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TRANSPLANTABLE OSTEOSARCOMA IN MICE

Structural Characterization of a Transplantable Osteosarcoma Obtained in an Allogenic System

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Light microscopic, histochemical and ultrastructural studies of a transplantable mouse osteosarcoma were carried out. The osteosarcoma grew in CBA mice after injection of cultured cells derived from a Dunn osteosarcoma. The tumour differed from the original Dunn osteosarcoma with respect to metastatic potential and structural features.

The transplantable tumour was an anaplastic, richly vascularized, fibroblastic osteosarcoma with alkaline phosphatase activity and rather sparse osteoid formation, resulting in death of the animals within 6 to 8 weeks. Virus particles were found intracellularly, mainly localized to cisterns of rough endoplasmic reticulum, and extracellularly often close to plasma membranes and collagen fibres. Signs suggestive of formation of collagen fibres by tumour cells were observed. A possible viral influence upon the tumour was suggested also by its growth behaviour *in vitro*.

The results indicate that this new transplantable tumour, obtained in an allogenic system, represents a clonal derivative of the original Dunn osteosarcoma.

Key words: bone neoplasm; electron miscroscopy; morphology; osteosarcoma, transplantable; virus particles.

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The poor results obtained with surgery or irradiation therapy for osteosarcomas, in particular those located proximal to the knee and elbow joints (cf. Larsson et al. 1978a) have focused attention upon the newer therapies such as chemotherapy and immunotherapy. Regression of human osteosarcoma has been described after adaptive and active immunotherapy (Marcove et al. 1971, Marsh et al. 1972), and the presence of tumour-specific antigens, capable of evoking both humoral (Lewis et al. 1969, Hellström & Hellström 1973) and cell-mediated antibody responses (cf. Hellström et al. 1971, Kumar et al. 1972), has been demonstrated in osteosarcoma.

The present study describes a transplantable murine osteosarcoma obtained after injections of cultivated tumour cells originating from the Dunn osteosarcoma*.

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While osteosarcoma rarely arises spontaneously in laboratory animals, the Dunn osteosarcoma is routinely transplantable both to C_3H and CBA mice and the pleomorphic, osteoid-producing tumour causes death of the host within 6 to 8 weeks after implantation (cf. Miller et al. 1976). Our transplantable osteosarcoma obtained in an allogenic system was readily transplantable and resulted in death of the animals by continuous local growth. The purpose of the present study was to characterize the transplanted tumour light microscopically and ultrastructurally. Subsequent studies will deal with the anti-tumoral effects evoked by immunotherapy with BCG and with injected killed tumour cells.

MATERIAL AND METHODS

Cell culture

The osteosarcoma cells were obtained as a cell line growing in monolayer. The cells were growing in 90 mm Nunclon plastic dishes (Nunc., Roskilde, Denmark). The medium used was Eagle's minimum essential medium (MEM) supplemented with antibiotics and 10 per cent foetal calf serum. The cultures were incubated in humidified air with 5 per cent CO_2 at 37°C. The cells were split in a 1:4 ratio every third or fourth day by trypsinization.

Cell number was estimated in an electronic cell counter (Linson Counter 411, Lonsom Instrument AB, Stockholm, Sweden). Cell viability was approximated by the trypan blue dye exclusion test. Mycoplasma could not be detected by standard culture methods.

Animals and single-cell injections

A total of 185 CBA mice*, 10 to 15 weeks old of both sexes, were used. Their body weight was 20 to 30 g. They were housed 5 or 10 in one cage and were kept on a standard laboratory ration** which, together with drinking water, was supplied without restriction.

Monocellular suspensions of cultivated cells were prepared by trypsinization. Approximately 2 $\times$ 10^6 viable tumour cells in 0.5 ml of serum-free MEM were injected subcutaneously into the flank of 150 of the animals. After 8 weeks, when none of the animals had presented a growing tumour, nor any tumour showing spontaneous regression, a second subcutaneous injection of 2 $\times$ 10^6 viable tumour cells was given to each animal. All animals were examined once a week for the occurrence of tumour.

After 6 months, a rapidly growing tumour occurred in one animal at the site of the injection, while none of the others showed any evidence of tumour. Thirty-five of the latter, non-tumour bearing mice were used for the experiment described below. The remaining 149 animals were observed for another 6 months without any evidence of tumour occurrence.

Transplantation of standardized tumour sections

The tumour-bearing animal was killed 6 weeks after the appearance of the tumour which had by then reached a considerable size. There were no metastases to the lungs or elsewhere. The solid tumour, which possessed some areas of bleeding, was dissected free under sterile conditions. Standardized rounded sections with a diameter of 3.0 mm and a thickness of 2.0 mm were cut randomly from the tumour with a special knife equipped with two scalpel blades mounted parallel, and one trepane. The wet weights of 20 sections showed a mean of 0.015 g with a coefficient of variation of 9.5 per cent.

The sections were transplanted subcutaneously to the dorsum of 35 animals which had been given injections of monocellular suspensions 6 months earlier, and to 35 intact control mice. The operations were performed under sterile conditions and light ether anaesthesia. Each animal received two standardized tumour sections i.e. 0.03 g of tumour tissue, and were then left for a planned observation period of 6 months during which they were examined individually once a week with regard to growth or regression of the tumour which could easily be felt below the skin.

Animals exhibiting growing tumours were sacrificed when the tumour had reached a considerable size that would have killed the animal within another 1 or 2 weeks. After careful dissection the tumour was completely removed and the wet weight recorded. Specimens were then taken for light and electron microscopic studies. Thorough autopsy examinations were performed and tissue suspected to contain metastases was removed for microscopic study.

In a comparative study on 15 separate mice, the incidence of growing tumour was studied after transplantation of 0.06 g of tumour tissue.

*Obtained from Karolinska Institutet, Stockholm, Sweden

**Obtained from Ewos Co., Södertälje, Sweden

Light microscopy and histochemistry

Specimens for light microscopy were fixed in 10 per cent neutral formalin and paraffin-embedded sections were stained with haematoxylin and eosin, van Gieson's stain, periodic acid-Schiff (P.A.S.), and Laidlaw's silver stain.

Histochemical demonstration of alkaline phosphatase was carried out on fresh frozen sections, using the method described by Barka & Anderson (1963).

Electron microscopy

The specimens taken for ultrastructural study were fixed in 2.5 per cent glutaraldehyde in 0.34 M Veronal acetate buffer adjusted to pH 7.4, followed by postfixation in 1 per cent osmium tetroxide in the same buffer. Embedding was carried out in Epon 812. Thick sections stained with toluidine-blue were used for identification of suitable areas for the thin sections which were stained with uranyl acetate and lead citrate prior to examination in a Siemens Elmiskop 1 A or 101.

RESULTS

Transplantation of standardized tumour sections

As reported above, injection of Dunn osteosarcoma cells derived from *in vitro* culture caused a growing tumour in only one out of 150 of our CBA mice. This tumour occurred first after 6 months of observation.

In contrast, transplantation of standardized sections constituting 0.03 g of this particular tumour gave rise to growing tumours in 28 out of 35 animals, i.e. 80 per cent. The tumours grew rapidly and we had to kill all tumour-bearing animals 6 to 8 weeks after transplantation, otherwise the animals would have died because of extensive local tumour growth. The other seven animals showed initial spontaneous disappearance of their tumour.

Transplantation of 0.06 of tumour tissue gave rise to growing tumour in all the animals, i.e. in 100 per cent.

Out of the 35 animals receiving transplanted tumour sections 6 months after the cultured cell injection there were five which developed growing tumours, i.e. 14 per cent. No evidence of tumour whatsoever was found at repeated examinations throughout the observation period of 6 months in the remaining 30 animals. The tumours thus showed spontaneous disappearance without any evidence of initial tumour growth.

Three of the mice with tumours in this group were killed 7 weeks after transplantation. At this time the tumours were considerably smaller than those observed at the same time after transplantation in the control animals. The other two tumour-bearing mice in the cell-injected group were killed 13 weeks after transplantation. At this time the tumour size was comparable to that observed in the control mice 6 to 8 weeks after transplantation.

Autopsy findings

Immediately after sacrifice, the tumours were dissected free and removed *in toto*. The wet weights of the tumours, tumour growth period, and tumour incidence are shown in Table 1.

The tumours had a smooth outer surface.

Table 1. Some data regarding the growth of transplantable osteosarcomas in cell-injected and control animals

Groups of animals	Incidence (per cent)	Tumour growth period (weeks)	Tumour wet weight (mean ± s.d.; grams)
Cell-injected	14	7–13	2.17±2.53
Control	80	6–8	4.33±2.69

The cut sections were solid with rather sharply demarcated areas of bleeding. There was no evidence of bone formation macroscopically. Metastases to lungs or elsewhere were not found in any of the animals from any of the groups.

Light microscopy and histochemistry

All growing, transplanted tumours, whether from the controls or the cell-injected mice, were essentially similar both as to light and electron microscopic features and alkaline phosphatase activity. Therefore no differentiation will be made in the description below between the two groups of mice.

The light microscopic studies disclosed solid tumours rich in cells which usually were closely packed, occasionally in a parallel and whorled arrangement. Fibroblast-like cells predominated. They were more or less spindle shaped and possessed a moderate amount of cytoplasm with slight or moderate staining affinity. The cell boundaries were rather indistinct. Most nuclei were oval or rounded (Figure 1), and contained one, two or more, moderately large, distinct nucleoli. The chromatin was finely dispersed and the nuclear membranes rather distinct. Other fibroblast-like cells possessed more or less elongated nuclei with coarse chromatin pattern and rather indistinct nucleoli (Figure 2).

Osteoblast-like cells could be seen in some portions of the tumours. These cells were more sparsely represented than the fibroblast-like cells. They were often irregularly shaped and possessed rounded, oval or irregular nuclei with moderately coarse chromatin pattern and one or more moderately distinct nucleoli. The nuclear membranes were easily discerned. A moderate amount of light staining cytoplasm was observed. The cell membranes were indistinct.

Mitotic figures typical and atypical were found both among the fibroblast-like and osteoblast-like cells, often in great numbers (Figure 3). No bone or cartilage was identified in the tumours.

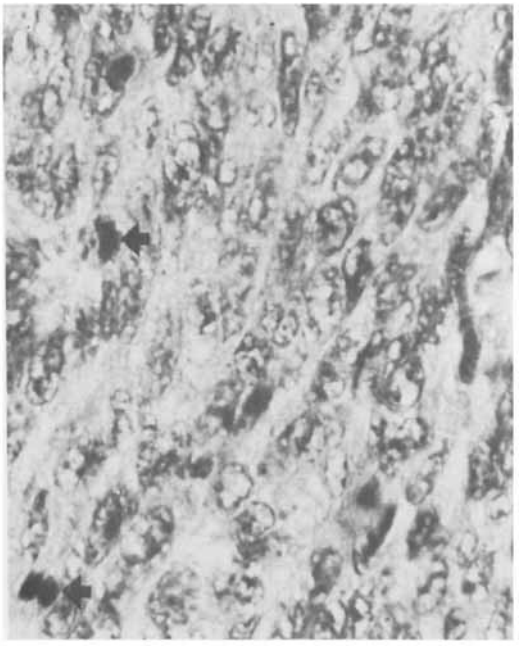

Figure 1. Photomicrograph of mouse osteosarcoma of fibroblastic type showing fibroblast-like cells with rather large, rounded or oval nuclei with moderately distinct nucleoli and mitotic figures (arrows) (Haematoxylin & eosin, × 500).

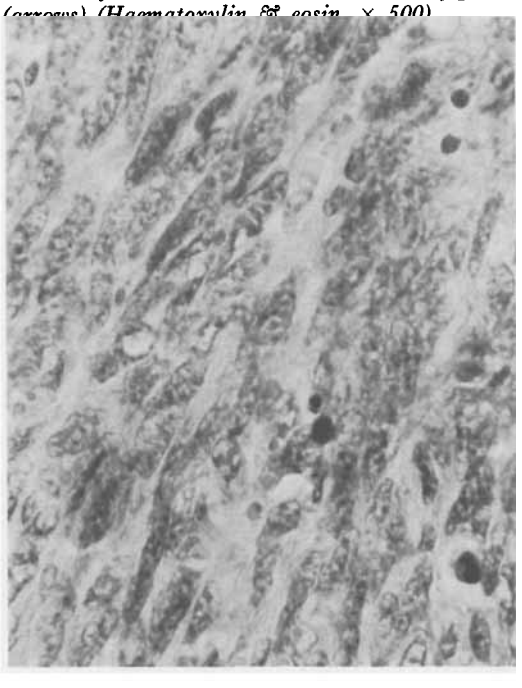

Figure 2. Mouse osteosarcoma demonstrating fibroblast-like cells with elongated nuclei possessing moderate chromatin density (Haematoxylin & eosin, × 500).

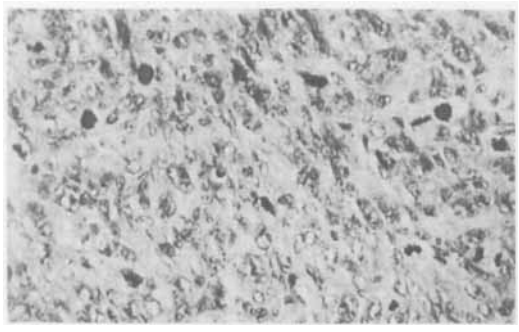

Figure 3. Area of mouse osteosarcoma demonstrating at least 12 mitotic figures (Haematoxylin & eosin, × 500).

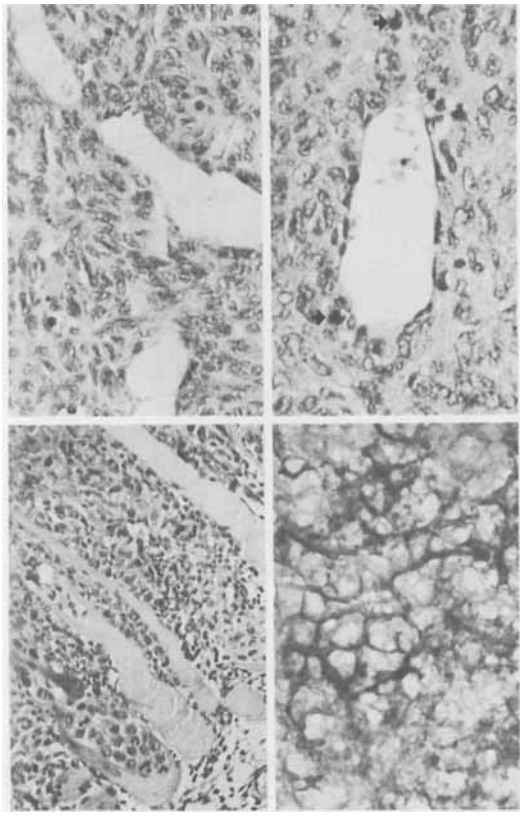

Figure 4. Rather wide blood spaces in mouse osteosarcoma (Haematoxylin & eosin, × 380).

Figure 5. Blood space in mouse osteosarcoma lined by endothelial-like cells. Erythrocytes are seen in the lumen. Mitotic figures are also demonstrated (arrow) (Haematoxylin & eosin, × 460).

Figure 6. Mouse osteosarcoma infiltrating among skeletal muscle fibres (Haematoxylin & eosin, × 270).

Figure 7. Alkaline phosphatase activity in mouse osteosarcoma (× 450).

In general, the polymorphism varied somewhat in different parts of the tumours. Most areas showed a moderate or high degree of polymorphism. Some portions of the tumours showed rich vascularization with the occurrence of widely anastomosing blood spaces containing erythrocytes (Figure 4). These blood spaces were mainly observed in areas with osteoblast-like cells. The vascular spaces were lined by more or less distinct endothelial cells (Figure 5). Areas of bleeding and necrosis could be observed in some parts of the tumours. The peripheral areas of the tumours occasionally exhibited a fibrous tissue capsule. Tumour infiltration was seen in parts of this capsule. Growth of tumour was also observed in adipose tissue and skeletal muscles (Figure 6).

Histochemistry disclosed unambiguous alkaline phosphatase activity in the tumours (Figure 7).

Electron microscopy

The findings made under the light microscope were verified ultrastructurally. Thus, there was a predominance of elongated fibroblast-like cells with rounded or elongated nuclei and a varying chromatin density and nucleolar size. The cytoplasm was moderately or sparsely developed and exhibited a moderate electron density, numerous free ribosomes and a rather prominent rough-surfaced endoplasmic reticulum. The cisterns of endoplasmic reticulum were either electron lucent or contained slightly electron dense flocculent material. The Golgi complex was rather inconspicuous. The mitochondria were medium-sized, and elongated or rounded, and showed distinct membranes and a moderate matriceal density. The cell membranes were irregular.

The osteoblast-like cells possessed rounded, oval or irregular nuclei with moderate chromatin density and nucleoli of varying size. A prominent rough-surfaced endoplasmic reticulum was observed. Amorphous masses of low density were seen in most cisterns of endoplasmic reticulum.

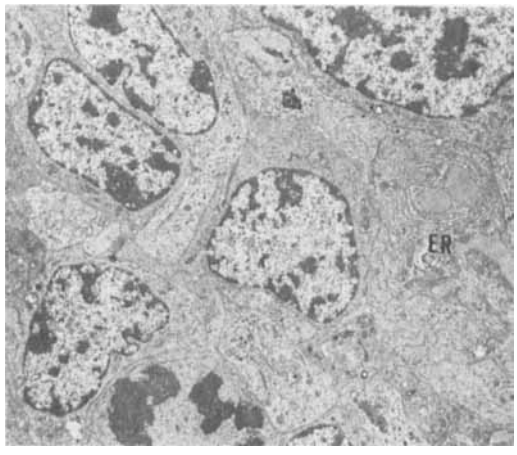

Figure 8. Electron micrograph of mouse osteosarcoma showing solid tumour tissue with one mitotic figure. Prominent whorled endoplasmic reticulum is seen in one of the tumour cells (ER) (× 3,000).

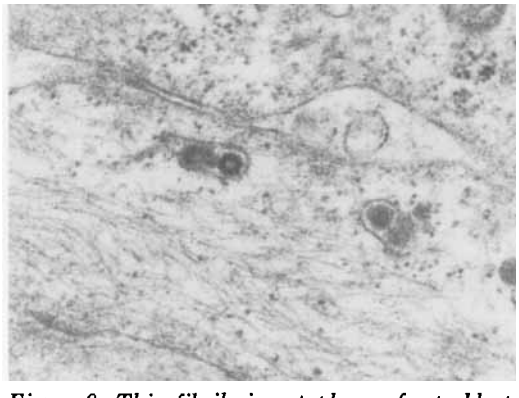

Figure 9. Thin fibrils in cytoplasm of osteoblast-like cell. Virus particles are seen in cisterns of endoplasmic reticulum (× 20,000).

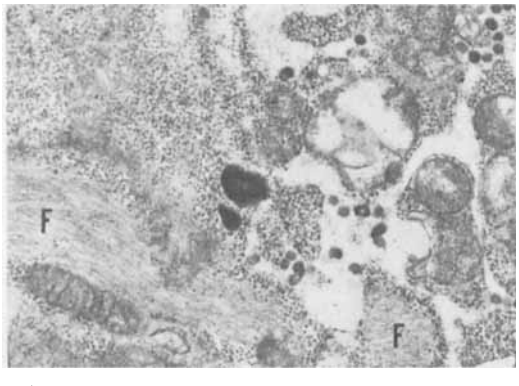

Figure 10. Cytoplasm of osteoblast-like cell containing bundles of fibrils (F). Virus particles are seen in cisterns of endoplasmic reticulum (× 10,000).

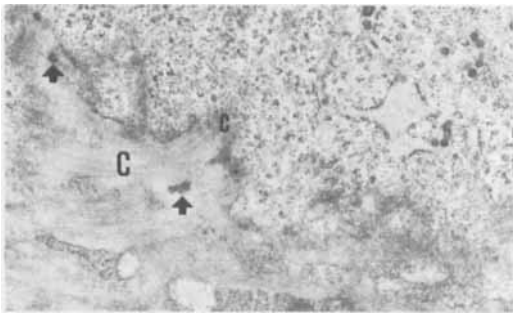

Figure 11. Extracellularly localized collagen fibres (C), at some places seemingly in contact with osteoblast-like cells (c). Virus particles are seen among the collagen fibres (arrows) and intracellularly in the cisterns of endoplasmic reticulum (× 8,000).

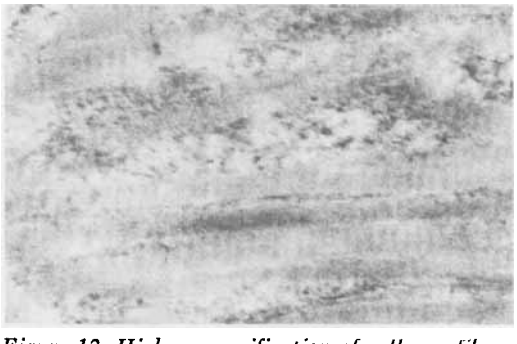

Figure 12. Higher magnification of collagen fibres at periphery of osteoblast-like cell (× 38,000).

The Golgi complex was rather large. The mitochondria were small or medium-sized and possessed distinct cristae. A moderate density was seen in the matrix of the mitochondria. The cytoplasmic ground substance exhibited varying electron density and a varying number of free ribosomes. Mitotic figures were frequently encountered among the tumour cells both of the fibroblast-like and osteoblast-like type (Figure 8).

Thin cytoplasmic fibrils of low or moderate density were seen in the cytoplasm of many osteoblast-like cells (Figure 9). In some cells bundles of fibrils were observed (Figure 10). Collagen fibres were seen extracellularly (Figure 11). Occasionally signs suggestive of formation of collagen fibres from osteoblast-like cells were found (Figures 11 and 12). Microtubules were observed in the osteoblast-like cells (Figure 14).

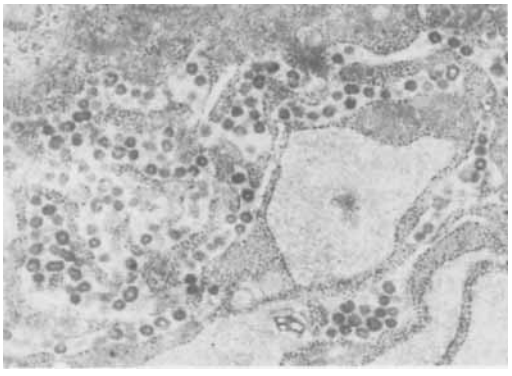

Figure 13. A rich number of virus particles in widened cisterns of rough endoplasmic reticulum (× 9,000).

Virus particles

A prominent ultrastructural feature was the presence of virus particles in the tumours. They occurred with varying frequency both in the fibroblast-like and in the osteoblast-like cells, as well as extracellularly. Most virus particles were localized to the cisterns of rough endoplasmic reticulum (Figures 9, 10, 11 and 13). A few virus particles were localized to electron lucent, smooth vacuoles in the cytoplasm of the tumour cells. The extracellular virus particles were found among the collagen fibres and often close to the plasma membranes. Budding of virus particles was seen both in endoplasmic reticulum (Figure 14) and extracellularly (Figure 15).

Growth in vitro

The original cell line grew as fibroblast-like cells in a monolayer. When confluency was achieved the cells grew in a criss-cross pattern with extensive piling up. The transplantable solid tumour was easily converted to a monolayer cell line again. This cell line had the same general culture characteristics as the original cell line. Mycoplasma could not be detected. However, in many dishes a spontaneous cytopathic effect (CPE) was observed, especially when

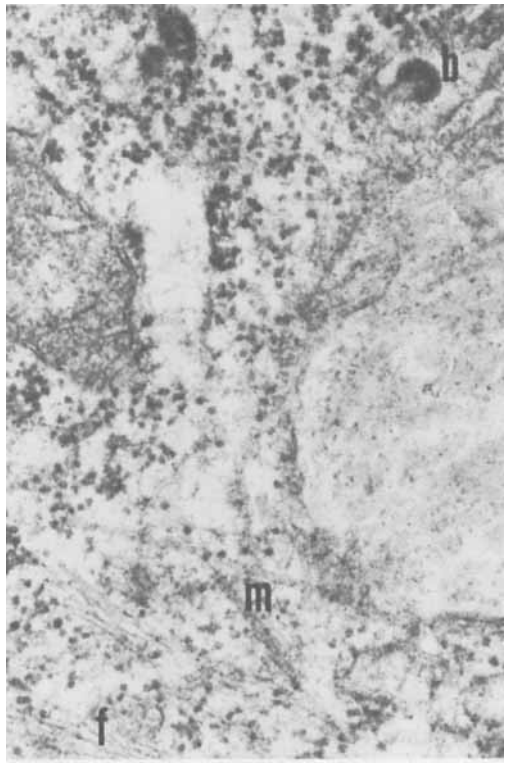

Figure 14. Portion of osteoblast-like cell demonstrating cytoplasmic fibrils (f), microtubules (m) and budding of virus particle (b) (× 22,000).

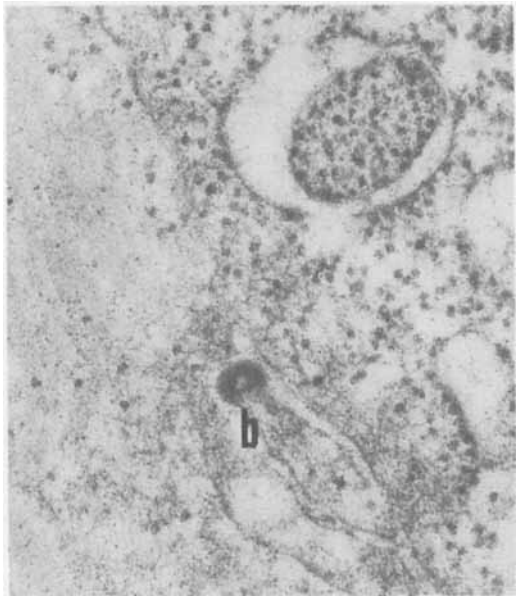

Figure 15. Peripheral portion of osteoblast-like cell demonstrating budding (b) of virus particle. Fibres are seen extracellularly (× 24,000).

the cells grew in the same medium for more than 2 days. In this study no attempts were made to isolate a virus from the cultivated cells.

DISCUSSION

The transplantable tumour in our mice displayed considerable anaplasia, high mitotic activity, unequivocal although relatively sparsely occurring osteoid, and clear alkaline phosphatase activity. Fibroblast-like cells predominated. Osteoblast-like cells showed signs suggestive of production of collagen fibres. No chondromatous components were found. We therefore classify the tumour as osteosarcoma of fibroblastic type.

The tumour described here exhibited only sparse bone formation and displayed some characteristics similar to those described for the so-called Ridgway osteosarcoma, which, however, is transplantable only in AKR mice (cf. Urist et al. 1977). Our tumour differed also from the metastasizing line of the Dunn osteosarcoma (cf. Miller et al. 1976).

Because of the rich vascularization of the tumour, a so-called teleangiectatic osteosarcoma could be considered. The presence, or at least the prognostic significance, of this type of osteosarcoma has been questioned in human bone pathology. In our opinion, a few human osteosarcomas are so distinctly teleangiectatic that their distinction from other types of osteosarcomas is warranted (Larsson et al. 1978b). The pattern of vascularization of the mouse osteosarcoma described here was not prominent enough for classification as teleangiectatic osteosarcoma. Thus, we regard our transplantable mouse tumour as an osteosarcoma of fibroblastic type with rich vascularization. The light microscopic, histochemical and ultrastructural features were not essentially different from those of human osteosarcoma.

A prominent ultrastructural feature in the neoplastic transplanted tissue was the occurrence of virus particles, which can be observed also in Dunn osteosarsarcoma. It is not known if the observed CPE in the cell cultures was due to the same virus, nor is it known if the observed virus particles represent a tumour virus activated in the allogenic system used. The particles might well represent a C-type RNA virus, which according to Urist et al. (1977) occurs in the Dunn osteosarcoma. Characterization of the virus and its ability to transform bone cells into tumour cells will be the subject of further study. Development of osteosarcoma in rats at the site of intratibial injection of murine sarcoma virus (Moloney) has recently been reported (Urovitz et al. 1976).

Injections of tumour cell suspension obtained from cultures of the original Dunn osteosarcoma more than 6 months before the transplantation evoked a high degree of resistance against the tumour by the host. There are good reasons to believe that this was of immunological nature, possibly because of incompatibility with respect to major transplantation antigens, assuming that the present tumour-host system is genetically different from the original Dunn osteosarcoma-host system (cf. Miller et al. 1976).

The osteosarcoma obtained was easily transplantable in the allogenic host. Take incidence could be increased from 80 to 100 per cent if the weight of the transplant was increased from 0.03 to 0.06 g. The reason for the increased transplantability is not known. One possible explanation is insufficient immunological defence in relation to the increase in tumour burden. The observation that the easily transplantable tumour was not accepted by animals which had received cell suspension 6 months earlier is no proof of occurrence of a specific immunological tumour defence. However, the results of our current subsequent studies indicate the presence of a specific immunological defence towards the tumour cells, although immunotherapy with BCG did not have any effect in stimulating the immuno-defence any further (to be published).

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