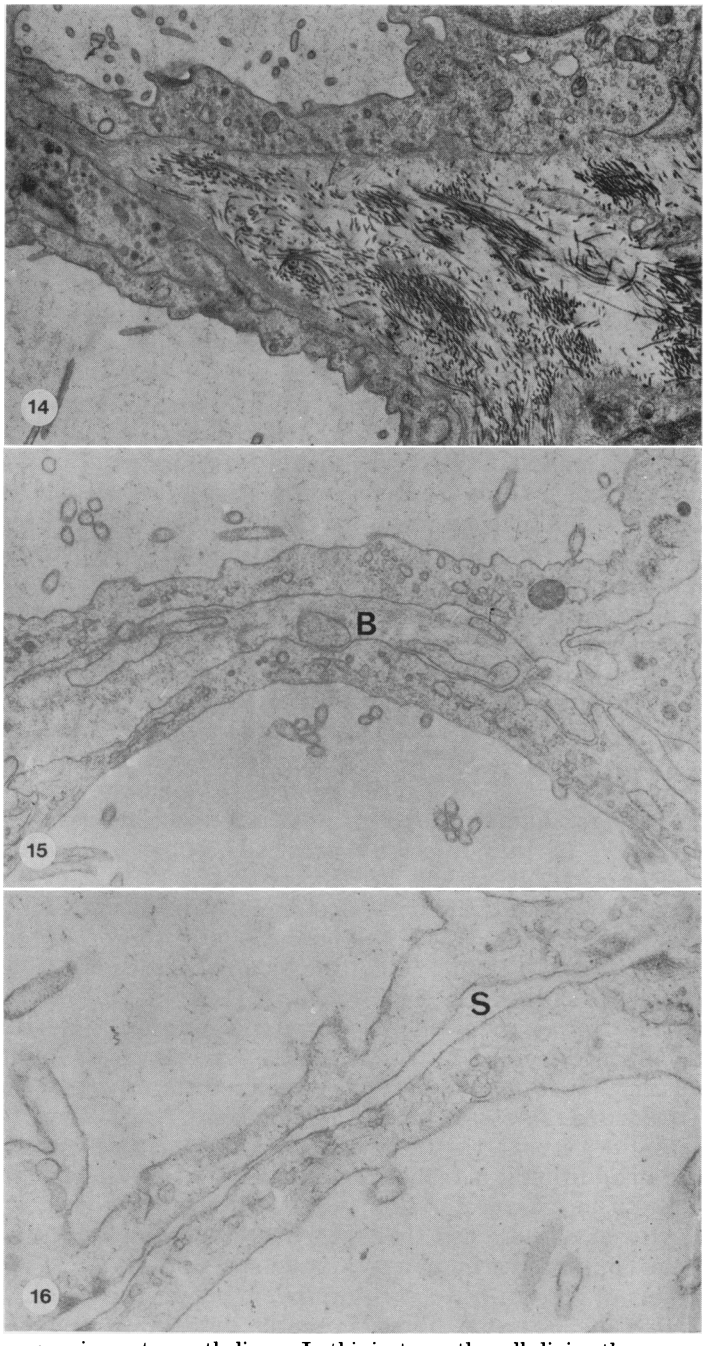

FIG. 14.—Tissue spaces in a rat mesothelioma. In this instance the cells lining the spaces are supported by collagen or reticulin fibres which are surfaced by basement-membrane material. × 16,800.

FIG. 15.—Thin layers of cytoplasm separating 2 intracellular tissue spaces. A layer of basement-membrane material (B) is present between the cells. × 24,000.

FIG. 16.—Extended layers of cell cytoplasm separating 2 intracellular tissue spaces. No supporting structure is present in the space between the cells (S). × 33,600.

Since with spaces up to 50 μm in diameter the whole circumference was seldom included within one electron microscope section, it was not possible to be certain what proportion of spaces in this tumour variety were intracellular. However, few junctions between the most flattened areas of cytoplasm were found and some spaces were certainly within the cytoplasm of one greatly extended cell. In some cases more than one enlarged space was within a single cell and the cytoplasmic lining was equally thin around both spaces (Fig. 13). Between the vesicularized cells varying levels of supporting structure were found. In some cases a skeleton of reticulin was present, and the cell cytoplasm was supported on this by a distinct basement membrane (Fig. 14). In other cases, however, the cells were supported by basement membrane only (Fig. 15) and some instances were found where no structure could be detected between the cytoplasmic layers of the adjacent cells (Fig. 16).

DISCUSSION

In the present study, tumours developed in approximately 33% of rats injected with asbestos and occurred at periods of 15–22 months after the start of the experiment. In a previous study (Davis, 1974) it was reported that in rats injected with the same asbestos dose in the peritoneal cavity, 60% of the animals developed tumours at periods ranging from 9 to 22 months after dusting. It would appear, therefore, that crocidolite asbestos is more carcinogenic in the peritoneal than the pleural cavity and in addition it acts more quickly in this site. The reasons for this difference are at present obscure but they may be of theoretical importance in understanding the process of foreign-body carcinogenicity induced by asbestos fibres, and variations of reactions within the site of implantation could be a useful field for further study.

Further differences are obvious in the histological patterns of the tumours produced. In the peritoneal cavity, almost all tumours followed one basic pattern which began with extremely small nodules consisting mainly of connecting tissue cells with a few layers of cells of epithelial type on their surfaces. Even in advanced tumours, which could sometimes cover large areas of the peritoneal cavity with widespread layers 2–3 mm thick, the same basic pattern was maintained. In the pleural cavity, however, only a few tumours showed this histological pattern alone. In the remaining tumours, although some areas of spindle-cell connective-tissue structure were always present in advanced cases, the epithelial tumour elements were present in a more differentiated state, as papillary or vesicular areas, than had usually been the case with tumours of the peritoneal cavity.

The earliest lesions of each tumour type, which were usually too small to be detected with the naked eye at necropsy, always showed the histological pattern of the advanced tumours, at least as far as the epithelial elements were concerned. Both the papillary and vesicular varieties appeared to originate from areas of the mesothelial surface where the epithelial cells had become rounded and hyperplastic, but with one type the cells grew in solid columns, whereas the other exhibited large tissue spaces from the first. The impression is gained that, while the peritoneal form of histological pattern is formed from cells of basically connective-tissue type that adopt a superficially epithelial form when growing on or near a free surface, the other 2 varieties develop from the true mesothelial layers of flattened epithelial cells. In one case the cells still show a potential for extending their cytoplasmic surface area to line tissue spaces even if the spaces are intracellular, but in the other this potential is greatly diminished and the cells remain rounded. However, animals with advanced tumours with epithelial areas of these 2 types were always found to have some spindle-cell-tumour elements and it is important to consider whether or not these cells are derived from early tumour cells

of epithelial type or whether they represent a separate example of neoplastic change. This latter possibility cannot be ruled out, but previous studies on the developmental potential of mesothelioma cells have shown that with this type of tissue, changes in cell pattern can occur. Thus Maximov (1927) showed that outgrowths from cultures of mesothelial surface could adopt a spindle-cell fibroblastic pattern, while Stout and Murray (1942) cultured pieces from a human mesothelioma that showed only spindle-shaped cells and observed outgrowth that appeared similar to normal mesothelial cells. Lewis (1923) examined mesenchyme rather than true mesothelial cells but showed that mesenchymal outgrowths from cultures of chick embryo heart showed all types of differentiation from bipolar and multipolar reticulum cells to flat mesothelial forms. It seems likely, therefore, that the mesothelial lining cells and all tumours to be classified as mesotheliomas develop from basically mesenchymal cells. With mesotheliomas the actual pattern of tumour histology that is found probably depends upon minor modifications of genetic expression that occur at the time of tumour transformation, but further changes within the overall mesenchymal pattern can occur in different areas of advanced tumours.

The present study has indicated that pleural mesotheliomas can begin from mesenchymal cells showing both epithelial (mesothelial) and connective-tissue form and this raises interesting problems regarding the methods by which asbestos fibres are able to bring about transformation. After injection the asbestos fibres are quickly surrounded by cells and embedded in areas of granulation tissue. It is unlikely that any free fibre exists within the pleural cavity for more than a few days after dust injection and there is no indication that the granulomas break down and release fibres at any point in time, although there is some turnover of cells within the granulomas (Davis, 1970). Many of the granuloma cells show ultrastructure dissimilar from that of both macrophages and fibroblasts and these could represent uncommitted mesenchymal cells. Transformation of these cells by the asbestos fibres with which they are in contact could result in the early connective-tissue forms of mesotheliomas. Where tumours appear to develop from cells on the mesothelial surfaces, however, the problem is more difficult since these sites could not have been in direct contact with asbestos fibres for some months. It seems worth considering whether the primary mesencyhmal cells partially transformed within granulomas might not migrate to the mesothelial surface and there adopt an epithelial pattern some time before transformation becomes complete. A situation similar to this has been reported by Brand, Buoen and Brand (1971) in studies of tumour genesis using implanted plastic discs. It was found that if discs with their surrounding tissue capsule from mice of the CBA HT6 strain were transplanted into normal CBA mice, tumours developed at the time that would normally have been expected in the original mice and from cells showing the same HT6 chromosome marker. From these studies it was concluded that partly transformed cells develop as a clone in the tissue capsule surrounding an implant within a few weeks of implantation even although no recognizable tumours develop for many months. However, in most experiments it was necessary to transplant the plastic disc as well as the capsule for subsequent tumour development to occur, which suggested that complete transformation requires the continuing presence of the original foreign body. If carcinogenesis by asbestos fibres is the result of similar processes to that involving plastic discs, then cells would need to remain in contact with asbestos fibres for long periods. However, Brand, Buoen and Brand (1967) have shown that capsules that have remained for a long period in contact with plastic discs may be transferred without the disc and still develop tumours in recipient mice. This appears to apply if capsule tissue is transferred within a few weeks of final tumour appear-

ance. These few weeks might be enough for cells finally committed to tumour formation to migrate from asbestos granulomas to mesothelial surfaces before ultimate tumour growth starts, but the whole relationship between asbestos and other types of foreign body carcinogenesis needs to be considered further.

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