Retrovirus-Induced Osteopetrosis in Mice

Ultrastructural Evidence of Early Virus Production in Osteoblasts and Osteocytes

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Newborn female strain NMRI mice were given injections of a mouse retrovirus (OA MuLV) known to induce osteopetrosis, osteoma, and lymphoma. Femur metaphyses and lumbar vertebrae were investigated ultrastructurally 3 d, 7 d and 28 d after infection. Budding, immature and mature virus was observed associated with osteoblasts and osteocytes, but not with osteoclasts or chondrocytes, 28d after infection with the virus. No production of virus particles was observed in bone-tissue in mock-treated controls. Thus, the primary target cell for OA virus in bone appears to belong to the osteoblastic/osteocytic cell lineage. (Am J Pathol 1986, 124:319-323)

AT PRESENT much research is devoted to elucidating the role of retroviruses in the etiology of cancer and other proliferative and nonneoplastic disorders. However, reports concerning viruses which specifically affect bone cells are fairly rare. Recently we described the characteristics of a murine retrovirus (OA MuLV) isolated from spontaneous osteomas in Strain 101 mice that is capable of inducing osteopetrosis, osteomas, and lymphoma.[1] Osteopetrosis was induced in more than 60% of infected NMRI mice. It developed preferentially in the femur metaphysis, os ileum, and lower part of the vertebral column. In the following we describe the results of an experiment aimed at identifying the primary target cell in bone for this virus after infection of newborn NMRI mice.

Materials and Methods

Virus

OA virus was isolated from spontaneous osteomas in two old female 101 strain mice and further propagated in NIH 3T3 (mouse embryo fibroblast) cells after 3 cycles of end-point dilution as described elsewhere.[1] Cell-culture supernatant was filtered through 0.22-μ Millipore filters prior to injection.

Animals

All animals were obtained from our own breeding colonies. Animals were maintained under specific pathogen-free conditions and fed food and water *ad libitum*.

Fifteen newborn female NMRI mice were given intraperitoneal injections of 100 μl OA-virus suspension. The same number of animals, mock-treated with Dulbecco's MEM, were kept as controls.

Three, 7, and 28 days after injection 5 infected and 5 control animals were killed, and tissue was removed for further analysis as described below.

Electron Microscopy

The distal part of the femur was removed from both legs of each animal and either divided in two lengthwise or left whole. One of the lower lumbar vertebrae was also removed and divided lengthwise or left whole. The pieces of bone were immediately fixed in 3% glu-

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taraldehyde in cacodylate buffer. Tissues were postfixed in chrome-osmium, dehydrated in alcohol, and embedded in Spurr resin ("hard") under a weak vacuum, according to standard procedures. All pieces of bone were flat-embedded. The pieces of femur were oriented such that the growth-plate could be cut longitudinally, and the lumbar vertebrae such that they could be cut transversely.

One-micron sections were cut and stained with toluidine blue for light-microscopic examination. The best oriented block of femur and vertebra per animal was selected and trimmed to include the top of the growth plate (femur), or bone marrow together with mature bone trabeculae (vertebra). Thin sections were cut with a diamond knife, mounted on single-hole grids with a formvar-film support, and stained with uranyl and lead in an LKB Ultrostainer. Sections were viewed in a Zeiss EM10CR electron microscope.

The entire area of each section (approximately 0.7 sq mm, containing an average of 1000–2000 bone cells) was scanned at a primary magnification of ×20,000, for the presence of ultrastructurally identifiable budding and free virus particles. The number of particles was scored semiquantitatively as follows: −, no particles found; +, occasional particles found after extensive search; ++, moderate numbers of particles found; +++, numerous particles readily found. The precise position of cells of interest within the femur metaphysis was determined as described elsewhere.[2]

Results

The presence of virus particles in bone tissue in the femurs is summarized in Table 1. No particles were observed at any time in femur bone tissue from control animals. Similarly, no particles were observed in treated animals in the first week after injection with OA virus. Four weeks after infection, however, large numbers of virus particles were observed budding from the surface of, and collected in pools around, osteoblasts and osteocytes (Figure 1B). Particles were never observed budding from the surface of osteoclasts or chondrocytes. Budding particles showed the typical structure described for C-type retroviruses. Extracellular particles were nearly all immature; ie, they consisted of three concentric electron-dense ring-shaped shells surrounding an electron-lucent core, the whole measuring approximately 100 nm in diameter. The position of a cross-sample of cells with budding virus in one section is shown in Figure 1A. In all preparations cells with budding virus were found more or less evenly distributed in the primary ossification center from just below the chondroid region to the area of increased bone marrow.

Table 1—Presence of C-Type Virus Particles in Osteoblasts and Osteocytes in Bone Tissue From Control NMRI Mice and Mice Given OA Virus Shortly After Birth

	C-type virus particles			
	Control mice		Infected mice	
Age of mice	Femurs	Lumbar vertebrae	Femurs	Lumbar vertebrae
3 days	−(5/5)	−(5/5)	−(5/5)	−(5/5)
7 days	−(5/5)	−(5/5)	−(5/5)	−(5/5)
28 days	−(5/5)	−(5/5)	+++(4/5) ++(1/5)	+++(4/5) + (1/5)

Virus particles were scored as follows: −, no particles; +, occasional particles; ++, moderate numbers of particles; +++, numerous particles.

The lumbar vertebrae contained a greater proportion of mature bone trabeculae, and this difference was reflected in the appearance of virus particles. Again, particles were not observed until 4 weeks after infection with OA virus (Table 1). Budding, immature, and mature particles were observed, the majority associated with osteocytes, the remainder with osteoblasts. The osteocyte lacunae were often filled with dense clusters of particles (Figure 2A). The mature particles showed the atypical morphologic characteristics of the original OA virus. The diameters of the particles varied from 90 to 150 nm, and the dense core was often poorly defined, sometimes filling the whole space within the outer envelope of the particles (Figure 2B). There were also some bizarre branched and cylindrical immature forms (Figure 2C).

Although some osteocyte lacunae contained large numbers of particles, there were others, both in the femurs and the lumbar vertebrae, which contained no particles at all. The distribution appeared to be random. Virus-filled and virus-empty lacunae were often close together.

As is generally the case in NMRI mice (unpublished observations), additional C-type particles with normal morphologic features were occasionally found associated with hematopoetic bone marrow cells in all samples—ie, both in control and infected animals and in femurs and lumbar vertebrae.

Discussion

The aim of this investigation was to identify the first cells in bone tissue to be infected after injection of OA MuLV in newborn mice. The main advantage of electron microscopy in such investigations is that identification of budding virus, even in low numbers, is sufficient to prove infection of cells. Although some of the pleomorphic mature virus particles might possibly be con-

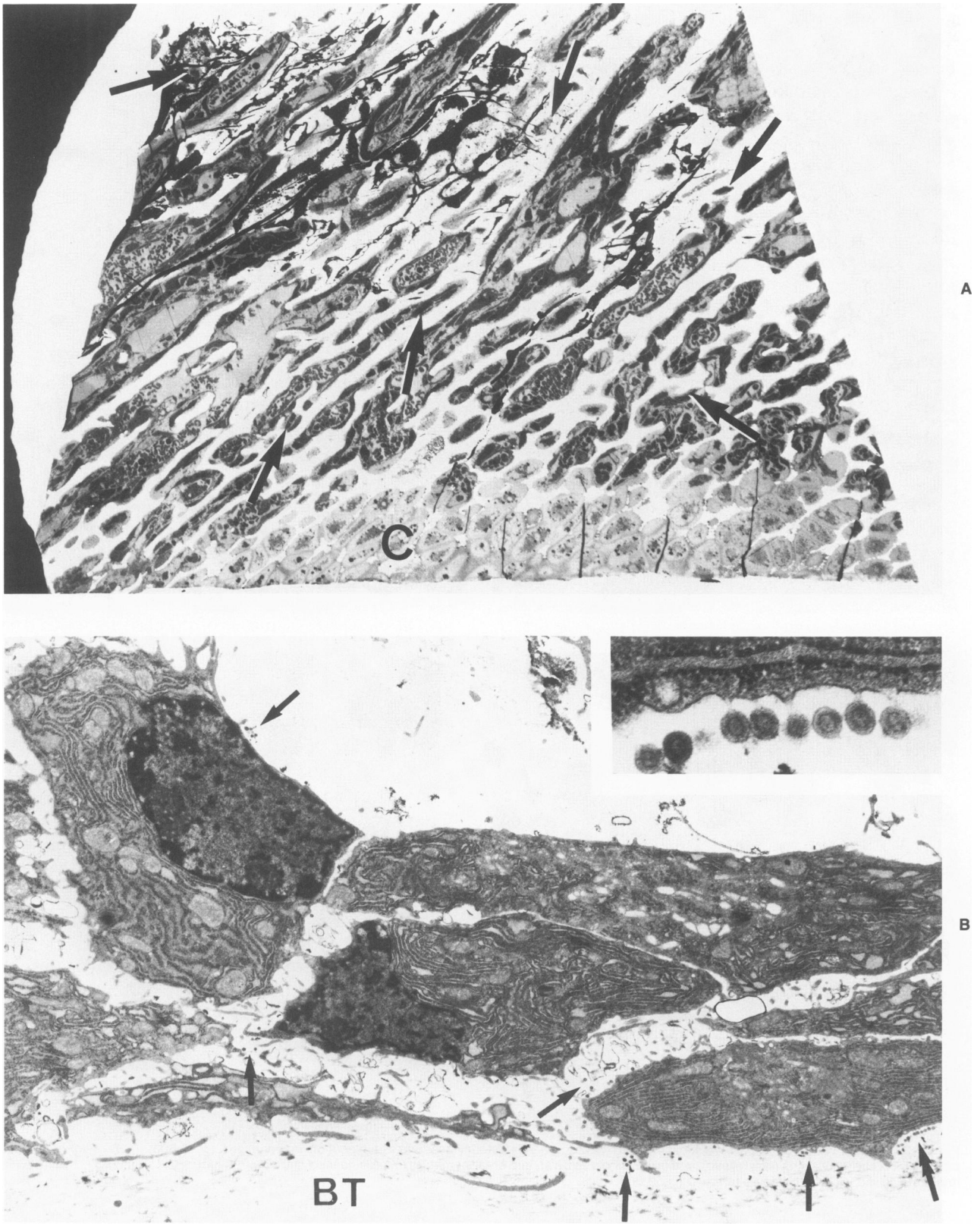

Figure 1—Proximal femur from a 4-week-old infected mouse. **A**—Zone of primary spongiosa showing the chondroid region (*C*) and position of a selection of virus-producing bone-tissue cells (*arrows*). (×200) **B**—Osteoblasts adjacent to bone trabeculae (*BT*) surrounded by budding and free C-type retrovirus particles (*arrows*). **Inset**—Budding virus. Enlargement of area shown by *double-headed arrow* in **A** (×5600; **inset**, ×58,000)

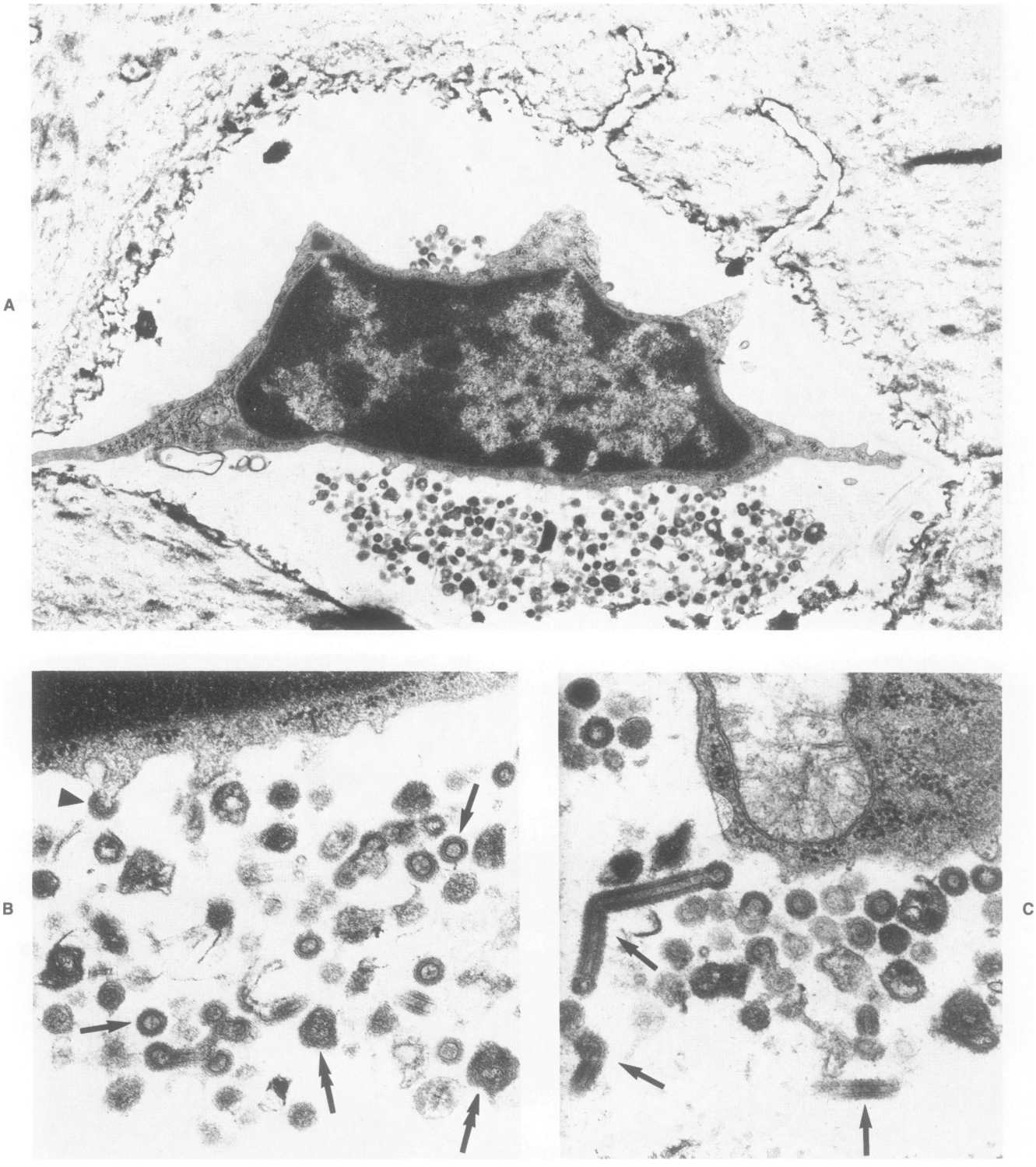

Figure 2—Lumbar vertebra from a 4-week-old infected mouse. **A**—Osteocyte lacuna with a pool of virus particles. (× 15,600) **B**—Higher magnification of **A** showing budding (*arrowhead*), immature (*single arrows*), and atypical mature (*double-headed arrows*) virus particles. (× 58,000) **C**—Bizarre immature forms of virus particles (*arrows*) (× 58,000)

fused with matrix vesicles, budding and immature virus particles can be identified unequivocally from their characteristic structure and morphologic features. The electron-microscopic results clearly showed that C-type retrovirus replicates in cells of the osteoblastic/osteocytic cell lineage 4 weeks after infection of the animals. No virus particles were ever observed budding from bone cells in control mice. It is also known that there is no spontaneous expression of infectious endogenous virus in bone tissue of normal NMRI mice at this time.[3]

Thus, the virus particles observed budding from osteoblasts and osteocytes in infected mice probably derive from the injected OA MuLV. This conclusion was further supported by the pleomorphic appearance of the mature particles, which is characteristic for OA virus.[1] Because cells of the osteoblastic/osteocytic cell lineage were the first to be productively infected with the injected virus, they probably represent the primary target for OA MuLV in skeletal tissue.

Infection of cells of the skeletal tissue was investigated in parallel experiments (reported in detail elsewhere[4]) using cell cultures derived from calvaria and humeri of infected and control animals. Expression of the retroviral protein p30 in these cells was demonstrated with the use of an immunoperoxidase technique. Viral protein was shown clearly to be expressed in cell cultures established from animals 7 and 28 days after infection, but not in those from controls, which indicated virus infection as soon as 7 days after injection.

There is a considerable delay between the expression of virus in osteoblastic and osteocytic cells observed here and the appearance of osteopetrosis first detected 6 months later.[1,5] The mechanism of osteopetrosis development during this time is not known. The most likely possibility is that functional alterations in the osteoblasts or osteocytes result in abnormal proliferation or increased bone matrix production. However, the mechanism could also be indirect, resulting, for example, from an osteoblast mediated effect on the bone-resorbing cell population.[6] Also, the possibility cannot be excluded that other cells such as osteoclasts are non-productively infected or become infected at a later stage and play an independent role in the development of the disease. The OA-induced osteopetrosis, however, appears to be very similar to the retrovirus-induced avian osteopetrosis studied by others in chickens.[7,8] Recent research has shown that virus expression in the chick is associated with lining osteoblasts and osteocytes,[9] the same location as that observed here for OA MuLV. In avian osteopetrosis the increased bone mass appears to result from increased bone deposition following stimulation of osteoblast and osteocyte activities in response to viral infection.[9] By analogy, this would also seem to be the most likely mechanism for the development of OA-induced osteopetrosis. In contrast, the congenital or "mammalian" form of osteopetrosis described in humans and studied in mice, rats, and rabbits appears to have quite different pathologic features.[10-12] It is caused by a genetically transmitted osteoclast defect which is manifested in reduced bone resorption and, thus, accumulation of bone mass.

The idea that bone cells in mammals might be a target for retroviruses has received increasing attention in recent years. Cells from patients with Gardner's syndrome, for example, have been shown to be more susceptible to transformation by retroviruses,[13] and it has been suggested that viruses might play a role in the etiology of Paget's disease of bone.[14] The association of viruses with a variety of bone diseases has been reviewed in considerable detail.[15] The system described here provides an opportunity for studying early changes in target cells in mice after infection with a retrovirus known to induce severe osteopetrosis.

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