Virchows Arch. A Path. Anat. and Histol. 364, 249—263 (1974)

The Mode of Growth of Experimental Metastases in Rabbit Femora

J. M. Faccini

University College Hospital Medical School, London

Received April 10, 1974

Summary. Implantation of the Brown-Pierce carcinoma into a burr hole made within the cortex of rabbit femora has produced an experimental model which imitates the situation that occurs with spontaneous skeletal metastases in humans and animals. By killing the animals at regular intervals after tumour inoculation it has proved possible to follow the mode of growth of this tumour within bone. Initially the tumour grew amid the repair tissue associated with the cortical defect. When the tumour mass had reached a significant size osteoclastic resorption of cortical bone was seen to occur both within the vicinity and at some distance from the tumour. When the resorption cavities thus produced were expanded sufficiently the tumour grew into them both by direct extension and embolisation—small emboli were found in enlarged resorption cavities at sites remote from the main tumour mass. In the bones from fluorotic animals, because expanded resorption cavities existed before the onset of the experiment, this tumour invasion of cortical bone occurred earlier. Electron microscopy revealed large numbers of autophagic vacuoles and lysosomes within the tumour cells and it is suggested that lysosomal enzymes play an important role in bone resorption associated with tumours.

The number of published pathological studies on the growth of metastases in bone is disproportionately small, considering the frequency with which human carcinomata metastasise to the skeleton. The mechanism whereby tumour metastases are able to resorb bone, furthermore, remains in dispute. The majority of authors favour a direct action of tumour cells on the bone matrix independent of the action of the normal processes of resorption in the presence of osteoclasts (Milch and Changus, 1956). Post mortem histological examination of bones from patients with skeletal metastases has shown, however, that cortical resorption cavities large enough to be recognisable macroscopically on radiographs do not always contain tumour cells although a metastasis is usually present in the adjacent medullary cavity; with subsequent enlargement these cavities, which are an exaggeration of normal Haversian remodelling, are found to contain tumour cells (Hodson *et al.*, 1970).

The following experiments were performed to follow, over a known time sequence, the effect on cortical bone of a tumour known to grow in the medullary cavity of rabbit femora after transplantation (Shivas *et al.*, 1963). In order to modify the process of resorption, three animals, that had previously received fluoride, were included in the study—the cortical endosteal bone of rabbits receiving large doses of fluoride contains expanded resorption cavities due to secondary hyperparathyroidism (Faccini, 1969).

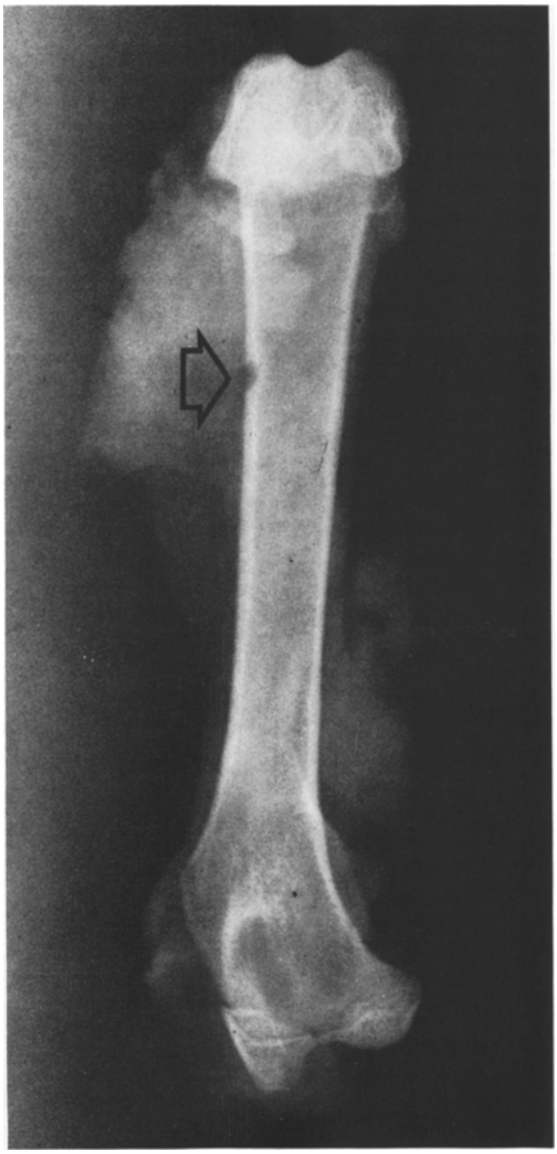

Fig. 1. Radiograph of femur from control animal killed 7 days after insertion of boiled tumour into burr hole in the cortex (arrow). There is some endosteal new bone formation closing off the medullary cavity

Materials and Methods

Twenty female New Zealand white rabbits aged between 4–6 months and weighing between 2–2$^1/_2$ kg were used for the experiment. Seventeen of the rabbits were inoculated with tumour and three acted as controls, receiving boiled tumour; the right femur of the experimental animals was inoculated with the tumour while the left served as further control bone. Three of the rabbits that received tumour had been given 200 ppm fluoride, as sodium

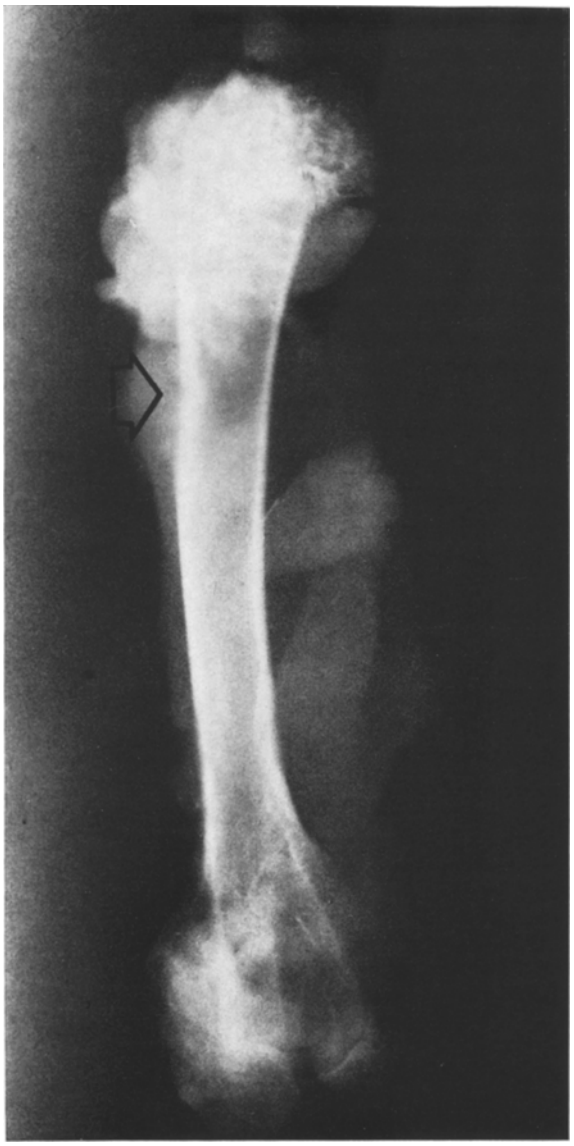

Fig. 2. Radiograph of control femur, 14 days after insertion of boiled tumour, showing the burr hole almost completely filled by new bone growth (arrow)

fluoride, in their drinking water over the previous eight weeks—they appeared healthy and were of average weight.

The tumour employed was the Brown-Pierce carcinoma—a squamous tumour of rabbits that has been maintained in serial passage for many years at the Chester Beatty Research Institute.

The method of inoculation was a simple modification of that used by Shivas *et al.* (1963). The animals were anaesthetised by intravenous pentothal and maintained, if necessary, with open ether. A longitudinal incision 4 cm long was made in the right thigh, the fascia incised

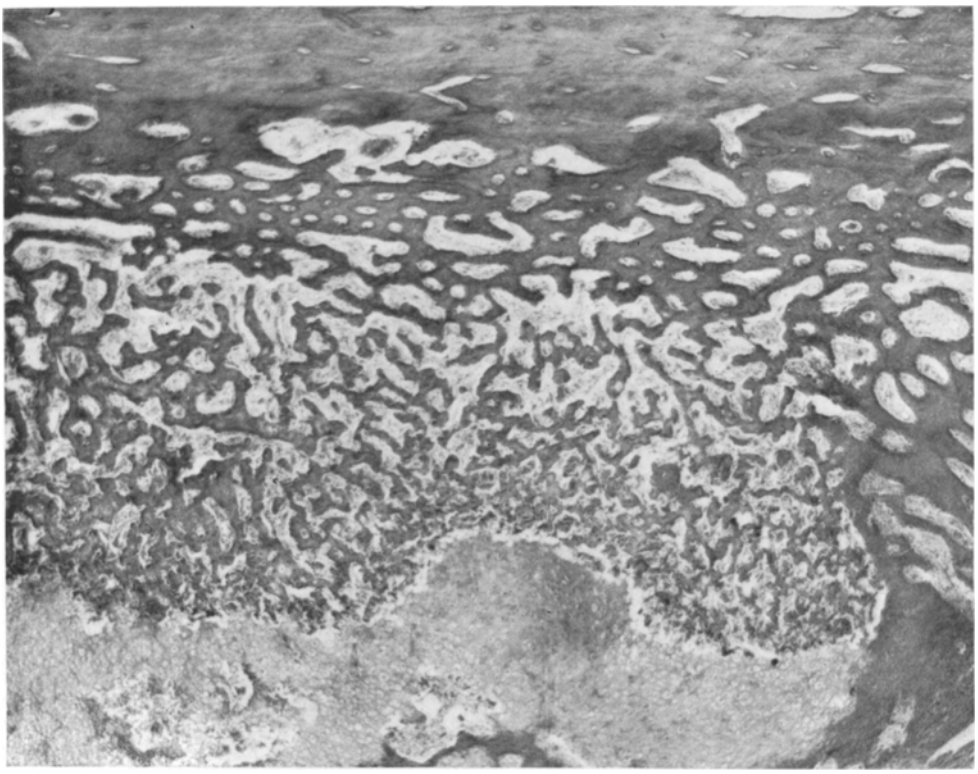

Fig. 3. Healing at the site of the cortical defect in the control bone at 14 days. Trabeculae of new bone are growing from the cortex above and are capped by cartilage below. H & E ×25

and the vasti separated by blunt dissection. The periosteum was exposed and incised longitudinally for a distance of approximately 5 mm. A hole through the cortex of the femur 2 cm from the lateral condyle was made using a sterile No. 1 rosehead dental burr, kept cool by dripping water. The size and position of the hole can be seen in Fig. 1. A piece of tumour approximately 1 mm cubed was inserted into the opening and left plugging it so that it presented on both the endosteal and periosteal surface of the cortex. The wound was closed in layers with catgut and silk sutures, the time of operation being of the order of five minutes. Exactly the same procedure was employed for the control animals except that the tumour was boiled for thirty minutes beforehand.

Six of the rabbits inoculated with live tumour and one with boiled tumour were killed after seven days. The other control rabbits plus six inoculated with live tumour (including the three fluorotic animals) were killed after fourteen days and the remaining five at twenty-one days.

Both femora were removed and fixed in cold formol calcium, after x-ray the bones were sawn longitudinally in half and decalcified in formol-formic acid solution.

In three instances the femora were sawn open *in situ*, immediately after death, and specimens of tumour and adjacent trabecular bone were fixed in cold 3.125 per cent gluteraldehyde in a 0.05 M phosphate buffer at pH 7.3 and post-fixed in osmium tetroxide for examination by electron microscopy. All bones were wax-embedded and stained with haematoxylin and eosin or the Gordon and Sweet method for reticulin and examined under normal and polarised light.

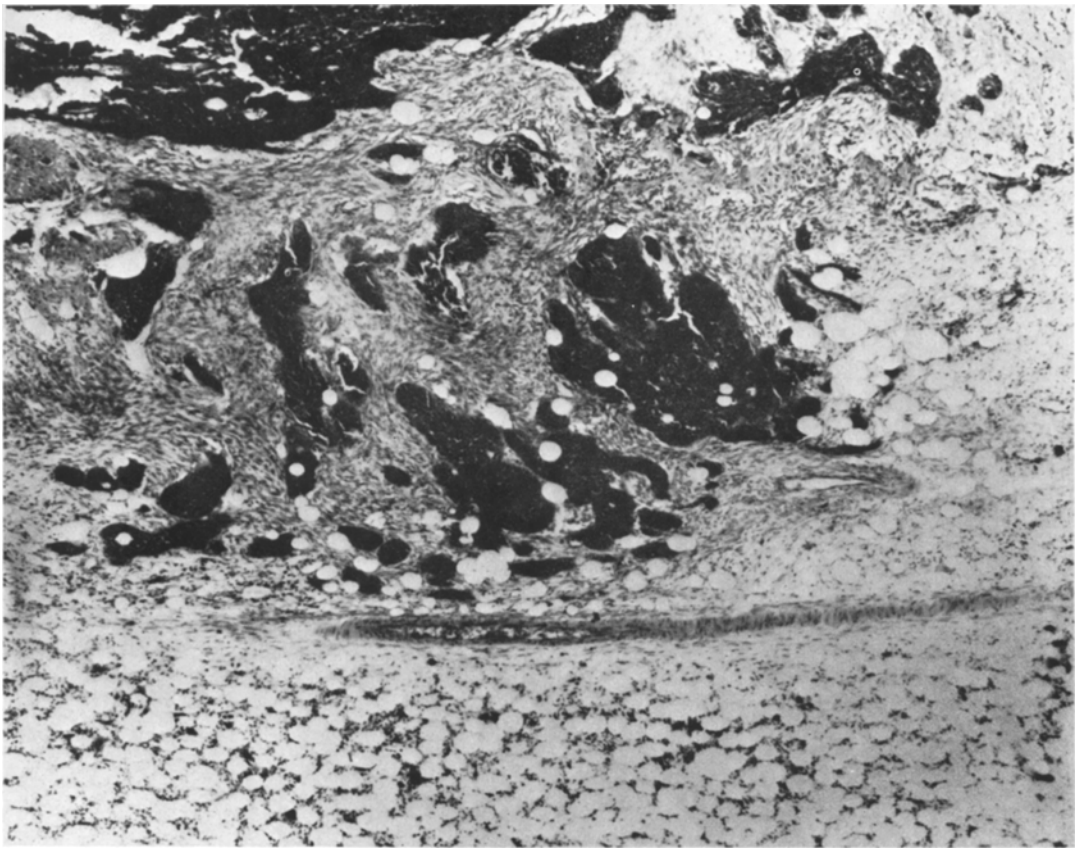

Fig. 4. Seven days after inoculation of tumour. Islands of tumour surrounded by fibrous tissue in the marrow cavity. H & E ×40

Results

A. Femora Inoculated with Boiled Tumour (Control Bone)

Radiographs of an animal killed after seven days and another after fourteen days are shown in Figs. 1 and 2 respectively. It can be seen that by seven days endosteal proliferation has closed the defect on the medullary surface and that this has increased by fourteen days, filling up the gap on the medullary side and producing a nodule of bone protruding into the medullary cavity. Histology showed considerable cartilage proliferation, and thin trabeculae of new bone can be seen to radiate from the periosteal surface (Fig. 3). The response to repair is well localised to the region of the defect extending for approximately 1.5 mm on the periosteal surface and 1 mm on the endosteal side. In one animal there was a definite focus of new bone formation on the other endosteal surface opposite the hole; there was no associated periosteal reaction on that side.

At fourteen days in one section two small islands of totally necrotic tumour could be seen in the periosteal fibrous tissue, but the remainder had apparently been removed.

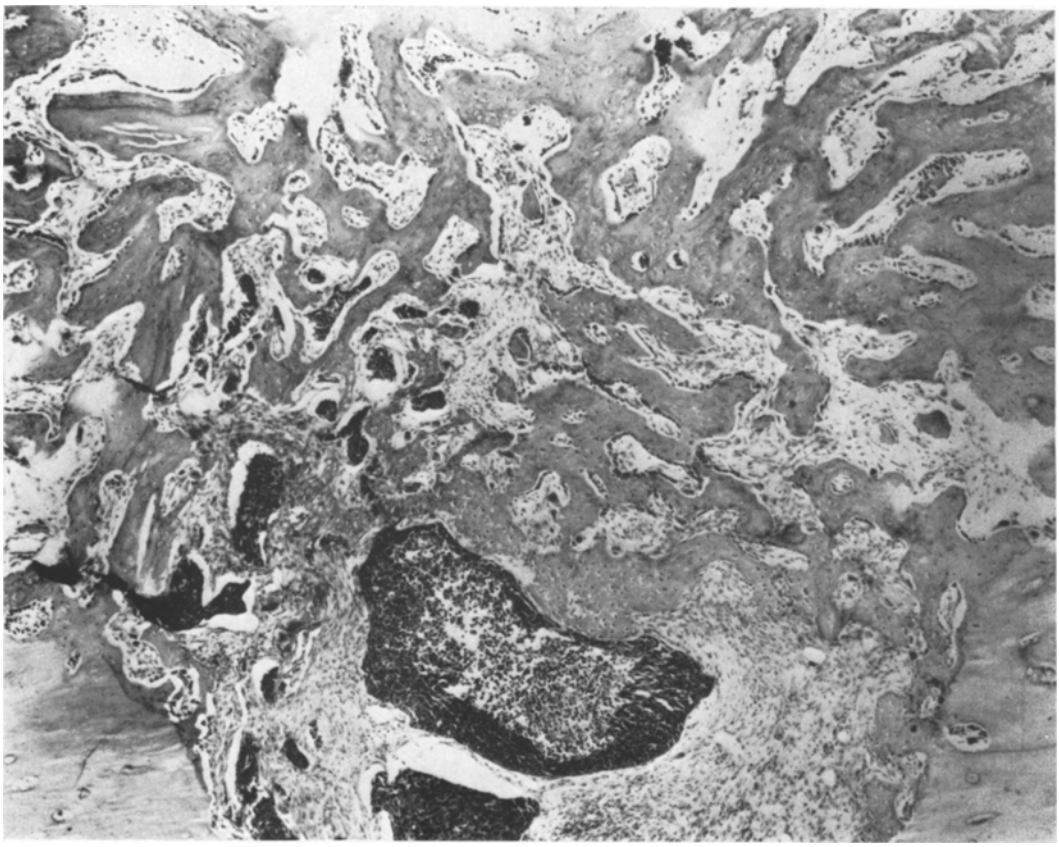

Fig. 5. Fourteen days after inoculation of tumour. Islands of tumour are present between trabeculae of new bone. The cortical defect below mostly contains fibrous tissue with tumour. H & E $\times 100$

B. Femora Inoculated with Live Tumour (Seven Days After Inoculation)

Viable tumour was present in all the animals killed after one week. There was a marked fibrous reaction within the cortical defect, around the periosteal surface and in the medullary cavity. The tumour had mainly formed discrete islands of cells separated by the fibrous tissue (Fig. 4) and there was new bone formation in the immediate vicinity. The extension of the tumour was greater on the periosteal surface than within the medulla and small emboli were present within parosteal blood vessels and lymphatics up to 1 cm from the site of inoculation and, in one animal, islands of tumour were to be found in the thigh muscles. Beneath the periosteum and adjacent to the fibrous reaction, spicules of new bone continued to be more extensive than within the shaft and had spread well beyond the site of inoculation into the soft tissues of the thigh and invading muscle. Periosteal new bone growth was abundant and had occurred also on the opposite side of the femur from the site of inoculation.

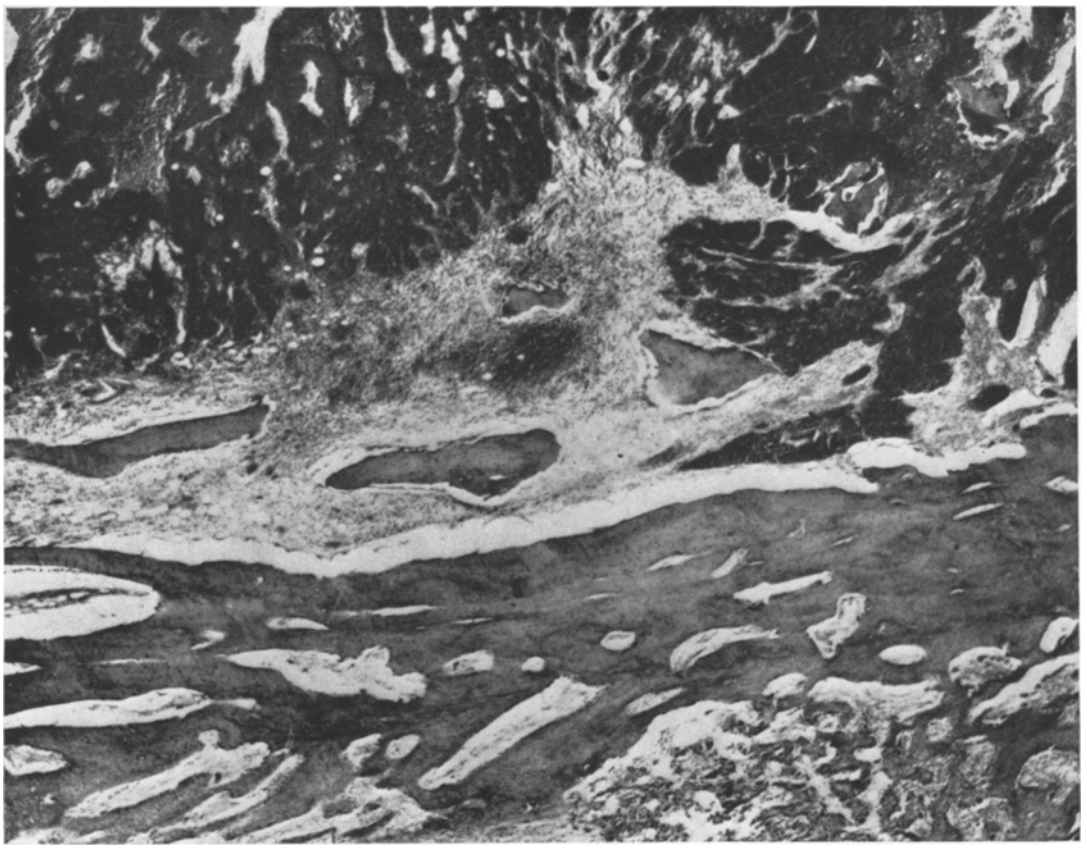

Fig. 6. Fourteen days after inoculation of tumour into fluorotic bone. The tumour fills the medullary cavity. There is no endosteal new bone growth but periosteal new bone growth is occurring (at bottom of picture). Note enlarged resorption cavities within the cortex. H & E ×100

In the femora from the animals that had received fluoride, in addition to the characteristic changes of fluorosis—widening of the shaft with thickening of cortical bone—there was considerably more tumour growth and resorption of bone. Tumour growth within the medulla extended distally to the metaphyseal vault and there was a complete lack of response by endosteal bone (Fig. 6); the only spongy bone present within the diaphysis in the region of the tumour was metaphyseal bone that had survived the normal processes of resorption prior to inoculation. This mature spongy bone, and the adjacent endosteal cortical bone, was now undergoing resorption by the tumour. Where the tumour was in direct contact with this resorbing bone, osteoclasts were not apparent but they could be discerned in resorbing bone, not adjacent to, but still in the vicinity of the tumour.

The earliest instance of invasion of the cortex by tumour was seen in this fluorotic bone: Fig. 7 shows a tumour embolus situated in a vascular canal within a large space in the cortex, in this instance, at a distance of 5 mm from the main body of the tumour.

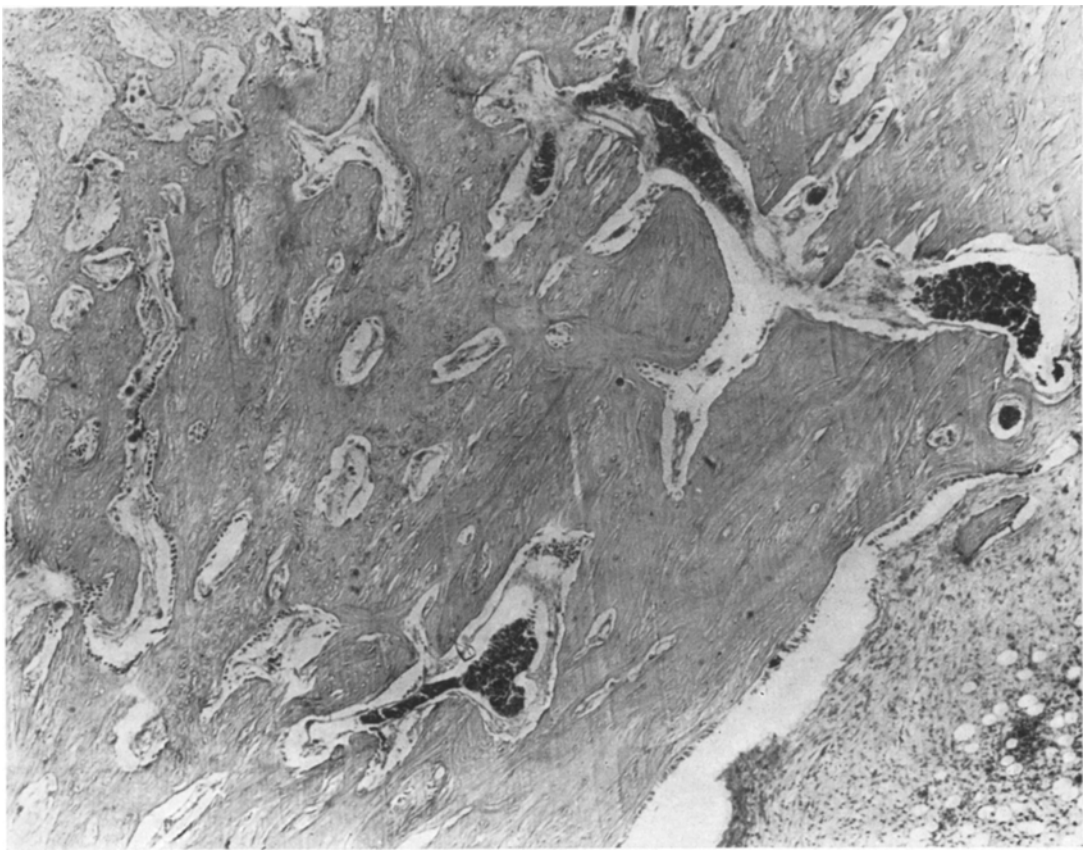

Fig. 7. Fourteen days after inoculation of tumour into fluorotic bone. Tumour emboli are present within resorption cavities in the cortex. There is no tumour in the immediately adjacent area of the medulla in the bottom right-hand corner. H & E ×100

In marked contrast to the absence of endosteal new bone growth and cartilage formation within the medulla, periosteal new bone growth was abundant. Conversely, tumour growth in this region was not as extensive as that in the medulla of these fluoride animals or periosteal tissue of the other animals; small emboli, however, could be discerned in the superficial tissues of the thigh.

21 Days after Inoculation. The tumour had taken in 4 of the 5 animals killed 21 days after the implantation of the tumour, and by this time there was a noticeable swelling on the lateral aspect of the thigh.

In the medullary cavity, the tumour, which now contained moderately large areas of necrosis, had reached the metaphyseal spongy bone but had not penetrated as far as the growth cartilage. Outside the femur it had extended as far as the distal epiphysis and had infiltrated the soft tissues including muscle extensively, and had encircled the femur to the medial aspect. Cartilage formation and new bone growth was considerably reduced and the picture was now one of considerable bone loss.

Fig. 8. Twenty-one days after inoculation of tumour. Tumour is located in the periosteum (above) and the medullary cavity (below); enlarged resorption cavities are present within the cortex and tumour emboli are present within some of them. H & E ×25

Resorption of cortical bone had occurred on both sides of the shaft. The tumour had grown along the endosteal and periosteal surfaces of the bone and filled the endosteal half of the cortical defect which showed a little evidence of repair. Wide resorption spaces had filled the cortex and the tumour had grown into these (Fig. 8). With regard to the mode of formation of these resorption cavities, it could be seen from those situated some distance from the main tumour mass that they were lined by conspicuous numbers of osteoclasts and simply contained blood vessels within loose connective tissue or in some instances marrow (Fig. 9). Fig. 10 shows an area of cortex which was remote from the main tumour mass, but contains tumour emboli within enlarged resorption cavities, a picture reminiscent of a spontaneous metastasis in human bone.

Besides enlarged resorption cavities, there was further evidence of bone removal around osteocyte lacunae, the dimensions of which were increased in size. These two parameters of increased resorption were not observed in the opposite femur taken from these animals killed 21 days after the inoculation of tumour, or in the femora of the control animals that received boiled tumour.

Electron microscopy of pieces of tumour from the medullary cavity showed cells with enlarged nuclei possessing a crenated border, prominent nucleoli and a

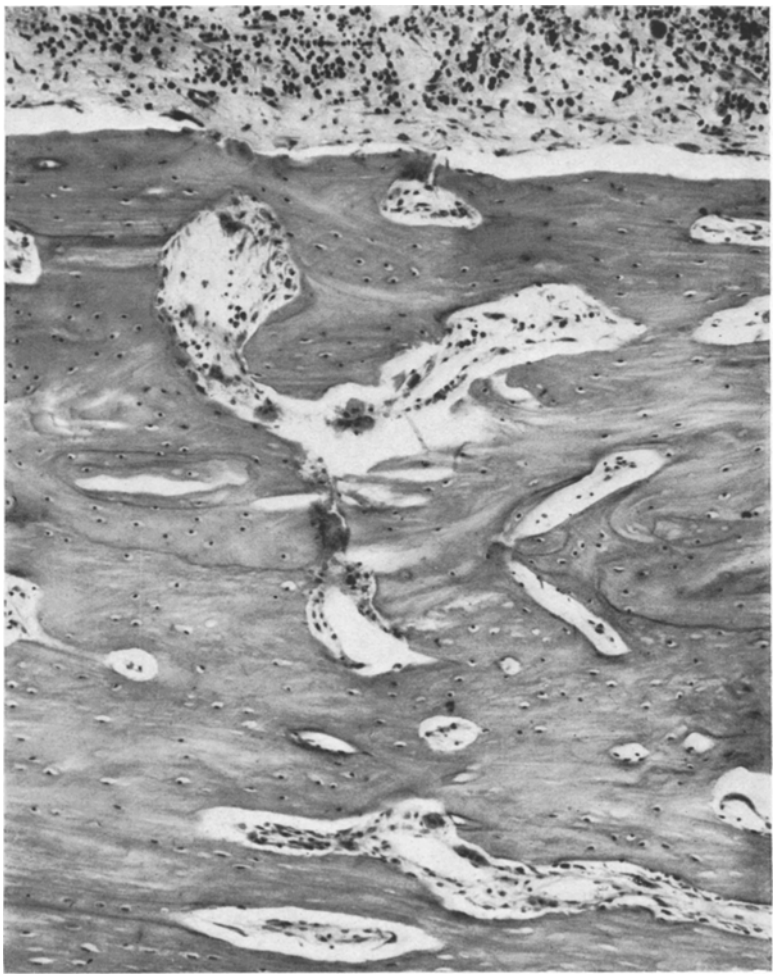

Fig. 9. Twenty-one days after tumour inoculation. Cortical bone showing resorption cavities with osteoclasts. There was no tumour in the immediately adjacent medulla. The lamellar character of the bone contrasts with the more woven type seen in the fluoride bone in Fig. 7. H & E ×400

dense chromatin pattern and frequent mitoses indicated by loss of the nuclear membrane and free chromatin in the cells. Many intracellular lysosomes and autophagic vacuoles were observed, irrespective of the size of the tumour mass or concomitant necrotic changes (Fig. 11).

Discussion

Implantation of the Browne-Pierce carcinoma into the femora of rabbits yielded viable tumour in 6 out of 6 animals at 7 days, 4 out of 6 at 14 days, and 4 out of 5 at 21 days. In the animals in which tumour survived it grew progressively

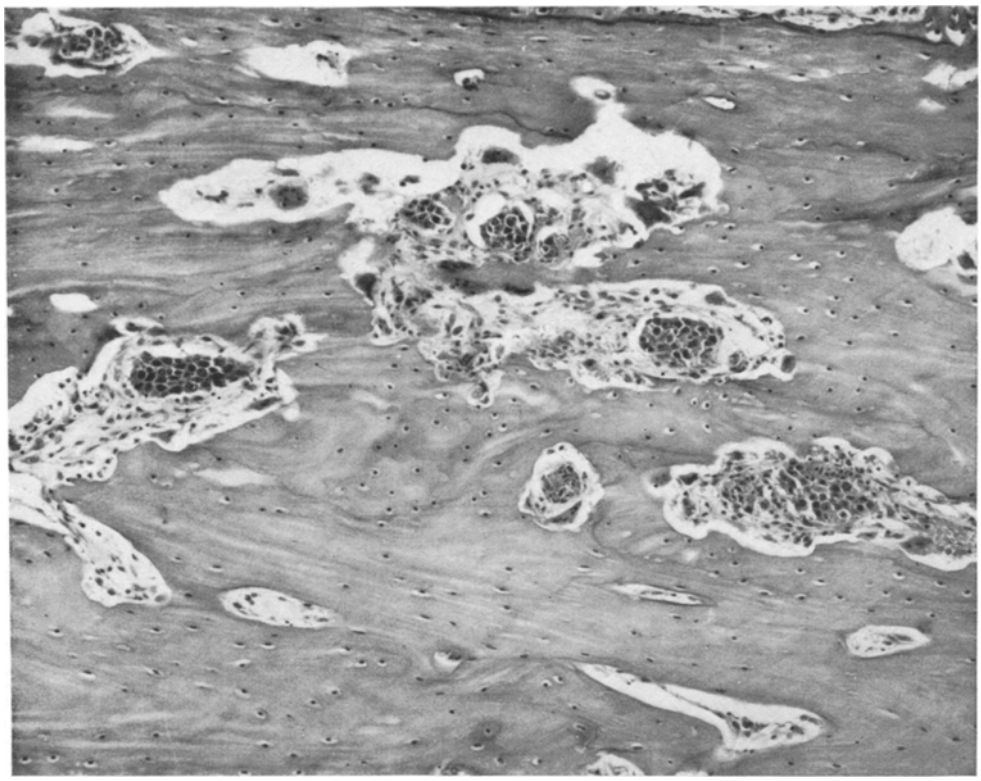

Fig. 10. Twenty-one days after tumour inoculation. Tumour emboli are present within enlarged resorption cavities in cortical bone. Numerous osteoclasts are present, some within Howship's lacunae. H & E ×400

within the medulla and especially in the paraosteal tissues. There was very considerable repair with new bone formation in the first two weeks. After three weeks this was much less, but resorption of the cortical bone became evident. In some cases the resorption appeared related to osteoclasts, in others to the presence of adjacent tumour. In animals inoculated with the tumour who had previously received fluoride, these changes were modified in that resorption was present in the cortical bone at 14 days, and new bone formation was absent within the medulla. Growth of tumour was extensive within the medulla and invaded the cortex.

Despite the fact that carcinomata in small mammals rarely, if ever, metastasise to bone, the implantation of the Brown Pierce carcinoma into the femora of rabbits has reproduced a picture similar to that seen in spontaneous metastases in the skeleton of humans or other larger mammals (Misdorp and Den Herder, 1966). The important points of similarity are the growth of tumour from the medulla into the cortex, including the formation of emboli well away from the main tumour mass, and the presence of enlarged resorption cavities within cortical bone. The definite time scale that the experimental model provides has shown

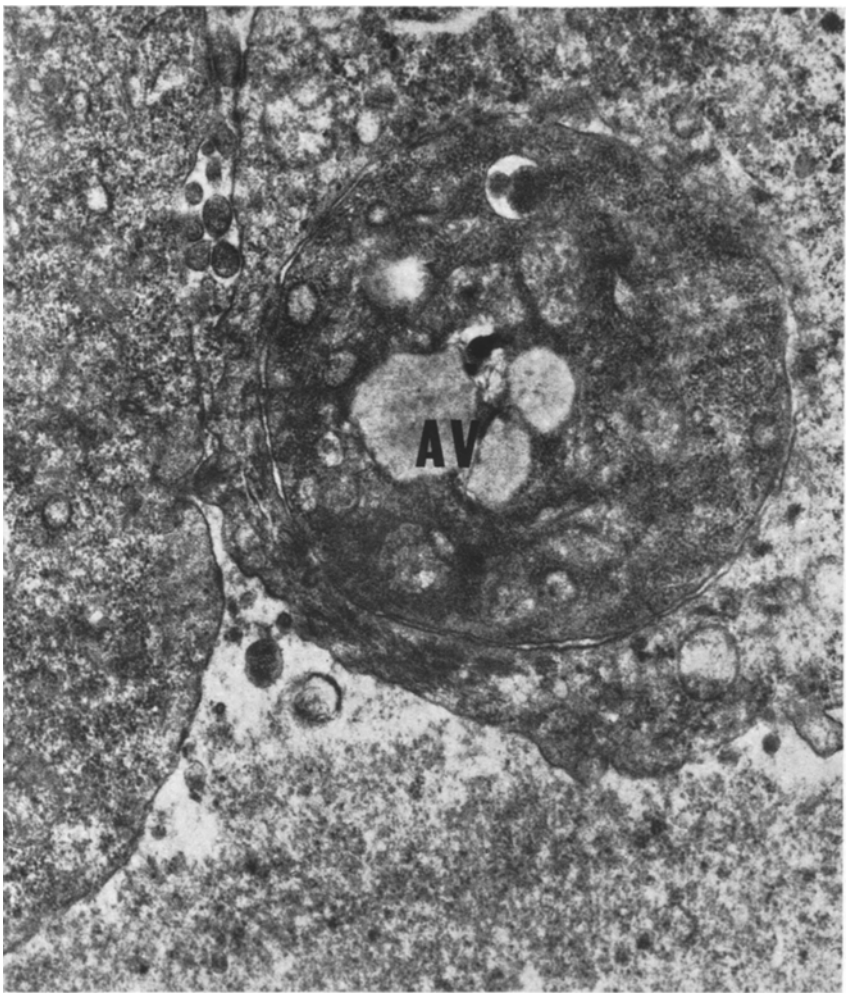

Fig. 11. Electron micrograph of part of cell from tumour showing autophagic vacuole (*AV*), completely separate from surrounding cytoplasm. Lead citrate and uranyl acetate ×15200

that it is not until tumour growth is extensive that resorption of cortical bone occurs; when this is present, invasion of the cortex is then seen, but not all resorption cavities are found to contain tumour; furthermore, the enlarged cortical cavities produced by fluoride enabled invasion to occur earlier, strongly suggesting that resorption occurs before the tumour grows into the cortex. Where the tumour was in direct contact with bone, especially on the endosteal surface of the cortex, osteoclasts were not seen which could mean that they were either replaced by tumour or were never there—the question of tumour cells directly resorbing bone in these circumstances is not, therefore, resolved, and further experiments are necessary.

A modification of the time scale and technique from that used by Shivas *et al.* (1963), who similarly implanted the Brown-Pierce carcinoma into rabbit femora, has resulted in an entirely different interpretation of the mechanism of growth of tumour in bone from that reached by these authors. They implanted the tumour bilaterally in all the animals they studied, pushing the tumour through into the medullary cavity and plugging the hole in the cortex with zinc oxide cement; they killed the animals from 3–14 weeks after inoculation. They concluded from this experiment that the cortex was not invaded until the main tumour mass reached it after filling the medullary cavity, and that invasion was the result of the "tissue pressure" being greater within the tumour than in the blood vessels within the cortex, they emphasised that "there was no evidence to suggest that osteoclasts played any significant part in bone destruction". This latter observation was also reached by Milch and Changus (1956), in a study of post mortem material, who digressed to invoke the arguments that osteoclasts are not involved in resorption. Milch and Changus (*ibid*) had concentrated their attention mainly to the changes observed near metastases in the medulla; however, examination of the cortex at some distance from tumour metastases will reveal evidence of resorption in the presence of osteoclasts in human bone (Faccini, unpublished observations).

A discussion on the role of osteoclasts in the process of resorption is not necessary to an argument on the mechanism of bone destruction in the presence of metastatic tumour; whether the osteoclast actively resorbs the bone or appears merely as the result of bone removal, it is ultimately associated with resorption. The finding of osteoclasts, therefore, in the vicinity of metastatic tumour is an indication of a stimulus to resorption associated with the presence of that tumour. The identification of osteoclasts, however, is not the only indication of increased resorption. In the animals in which tumour had invaded the cortex there were changes not seen in the cortices of the control bone: enlarged resorption cavities containing marrow elements or blood vessels, and widened osteocyte lacunae—an indication of bone removal (Belanger *et al.*, 1963). If is of interest in this respect that Shivas *et al.* (1963) describe the absence of osteocytes in cortical bone near the margin of growing tumour; they do not comment on this but Belanger and his co-workers believe that osteolysis is eventually associated with the death of osteocytes (Belanger *et al.*, 1963, 1966). It would appear from these experiments that the tumour does not invade cortical bone until large resorption cavities exist to receive it: this phenomenon was seen after three weeks when the volume of tumour was considerable, but it also occurred after two weeks in the fluorotic bone, owing to the fact that resorption cavities were already present before the experimental inoculation of tumour.

The mechanism whereby metastatic tumour produces osteolysis remains speculative, but the fact that cortical bone is resorbed in the vicinity of tumour cells while not in contact with them suggests a humoral process. Milch and Changus (1956), while deciding against such a process, do concede that there is frequently a gap between the tumour and the resorbing bone.

There is strong evidence that resorption of bone is basically a removal of matrix (Belanger, 1965) by lysosomal enzymes (Vaes, 1965) and degenerating malignant cells are known to contain increased numbers of lysosomes (Anton and

Brandes, 1968). Such cells in this tumour were observed to contain lysosomes and autophagic vacuoles—the latter a rich source of lysosomal enzymes found to be capable of degrading cartilage matrix (Dingle *et al.*, 1969). Furthermore, direct evidence for lysosomal release being involved in invasion of cartilage by tumour in the xiphisternum of rats has been shown in a histochemical study by Poole (1970).

An alternative mechanism has been proposed by Tashjian *et al.* (1972): they showed that bone resorption in tissue culture by the $HSDM_1$ fibrosarcoma of mice was most probably due to the release of prostaglandin E_2 by the tumour—prostaglandins are a well known stimulus of bone resorption in tissue culture (Klein and Raisz, 1970). This does not explain all the phenomena of bone resorption by tumours, however, as the same authors found that the subcutaneous injection of prostaglandin E_2 *in vivo* failed to reproduce the hypercalcaemia that accompanies the inoculation of the $HSDM_1$ tumour *in vivo*. It is unlikely that a universal mediator of calcium mobilisation common to all malignant tumours exists as some tumours associated with hyperparathyroidism are known to produce parathyroid hormone (Sherwood *et al.*, 1967).

The exact mechanism whereby tumours resorb bone is, therefore, in doubt but the experiment reported here has shown that they are capable of doing so by a humoral process supporting the *in vitro* studies in the literature.

When a resorption cavity produced by this process is large enough the tumour will invade cortical bone.

The author is extremely grateful to Dr. R. L. Carter of the Chester Beatty Research Institute who as well as supplyng the Brown Pierce Carcinoma acted as anaesthetist in the experiments, and to Professor J. F. Smith for his help and encouragement.

References

Anton, E., Brandes, D.: Lysosomes in mice. Mammary tumours treated with cyclophosphamide, Cancer (Philad.) **21**, 483–500 (1968)

Belanger, L. F.: In The parathyroid glands, ultrastructure secretion and function, ed. Gaillard, P. J., Talmage, R. V., and Budy, A. M. Chicago: Chicago Univ. Press 1965

Belanger, L. F., Robichon, J., Migicovsky, B. B., Copp, D. H., Vincent, J.: In Mechanisms of hard tissue destruction, ed. Sognnaes, R. F. Washington D.C.: Am. Ass. Adv. Sci. 1963

Belanger, L. F., Semba, T., Tolnai, S., Copp, D. H., Rook, L., Gries, C.: 3rd European Symposium on calcified tissues, ed. Fleisch, H., Blackwood, H. J. J., and Owen, M. Berlin-Heidelberg-New York: Springer 1966

Dingle, J. T., Fell, H. B., Glauert, A. M.: Endocytosis of sugars in embryonic skeletal tissues in organ culture IV. Lysosomal and other biochemical effects. J. Cell Sci. **4**, 139–154 (1969)

Faccini, J. M.: Fluoride and bone. Calcif. Tiss. Res. **3**, 1–16 (1969)

Hodson, C. J., Faccini, J. M., Craven, J. D.: Para haversian canal resorption in Symposium Ossium, ed. Jellife, A. M., and Strickland, B. London: E. & S. Livingstone 1970

Klein, D. C., Raisz, L. G.: Prostoglandins: stimulation of bone resorption in tissue culture. Endocrinology **86**, 1436–1440 (1970)

Milch, R. A., Changus, G. W.: Response of bone to tumour invasion. Cancer (Philad.) **9**, 340–351 (1956)

Misdorp, W., Den Herder, B. A.: Bone metastases in mammary cancer. Brit. J. Cancer **20**, 496–503 (1966)

Poole, A. R.: Invasion of cartilage by an experimental rat tumour. Cancer Res. **30**, 2252–2259 (1970)

Sherwood, L. M., O'Riordan, J. L. M., Aurbach, G. M., Potts, J. T., Jr.: Production of parathyroid hormone by non-parathyroid tumours. J. clin. Endocr. **27**, 140–146 (1967)

Shivas, A. A., Black, W. J., Finlayson, N. D.: The growth of the Brown-Pearce carcinoma in the medullary cavity of the rabbit femur. Brit. J. Cancer **17**, 711–714 (1963)

Tashjian, A. H., Jr., Voelkel, E. F., Levine, L., Goldhaber, P.: Evidence that bone resorption-stimulating factor produced by mouse fibrosarcoma cells is prostagland in E_2. J. exped. M. **136**, 1329–1343 (1972)

Vaes, G.: 2nd European Symposium on calcified tissues, ed. Richelle, L. J., and Dallemagne, M. J. Université de Liège **1965**

Dr. J. M. Faccini
Dept. of Morbid Anatomy
U.C.H. Medical School
University Street
London, WC1E 6JJ
England