FROM THE DIVISION OF RADIATION BIOLOGY, THE SWEDISH RESEARCH INSTITUTE OF NATIONAL DEFENCE, 172 04 SUNDBYBERG, AND THE DEPARTMENT OF PATHOLOGY, SÖDERSJUKHUSET, 100 64 STOCKHOLM, SWEDEN.

ULTRASTRUCTURE OF ^{90}Sr INDUCED OSTEOSARCOMAS AND EARLY PHASES OF THEIR DEVELOPMENT

A. NILSSON, P. SUNDELIN and INGER SJÖDÉN

Osteosarcomas induced by ^{90}Sr are of predominantly osteoblastic or fibroblastic types. The majority of the former arise along endosteal linings from fusiform cells in the osteogenic connective tissue and the latter from reticular cells of the medullary cavity (NILSSON 1962). The development of both these types is well known and seems to offer good opportunity to investigate ultrastructural phenomena during the early stages of cellular transformation leading to overt osteosarcomas. SUNDELIN & NILSSON (1967) have investigated these events by light microscopy and UV-spectrophotometry and to some extent also by electron microscopy. Systematic examination of radiation induced cellular changes on the ultramicroscopical level seem to offer information of importance for the understanding of radiation induced oncogenesis. The ultrastructural characteristics of ^{90}Sr induced malignancy in osseous and reticular tissues have therefore been investigated.

Material and Methods. One hundred CBA male mice, 75 days old, were injected intraperitoneally with 0.8 μCi ^{90}Sr/g body weight. In order to collect microscopic bone tumours in different stages of development, a total of 50 mice

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were killed in groups of five animals between 200 and 350 days after the injection of ^{90}Sr. The mice were killed by cervical dislocation and the time interval between each group was 15 days. Both femurs from each mouse were dissected free and were cut longitudinally along the median plane. One part of the femur was then fixed in Stieve's fluid for light microscopy and the other one in glutaraldehyde for electron microscopy. After decalcification in 20 % formic acid conventional light microscopic methods were used and the sections were stained according to the van Gieson method. By the aid of these sections, material representing different light microscopic stages of the development of predominantly fibroblastic osteosarcomas were selected for the electron microscopic examination. The material was classified according to SUNDELIN & NILSSON (1967), by the light microscopic appearance of the reticular cells as follows:

Stage 0: Hypoplastic bone marrow with morphologically normal reticular cells (Fig. 1 a).

Stage 1: Hypoplastic bone marrow with swelling and increasing number of reticular cells.

Stage 2: Hypoplastic bone marrow with proliferation of reticular cells with the histologic appearance of fibrosis without neoplastic character (Fig. 1 b).

Stage 3: Solitary or multiple small buds of tightly packed morphologically malignant cells inside an apparently non-neoplastic fibrous tissue (Fig. 1 c).

Stages 4, 5: Fibroblastic osteosarcomas situated completely within the bone marrow, the different stages being distinguished by the size, cellularity and pleomorphism of the tumour (Fig. 1 d).

Stage 6: Tumours infiltrating and breaking through the compact bone (Fig. 1 e).

Stage 7: Overt, paraosteal tumours.

After light microscopic examination of the longitudinal half parts of the femurs, the topographical site of microscopic tumours or their early stages was determined. From the counter-part fixed in glutaraldehyde (3 % aqueous solution buffered with Na-Cacodylat 0.067 M, pH 7.4) the corresponding region

Fig. 1. a) Stage 0. Aplastic fatty marrow with morphologically normal reticular cells. Left femur, mouse 244 days after injection of 0.8 μCi ^{90}Sr/g body weight. Van Gieson × 400. b) Stage II. Intramedullary, non-neoplastic diffuse proliferation of elongated swollen cells. Humerus, mouse 212 days after injection of 0.8 μCi ^{90}Sr/g body weight. Van Gieson × 400. c) Stage III. Solitary neoplastic bud inside a non-neoplastic fibroblastic tissue, diaphysis left femur, mouse 270 days after injection of 0.8 μCi ^{90}Sr/g body weight. Van Gieson × 100. d) Stage IV. Fibroblastic osteosarcoma almost completely occupying the medullary cavity. Femur, mouse 308 days after injection of 0.8 μCi ^{90}Sr/g body weight. Van Gieson × 400. e) Stage VI. Intramedullary fibroblastic osteosarcoma breaking through compact bone (right, lower corner). Tibia, mouse 218 days after injection of 0.8 μCi ^{90}Sr/g body weight. Van Gieson × 400.

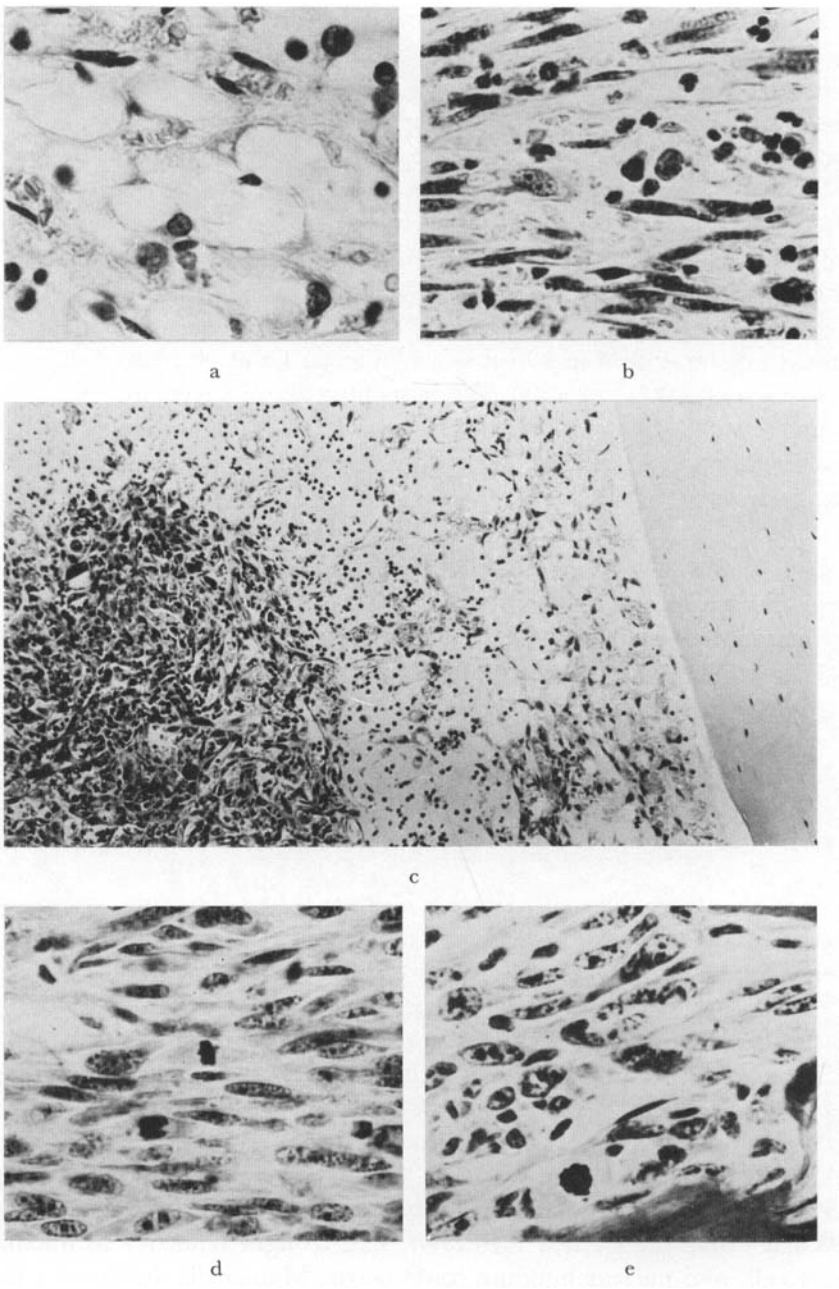


Fig. 1. (For legend see opposite page.)

was cut out and postfixed in osmium tetroxide according to MILLONIG (1962) (2 % solution buffered with phosphate buffer; pH 7.3, for one hour at 4° C). The material was dehydrated in ethanol and embedded in Epon (LUFT 1961). One microne sections for survey purposes were stained with 0.2 % toluidin blue (BJÖRKMAN 1962) for light microscopy to detect the relevant piece of tissue. For electron microscopy of this piece sections of about 700 to 900 Å were collected on 200 to 400 mesh copper grids and stained with 6 % uranyl acetate in methanol for 5 minutes (STEMPAK & WARD 1964) and subsequently with 0.2 % lead citrate for one minute (VENABLE 1965). The occurrence of lysosomes was verified with the BARKA & ANDERSSON (1963) method for acid phosphate. The specimens were examined in a Siemen's Elmiscope 1A at 60 kV and at a primary magnification of 1 000 to 20 000. The remaining 50 mice were allowed to survive until macroscopic tumours were detected. Both fibroblastic and osteoblastic osteosarcomas were selected and prepared for light and electron microscopy in the same way as described above.

Results

From the 50 mice killed between 200 and 350 days after ^{90}Sr injection the ultrastructure of 22 femurs with fibroblastic osteosarcomas or typical histologic changes preceding tumour formation were examined. The number of femurs at successive stages were:

Stage	0	1	2	3	4	5	6	7
Number of femurs	3	3	3	2	2	1	3	5

Fibroblastic osteosarcomas

Nuclear structure. The reticular cells of stage 0 had the same appearances as normal reticular cells with elongated nuclei and smooth nuclear membrane without invaginations. The nuclear chromatine had usually a clear, light network, which was finely granular and evenly distributed with only slight condensation along the nuclear membrane. Most nuclei contained no perichromatine granules but in a few cells small clusters of coarse granules were found (Fig. 2).

The nucleoli, one or two per cell nucleus, were small, generally round and granular in type, in some cases with invaginations. No vesicular nucleoli were found.

In cell nuclei from stage I and II—representing a non-neoplastic proliferation of reticular cells—the nuclear membrane had a slight tendency to folding and in a few cells also nuclear budding could occur. Many cells displayed a marked thickening of the nuclear membrane described as fibrous lamina (FAWCETT 1966) or internal dense lamella (KALIAFAT et coll. 1967) (Fig. 3). The nucleus

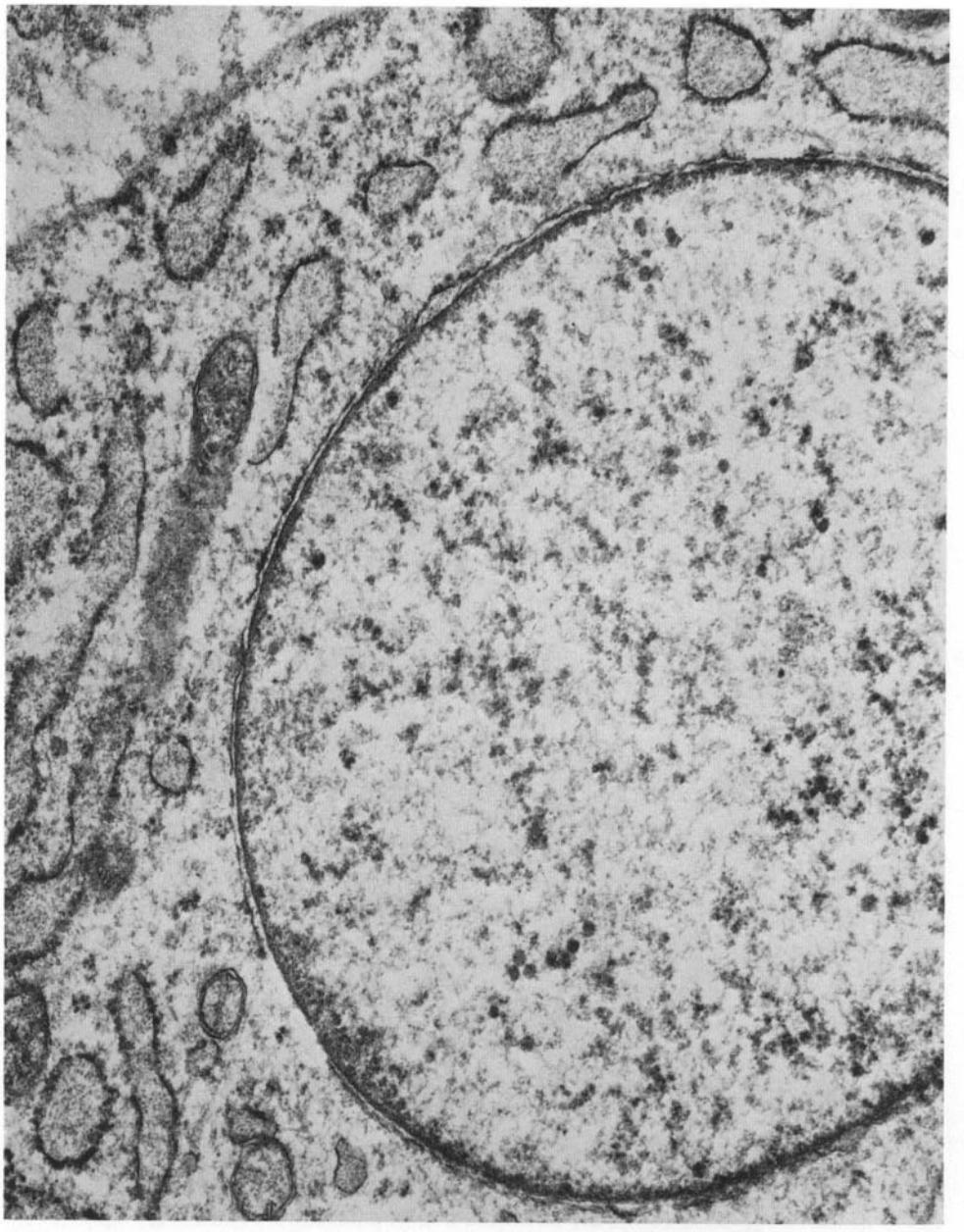


Fig. 2. Stage 0. Nucleus with light network of chromatine and small clusters of perichromatine granules. Slight condensation of nuclear chromatine along smooth nuclear membrane. Slightly dilatated rough endoplasmic reticulum. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 32 000.

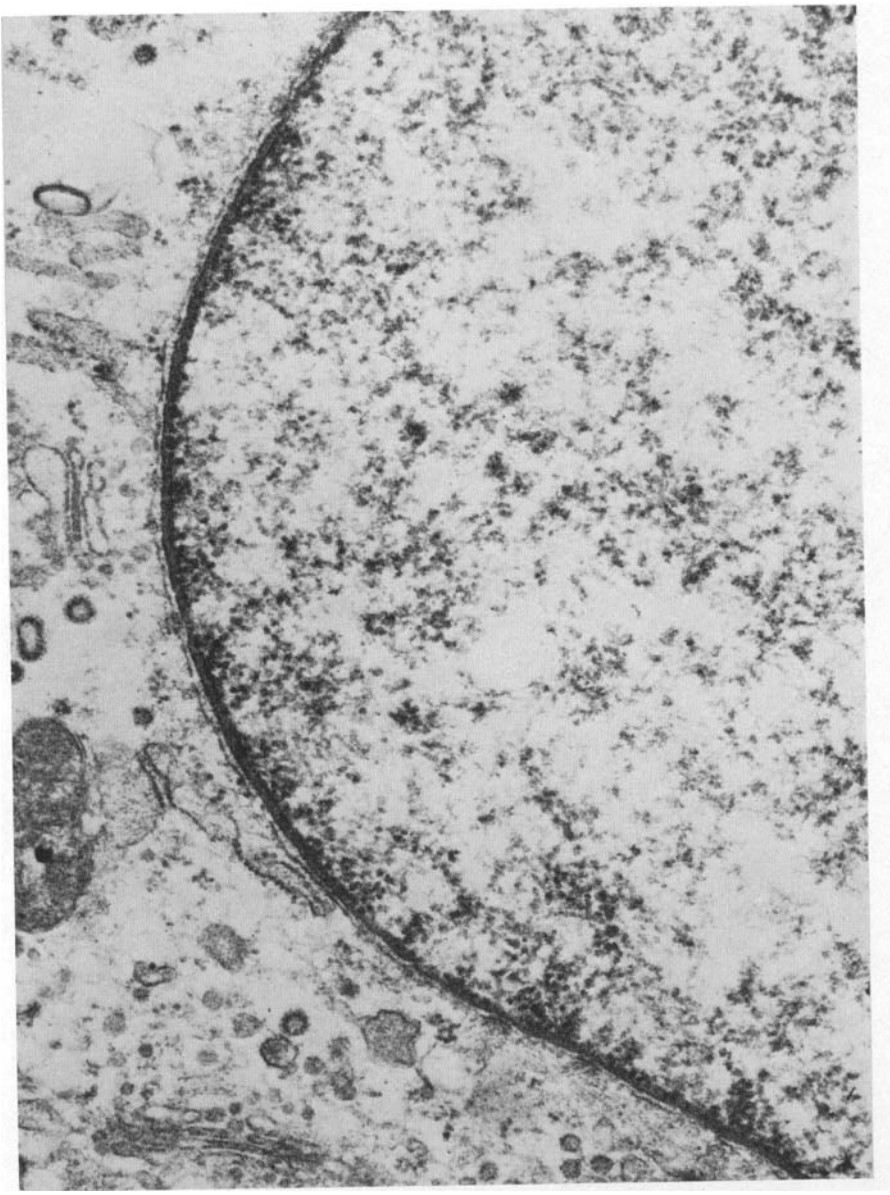


Fig. 3. Stage II. Fibrous lamination of the nuclear membrane. Left femur, mouse 314 days after injection of ^{90}Sr. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 32 000.

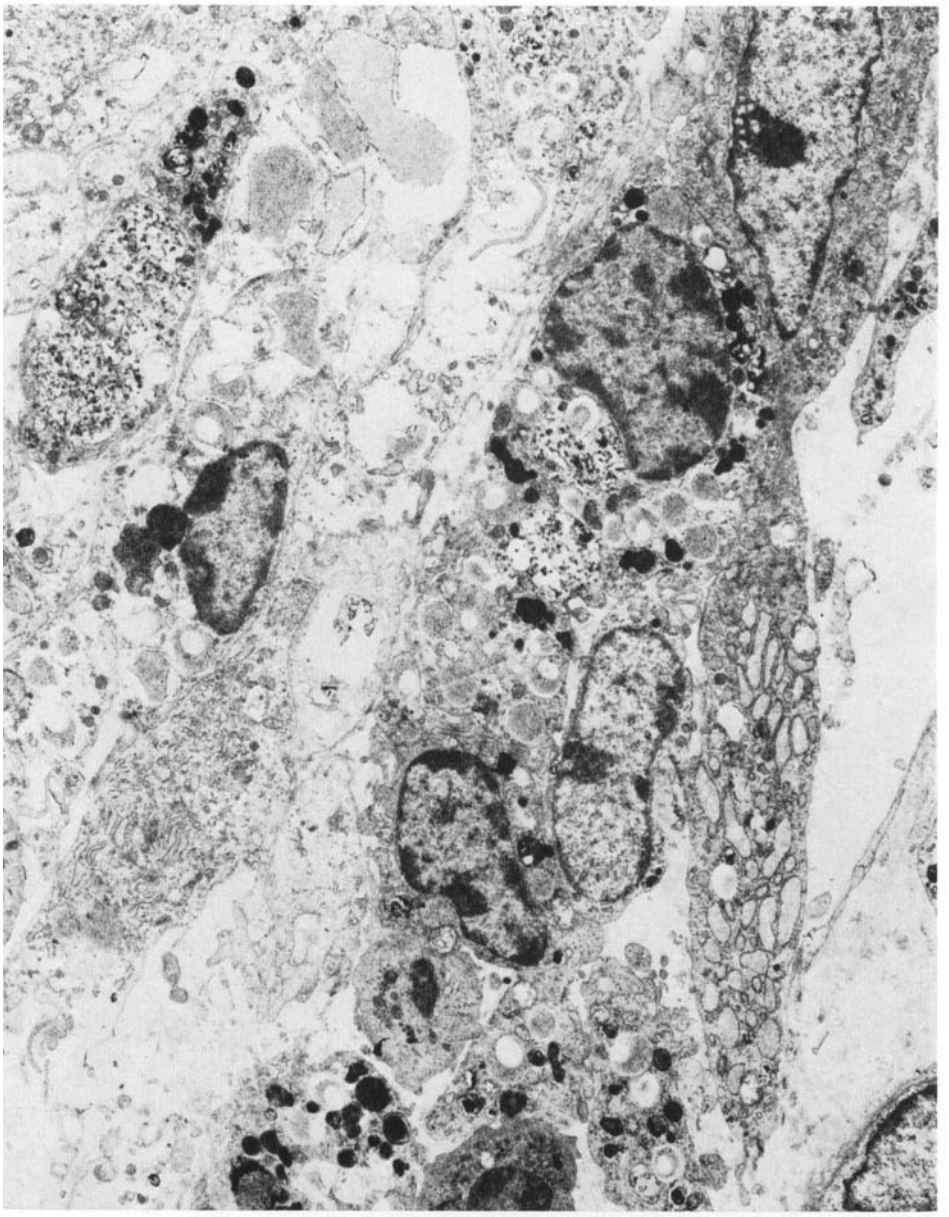


Fig. 4. Stage III. Electron micrograph from a neoplastic bud of the same type as shown in Fig. 1 c. Femur, mouse 317 days after injection of ^{90}Sr. The density of the nuclear chromatine varies from cell to cell. Strong condensation of chromatine along nuclear membrane and tendency to folding of nuclear membrane. Abundant dilated endoplasmic reticulum in some cells. Occurrence of fat globules and abundance of lysosome like structures. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 4 000.

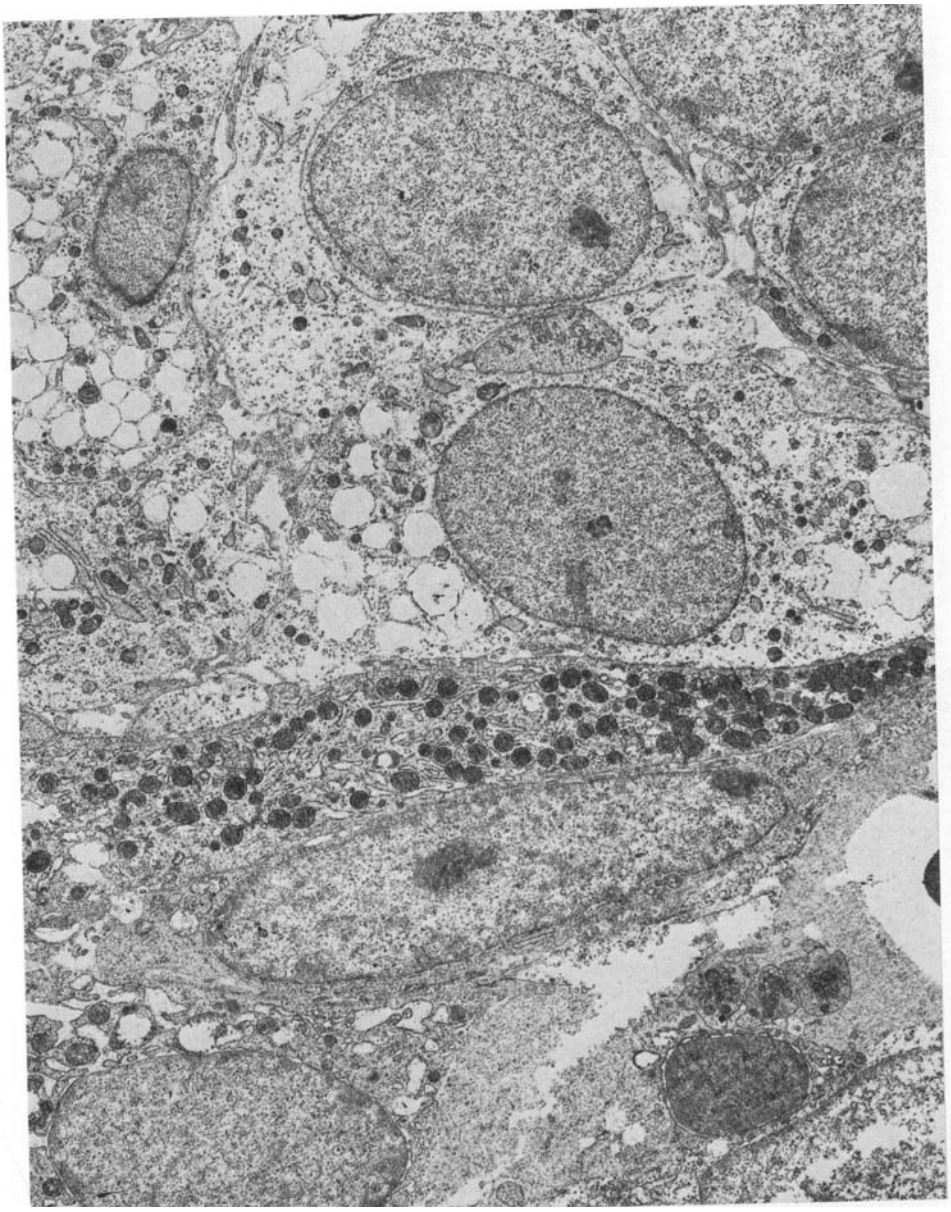


Fig. 5. Stage IV. Chromatine margination less marked but nuclear chromatine more evenly distributed and more finely granular than in earlier stages. In upper part of figure anaplastic cells with scanty cytoplasmic organelles. Abundance of type 4 mitochondria in one cell. Femur, mouse 355 days after injection of ^{90}Sr. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 4 200.

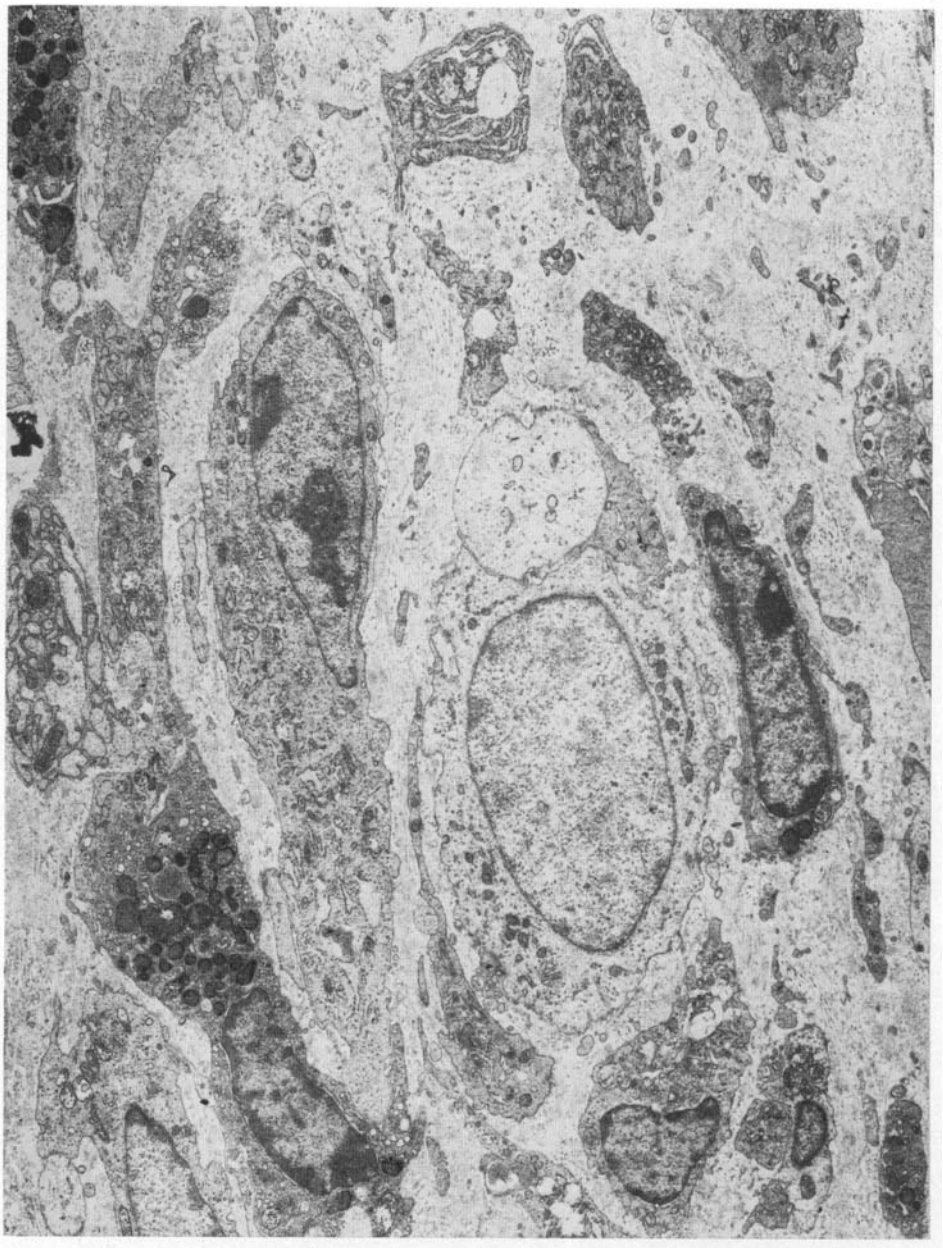


Fig. 6. Stage V. Cellular pleomorphism. Numerous cytosomes. Left femur, mouse 317 days after injection of ^{90}Sr. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 4 000.

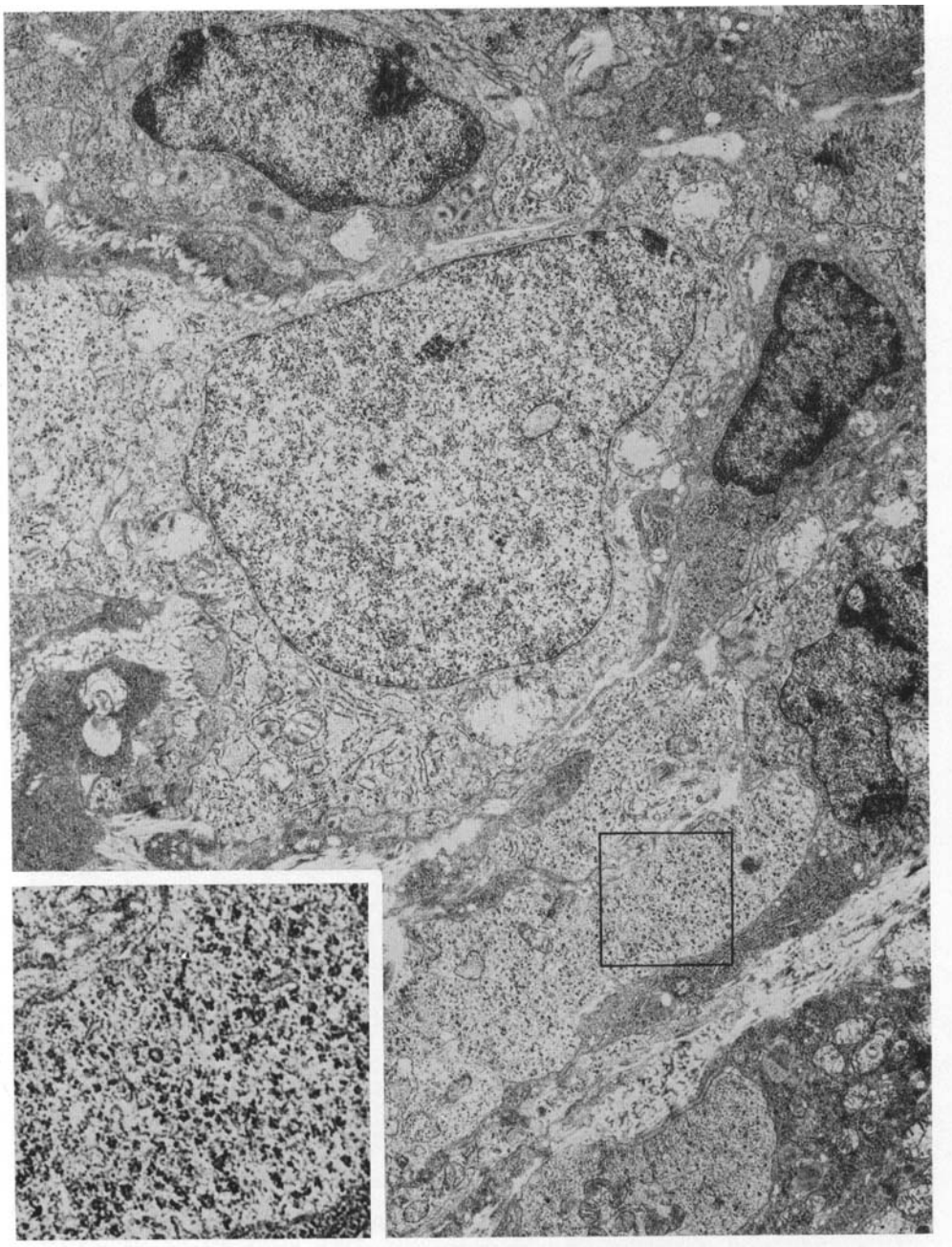


Fig. 7. (For legend see opposite page.)

generally appeared slightly more dense than in normal cells and had a coarse network of chromatine which was sometimes clumped into patches. Nuclear chromatine also began to condense along the nuclear membrane—in some cases being fairly insignificant, in others, however, evident. Accumulations of coarse perichromatine granules were larger and more numerous than had been seen in earlier stages.

Cell nuclei from stage III—small circumscript neoplastic condensations of reticular cells displaying polymorphism (Fig. 4)—had generally, compared with those from stage I and II, a denser and more finely granular chromatine, although many nuclei also contained greater patches of chromatine material. The nuclear infoldings were more numerous and accentuated. An evident chromatine margination still persisted in numerous cells.

The cell nuclei from stages IV and V—intramedullary tumours—were generally characterized by a strikingly increased cellular pleomorphism and anaplasia (Fig. 5). The nuclei were large. In many cells the nuclear membrane was more heavily folded than in the preceding stages whereas in others the membrane was very smooth. Some of the cells of stage IV had the highest nuclear density seen in the whole material, with an extremely fine granular chromatine. The condensation of chromatine along the nuclear membrane had disappeared in many cells but persisted to a varying degree in others (Fig. 6). Nucleolar inclusions and invaginations were seen in some of the cells from stage II and III but they were more common in cells belonging to tumours of stage IV and V.

The nucleoli from stage VI—penetrating—and VII—overt tumours— were larger than those of earlier stages but their size and shape varied considerably.

In these two stages two separate types of cells could be identified (Fig. 7). In one of these the cells were anaplastic with large nuclei having smooth margination without folding. The nuclear structure was loose with small numbers of granules. There was no granular condensation along the nuclear membrane and the pores in the membrane appeared few and narrow. The nucleoli of these cells varied considerably in size and shape. In the other cell type of stages VI and VII the cells had nucleoli which resembled those of cells from stage V. They had dense chromatine, heavily folded nuclear membrane and a varying degree of chromatine condensation (Fig. 8).

Cytoplasmic structures. The endoplasmic reticulum (ER) was generally of rough type at all stages. It was rather scanty in cells of stages 0—II. In stage III

Fig. 7. Overt fibroblastic osteosarcoma, femur, mouse 310 days after injection of ^{90}Sr. Anaplastic cell in the upper center of the figure with smooth nuclear membrane, insignificant chromatine margination and quite loosely arranged chromatine granulation of nucleus, poorly developed endoplasmic reticulum hardly detectable Golgi vesicles and few mitochondria but quite abundant polyribosomes (inserted). Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 5 600. Inserted figure × 16 800.

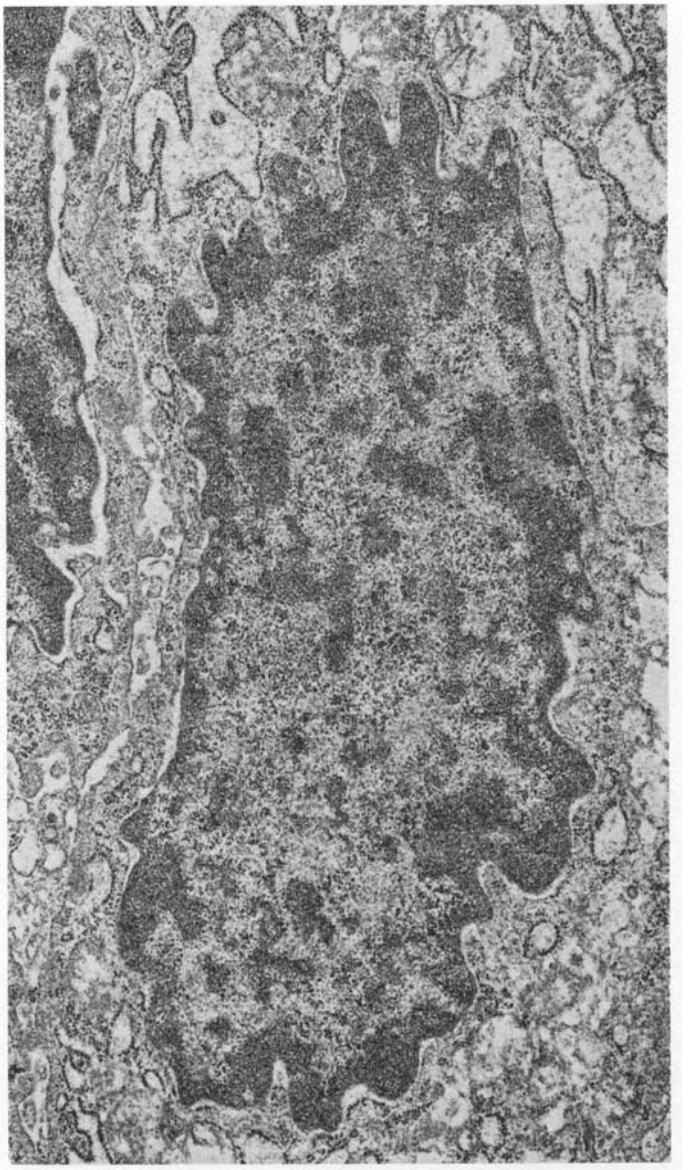


Fig. 8. Overt fibroblastic osteosarcoma. Heavily folded nuclear membrane (in contrast to Fig. 12) and evident chromatine margination. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 16 000.

the cells appeared with intensely basophilic cytoplasm in the light microscope and electron microscopy revealed abundance of ER in the cytoplasm of nearly all cells from this stage (Fig. 4). Cells from stage IV and later disclosed increasing intercellular variation with regard to this parameter. Some of the cells from stage VI had more rough ER than any other cells in the whole material. These were of the type described above which had dense nuclei with heavily folded nuclear membrane. The anaplastic cell type from stage VI and VII tumours had scanty ER which was often degranulated (Fig. 7).

Occasionally dilated ER could be found in cells from all stages. In cells from stages III and higher it was, however, a regular finding, and cisterns formed by dilated ER often contained dense material (Fig. 9).

The mean number of bound ribosomes per cell as estimated in arbitrary units increased gradually from stage 0 to IV and the number of free ribosomes increased from 0 to V. In overt tumours the number decreased, but there was an increasing intercellular variation. The majority of free ribosomes were polysomes at all stages, but single ribosomes were also found in most cells. In anaplastic cells of stages VI and VII, however, the dominance of polysomes was greatest (Fig. 7).

The number of mitochondria was counted in 22 to 61 cells at each stage. The numbers per cell varied within each stage group but there was a tendency towards increasing numbers from stage 0 to IV and a gradual slight decline in later stages. Anaplastic cells of stage VI and VII had few large mitochondria.

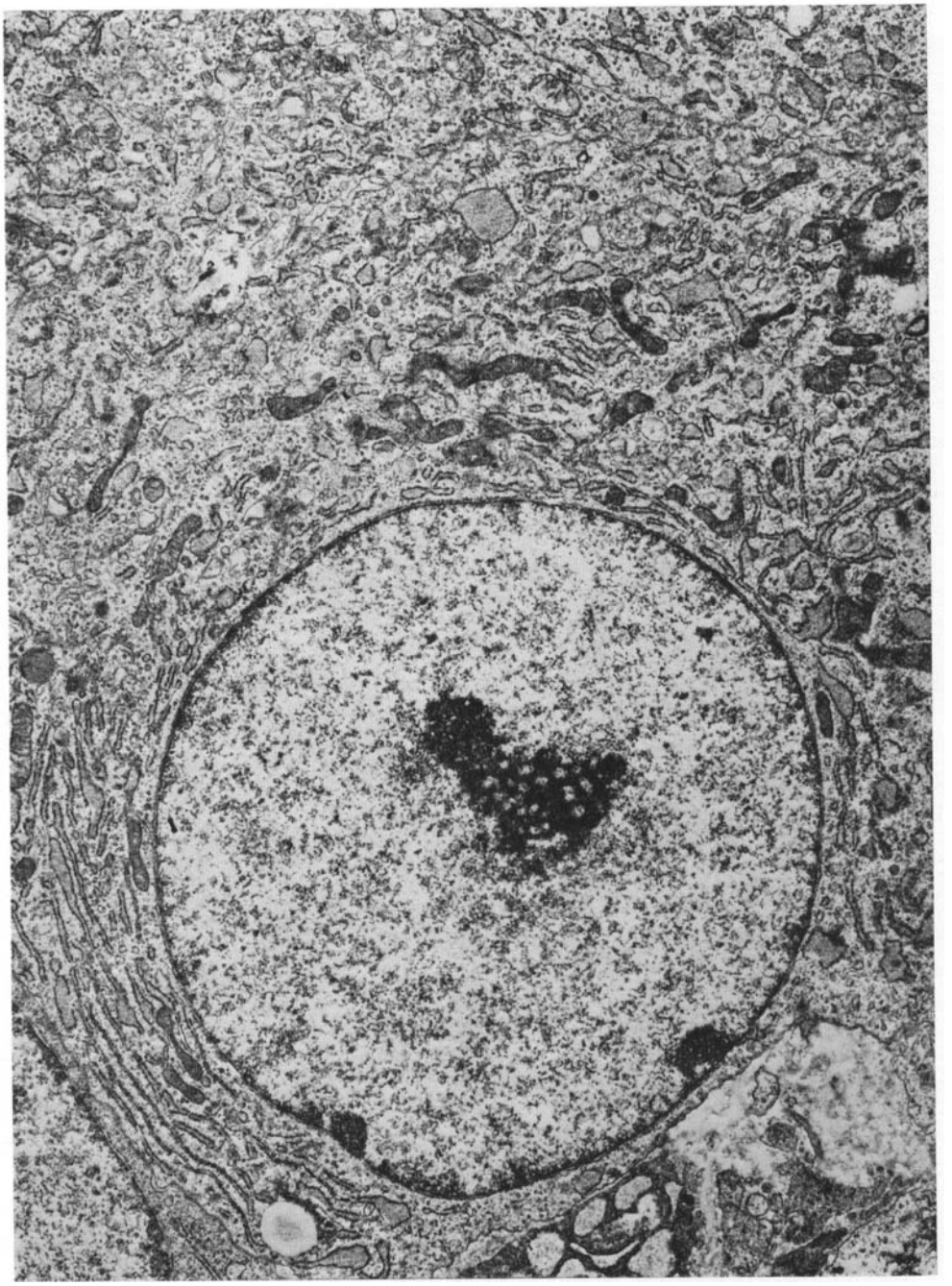
Lysosomes giving strong reaction for acid phosphate were found in all stages but were especially numerous in stage IV. The shape of the Golgi apparatus characterized as mainly tubular, mainly lamellar or mixed and its size was graded from 1 to 3 in each cell. There was a great intercellular variation within each stage group and no systematic variation was found when the stage groups were compared. In the anaplastic cells of stage VI and VII the Golgi apparatus was usually insignificant. In many cells of stage III to VII an abundance of small vesicles in the Golgi zone and its surroundings occurred. In the Golgi zone and its immediate surrounding multivesicular bodies (HAGUENAU 1969) were invariably found from stage I to VII. They were never numerous and did not evidently predominate a particular stage.

Occasionally microtubuli were found in cells from all stages but were most abundant in cells of higher stages.

In many cells in the higher intramedullary stages and in overt tumours numerous intracellular fibrils occurred (Fig. 10) (ROSS & BENDITT 1965).

Osteoblastic osteosarcomas

The osteoblastic tumours induced by ^{90}Sr originate from the endosteal linings of the bone and they successively fill up the marrow cavity or part of it before



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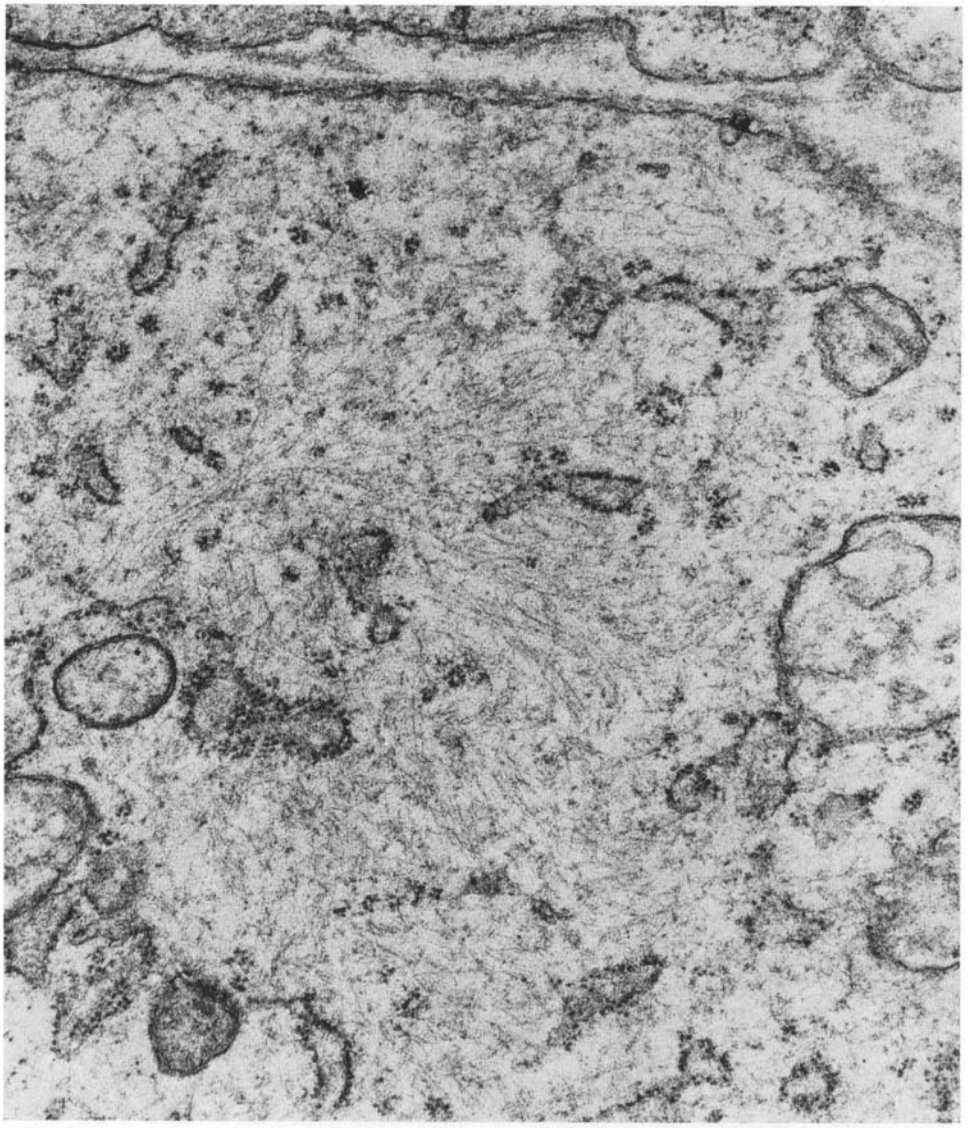

Fig. 10. Femur, stage V, mouse 317 days after injection of ^{90}Sr. Numerous intracellular fibrils. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 30 000.

Fig. 9. Stage IV. Femur, mouse 335 days after injection of ^{90}Sr. Abundance of rough endoplasmic reticulum with 'lacs' containing electron dense material. Numerous large, elongated and twisted type 4 mitochondria. Nucleolus with distinct nucleonemata. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 7 500.

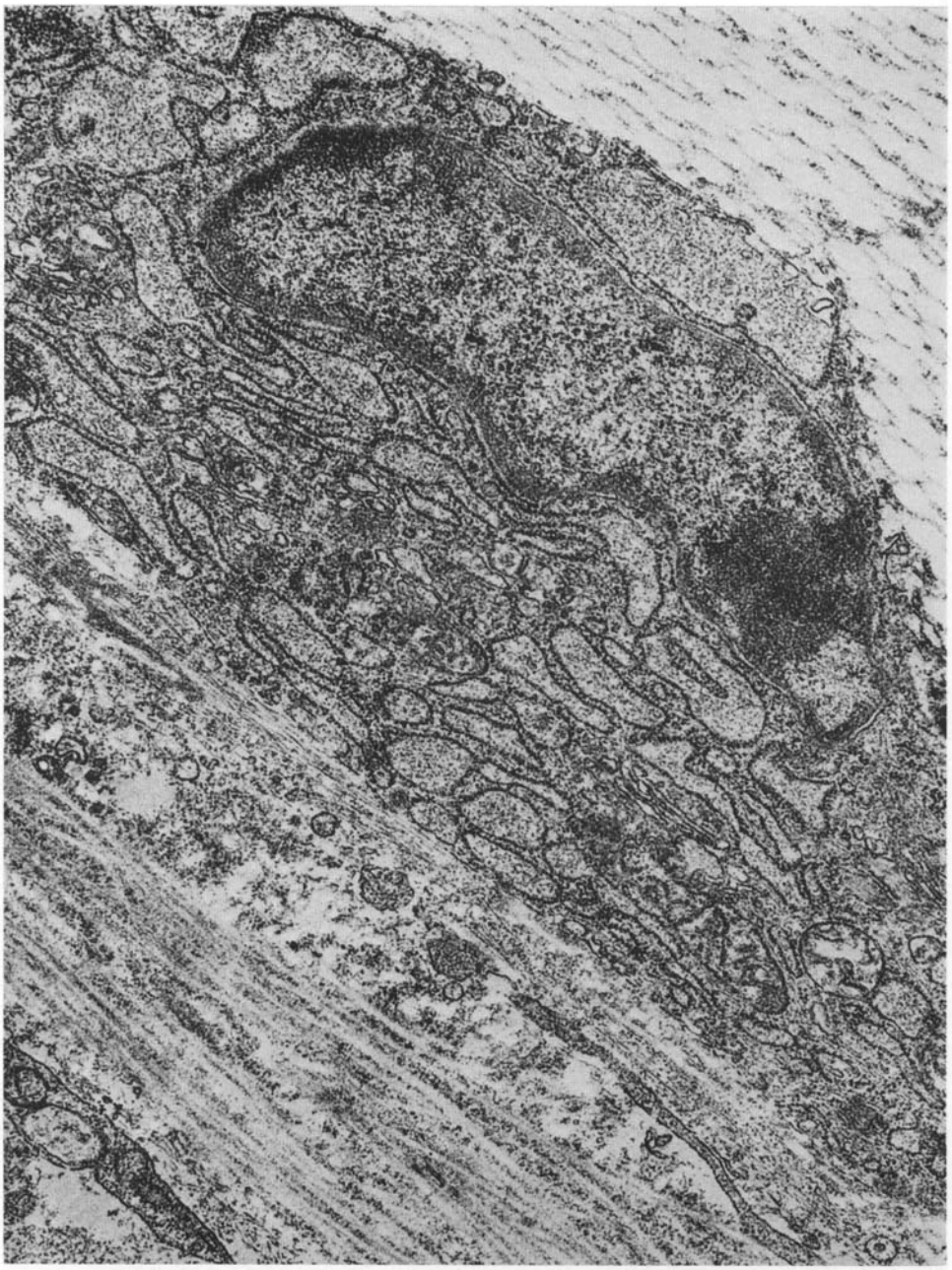

Fig. 11. Overt osteoblastic osteosarcoma 278 days after injection of ^{90}Sr. Well developed dilated rough endoplasmic reticulum. Abundance of ribosomes. Intercellular deposits of collagen. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 16 000.

they break through the compact bone. They grow slowly and particularly their central parts contain considerable amounts of bone and osteoid tissue. Because of their hardness these tumours were difficult to cut in the ultrotome and most material examined originates from peripheral parts of the tumours.

Nuclear structure. The nuclear membrane was generally heavily folded. It had normal thickness and fibrous lamination was never found. Nuclear chromatine was finely granular and a great proportion of it was condensed along the nuclear membrane. The nucleoli, one or two per cell nucleus, were generally fairly small.

Cytoplasmic structures. The cytoplasm of osteoblastic osteosarcoma cells contained moderate amounts of ER which was generally of rough type (Fig. 11). In many cells dilated ER formed large cisterns, some of which contained electron dense material (Fig. 12). The ER was not quite as abundant as in stage V fibroblastic tumours but the cisterns were generally larger in osteoblastic osteosarcomas.

Free ribosomes were numerous and a great proportion of them were polysomes.

Intracytoplasmic short thread like condensations were found in some cells (Fig. 13). Numerous filamentous structures without detectable periodic banding were evident as well as extracytoplasmic collagen fibrils.

Regressive changes in these cells were not as marked as in fibroblastic tumours. This can be due to their slower growth, their greater vascularization or the fact that the material investigated originates from peripheral parts of the tumours.

Discussion

The electron microscopy of the transformation of reticular cells into malignant clones and overt tumours has demonstrated many fine structural changes, some of which, however, seem to be unspecified for the oncogenesis. Most of the ultrastructural observations made do not significantly or specifically differ from those observed in tumours induced by other means (see HAGUENAU 1969, BERNHARD 1969).

A clear cellular pleomorphism was identified in stage III and by increasing stage of tumour development it became successively more accentuated. During tumour progression the characteristics of the cells differed more and more with respect to every parameter with increasing stage of development. In stage VI and VII an increasing population of anaplastic cells were encountered.

When examined in the electron microscope single reticular cells of stage 0 did not differ in any specific way from normal reticular cells and no sign of radiation injury could be detected.

The earliest ultrastructural changes were found in proliferating cells in stage I in the form of folding of the nuclear membrane. In the following stages the

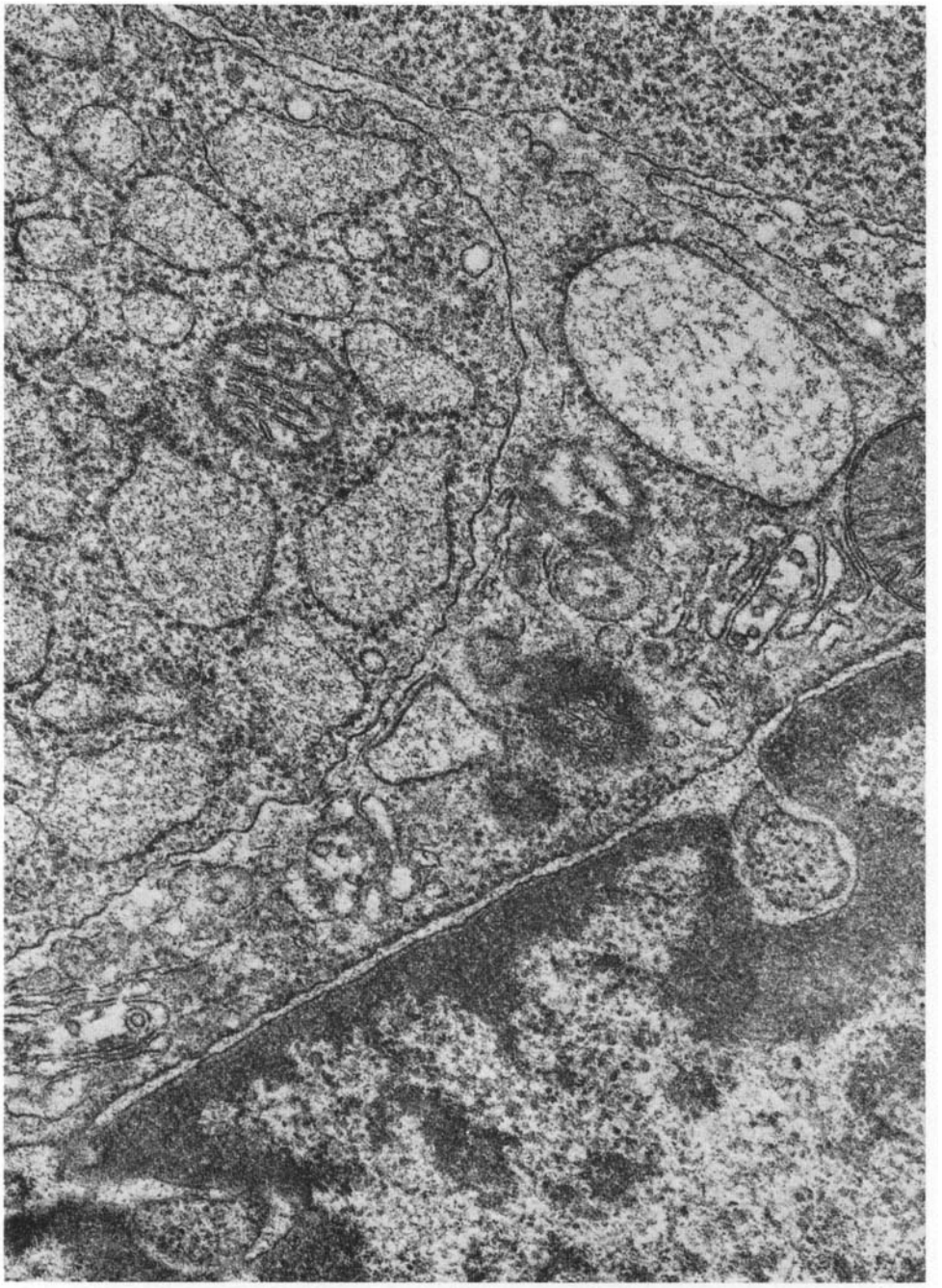

Fig. 12. (For legend see opposite page.)

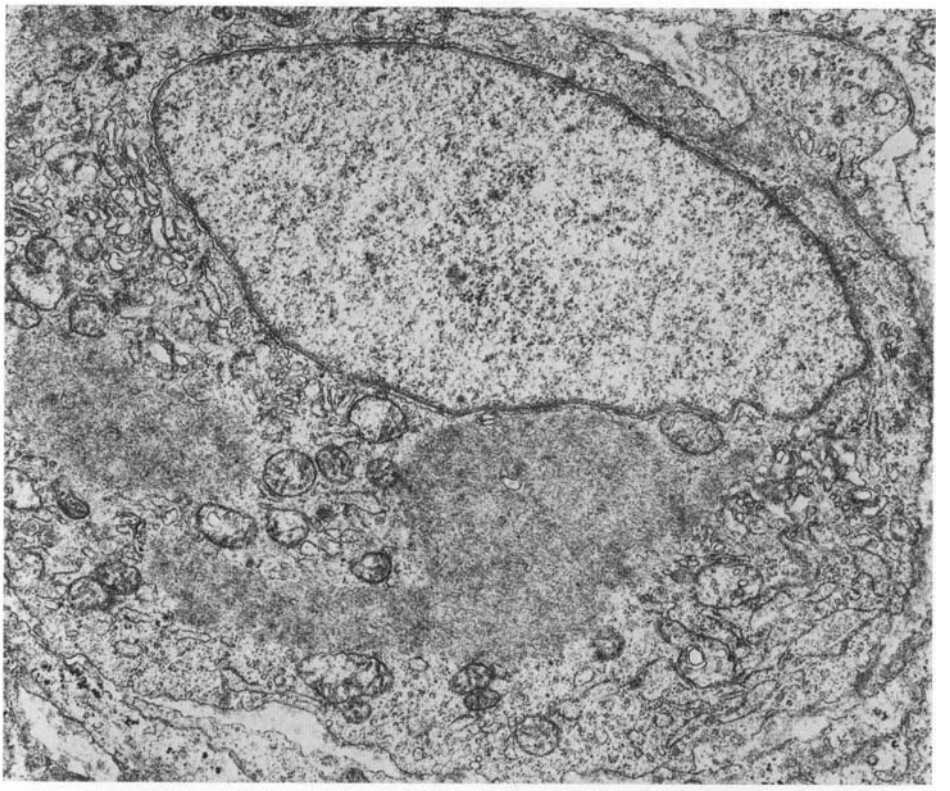

Fig. 13. Overt osteoblastic osteosarcoma 237 days after injection of ^{90}Sr. Great intracellular accumulation of threadlike structures. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 12 000.

reticular cells gradually adopted ultrastructural traits which characterize cancer cells: hypertrophy of the nucleus and the nucleolus, irregularities in the chromatine distribution, increase in ribosomes—especially polysomes—and increase in number of mitochondria (BERNHARD 1969, HAGUENAU 1969) etc.

It is evident that the peak number of mitochondria per cell which is found in stage IV slightly precedes the peak number of ribosomes (stage V) and that the peak number of ribosomes coincides with maximum cytoplasmic ultraviolet

Fig. 12. Overt osteoblastic osteosarcoma 335 days after injection of ^{90}Sr. Bottom right: Nucleus with deep invagination of nuclear membrane. Heavy chromatine margination along nuclear membrane. Center left: Strongly dilated rough endoplasmic reticulum. Top right: Numerous polyribosomes. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 32 000.

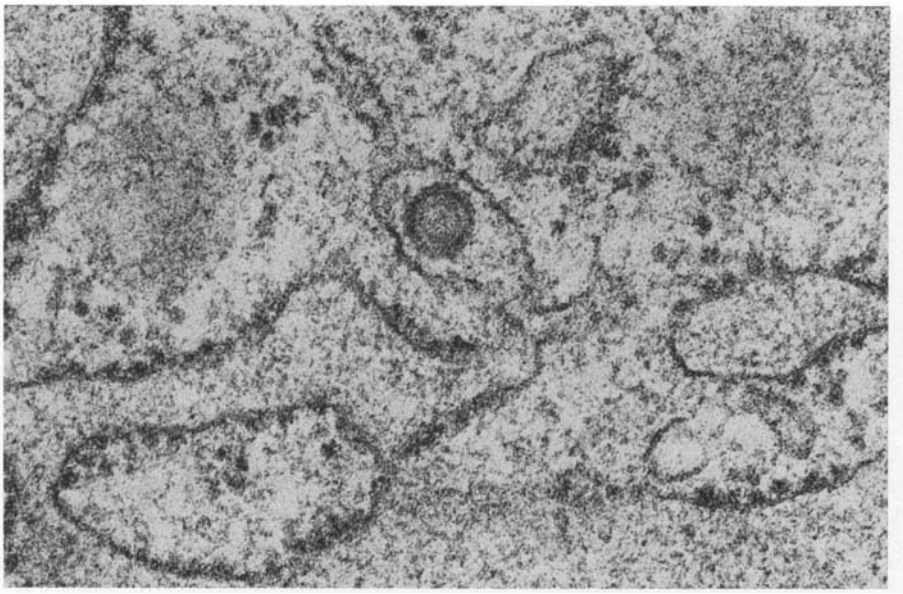

Fig. 14. Overt fibroblastic osteosarcoma, stage VII, from a first generation graft harvested 34 days after transplantation. The primary tumour which appeared 311 days after injection of ^{90}Sr was transplanted to new mice subcutaneously. Virus like particle inside endoplasmic reticulum. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 64 000.

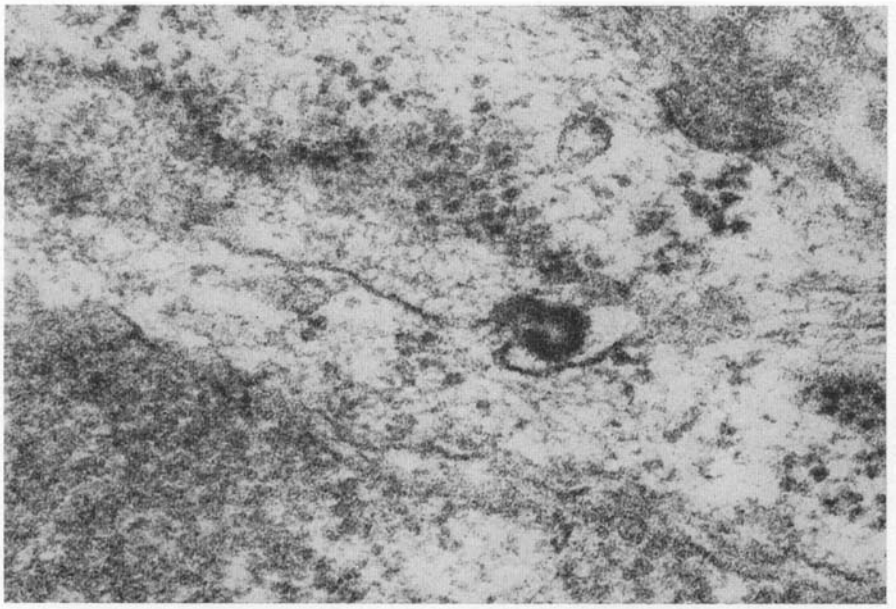

Fig. 15. Same primary tumour as in Fig. 13, but harvested 24 days after transplantation. Budding of a virus like particle from degranulated part of endoplasmic reticulum. Glutaraldehyde, osmium tetroxide, Epon, uranyl acetate, lead citrate × 84 000.

extinction (SUNDELIN & NILSSON 1967). Also the peak number of lysosome is found in cells around this stage (stage VI). Obviously these changes are reflexions of maximum proliferative activity at stages IV and V. At higher stages the intercellular variation with regard to all these parameters gradually increases.

The nature of the intracytoplasmic fibrillar structures found is not clear but it seems quite plausible to anticipate that these fibrils are identical with those described in fibroblasts by ROSS & BENDITT (1965).

Virus like particles budding from a degranulated part of the endoplasmic reticulum have been found in three separate tumours all of which belonged to stage VII (Figs 14, 15). No virus like particles were found in tumours of earlier stages. As we never have been able to transfer tumours with cell-free tumour extract (NILSSON 1962) the possible role of virus in tumourogenesis cannot be stated. The weak transplantation antigenicity which has been found in these tumours (NILSSON & RÉVÉSZ 1972) might, however, indicate that the virus is not playing the role as 'driver' but rather as 'passenger', particularly since it also might be anticipated that the antiviral defence inside a tumour is severely impaired thereby creating a 'safe heaven' for many types of virus.

Acknowledgement

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SUMMARY

The ultrastructure of radiostrontium induced fibroblastic and osteoblastic osteosarcomas and early phases of their development was investigated in CBA-mice. The material was classified by light microscopy into eight different stages of development. Fine structural changes were demonstrated such as folding of the nuclear membrane, hypertrophy of nucleus and nucleolus, disturbed chromatine distribution, increase in number of polysomes and mitochondria etc. Virus like particles were found rarely in overt tumours. Their role as 'driver' or 'passenger' is discussed.

ZUSAMMENFASSUNG

Es wurde die Ultrastruktur der durch Radiostrontium induzierten fibroblastischen und osteoblastischen Osteosarkome und die frühen Phasen deren Entwicklung bei CBA-Mäusen untersucht. Das Material wurde mittels Lichtmikroskopie in acht verschiedene Entwicklungsstadien eingeteilt. Es wurden feine strukturelle Veränderungen wie Faltung der Membran des Nukleus, Hypertrophie von Nukleus und Nukleolus, gestörte Chromatinverteilung, Anstieg der Zahl von Polysomen, Mitochondrien etc. gezeigt. Virus-ähnliche Partikel wurden vereinzelt in offenbaren Tumoren gefunden. Deren Rolle als 'Führer' oder 'Passagier' wird diskutiert.

RÉSUMÉ

Les auteurs ont étudié l'ultrastructure des ostéosarcomes fibroblastiques et ostéoblastiques induits par le radiostrontium et les premières phases de leur développement sur des souris CBA. Ce matériel a été classé par la microscopie optique en huit stades différents de développement. Les auteurs ont mis en évidence de fines modifications structurales telles que le plissement de la membrane nucléaire, l'hypertrophie du noyau et du nucléole, une perturbation de la distribution de la chromatine, une augmentation du nombre des polysomes et des mitochondries etc. Ils ont rarement trouvé des particules d'aspect viral dans les tumeurs évidentes. Ils discutent leur rôle de 'conducteur' ou de 'passager'.

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