Virus-Host Interactions in the Development of Avian Leukosis Virus-Induced Osteopetrosis: a Dissertation

VIRUS-HOST INTERACTIONS IN THE DEVELOPMENT OF AVIAN LEUKOSIS VIRUS-INDUCED OSTEOPETROSIS

A Dissertation Presented

By

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ABSTRACT

Avian leukosis virus (ALV)-induced osteopetrosis is a proliferative disorder of the bone affecting the growth and differentiation of osteoblasts. Osteopetrosis is a polyclonal disease in which cells of the bone contain, on average, multiple viral DNA copies. Osteopetrotic bone is also characterized by the accumulation of unintegrated viral DNA, suggesting an atypical life cycle of the virus in the infected osteoblasts. To better understand virus-host interactions in the induction of osteopetrosis by ALVs, infected chick osteoblast cultures and osteopetrotic bone were examined for aspects of the virus life cycle and effects of infection on osteoblast function.

Levels of infection and virus expression were compared in cultured osteoblasts and osteopetrotic bone. Osteopetrotic bone contained higher levels of viral DNA and correspondingly higher levels of viral proteins than infected osteoblast cultures, suggesting a higher viral load in the diseased bone. A significant level of mature Gag protein was present in the bone, suggesting the accumulation of mature virus particles in the diseased bone. It is possible that the accumulation of virus could facilitate the high levels of infection observed in the diseased bone.

The mechanism by which unintegrated viral DNA persisted in osteopetrotic bone was investigated by examining the susceptibility of infected osteoblasts to superinfection. The results indicated that, in culture, infected osteoblasts were able to establish interference to superinfection. This suggests that the persistence of unintegrated viral

DNA in osteopetrotic bone may not result from the continuing infection of productively infected osteoblasts.

The effect of virus infection on osteoblast function was examined in the diseased bone and in osteoblast cultures. In infected chickens, osteoblast activity, as evidenced by the expression of osteoblast phenotypic markers, was increased only in chickens developing severe osteopetrosis. In culture, virus infection had no apparent effect on either the proliferation or differentiation of osteoblasts. This indicates that infection was itself not sufficient to perturb osteoblast function. Furthermore, it suggested that additional components of the bone may be required for ALV infection to induce the abnormal activity of osteoblasts observed in osteopetrosis.

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CHAPTER I

INTRODUCTION

Avian leukosis virus (ALV)-induced osteopetrosis is a proliferative disorder resulting in the enlargement of affected bones. The disease is characterized by the nonclonal proliferation of osteoblasts, the primary cell type involved in bone formation. Osteopetrosis is induced in chickens by infection with avian leukosis viruses (ALVs), which are nontransforming, replication competent retroviruses (for reviews, see Simpson & Sanger, 1968; Smith, 1982).

Bone pathology. ALV-induced osteopetrosis results in substantial increases in bone formation, most noticeable in the long bones of infected chickens. Bone width can be increased 2-6 fold by 4 months after infection (Banes & Smith, 1977). Bone length is either normal or slightly shortened. The earliest lesions are typically detected in the tibia, arising at mid-diaphysis. The abnormal growth of bone quickly proceeds to encompass the entire shaft of the bone. Osteopetrotic lesions do not always involve the entire perimeter of the bone and can result in asymmetrical thickening of the bones (Sanger et al., 1966b). At later stages, the endosteum can become affected, causing obstruction of the medullary cavity. In advanced cases, other regions of the skeleton, including the sternum and calvaria, may also become affected (Holmes, 1961).

The increase in bone mass causes osteopetrotic bones to appear more opaque by X-ray analysis than normal cortical bone. The increase in bone opacity suggests an increase in the density of osteopetrotic bone. In actuality, the new bone produced in osteopetrotic lesions is, per unit volume, less dense than the underlying compact bone

(Biltz & Pellegrino, 1965). However, early characterization of the disorder was based on the X-ray analysis and led to its classification as "osteopetrosis" or petrification of the bone (Jungherr & Landauer, 1938).

Osteopetrotic lesions are characterized by an increase in the number of osteoblasts in the endoperiosteum and by the production of immature woven bone. The newly formed bone is more cellular than normal bone and is highly basophilic, in contrast to the eosinophilic staining of osteoid and compact bone (Sanger et al., 1966b; Boyde et al., 1978). The architectural arrangement of the diseased bone is disorganized as compared with normal bone. Vascular spaces that normally become remodelled into Haversian canals remain open and are larger than normal (Sanger et al., 1966b). These spaces are misaligned and oriented at right angles to the long axis of the bone. The lacunae of the bone are unusually large, irregularly spaced and frequently conjoined (Boyde et al., 1978). Although the collagen content of osteopetrotic bone is normal (Banes & Smith, 1977), collagen fibrils are randomly oriented in osteopetrotic lesions, resulting in a disorganized matrix (Banes et al., 1978; Boyde et al., 1978). Moreover, osteopetrotic bone is less dense than normal bone and is poorly mineralized (Biltz & Pellegrino, 1965).

Osteopetrotic lesions result from the hyperplasia of osteoblasts in the periosteum (Boyd et al., 1978; Graf & Channin, 1987). The periosteum of osteopetrotic bone becomes thickened due to an accumulation of proliferating osteoblasts (Smith, 1982). Osteoblasts in the endoperiosteum undergo hyperplasia, hypertrophy and become more basophilic in staining (Sanger et al., 1966b). Morphometric analysis of osteopetrotic bone indicates that the number of osteoblasts is increased 9 fold by 4 weeks after infection (Schmidt et al.,

1981). Ultrastructural studies reveal a margination of chromatin in the nuclei of periosteal osteoblasts and a thickening of regions of the plasma membranes (Simpson & Sanger, 1966). The cisternae of the rough endoplasmic reticulum are dilated and more abundant than normal (Boyde et al., 1978; Powers et al., 1987), suggesting that the metabolic activity in infected osteoblasts may be increased.

Electron microscopy studies on osteopetrotic bone reveal the production of virus from infected osteoblasts and osteocytes, as evidenced by the presence of virus particles attached to and budding from the cell membranes (Simpson & Sanger, 1966; Frank & Franklin, 1982). Virus particles are present in the osteoid adjacent to osteoblasts, embedded within the bone trabeculae, as well as clustered in the periosteocytic spaces of the lacunae (Simpson & Sanger, 1966; Boyde et al., 1978; Frank & Franklin, 1982). Virus particles appear to accumulate within the bone lacunae as the disease progresses, since increased numbers of virus particles are observed within the lacunae with time after infection (Frank & Franklin, 1982). As in other ALV infections, viral particles are only rarely observed intracellularly.

It has been proposed that the increase in bone formation in ALV-induced osteopetrosis could reflect deficiencies in bone resorption, the principal activity of the osteoclast. However, several studies suggest the normal functioning of osteoclasts in osteopetrotic bone. Multinucleated, acid phosphatase positive osteoclasts are present along the bone surface, residing in Howship's lacunae (Frank & Franklin, 1982). Ultrastructurally studies have indicated that osteoclasts in osteopetrotic bone contain well differentiated ruffled borders which appear to be active in bone resorption (Frank &

Franklin, 1982). Morphometric analysis indicates that the total number of osteoclasts per bone is increased in osteopetrotic bone, suggesting that virus infection does not kill osteoclasts (Schmidt et al., 1981). Furthermore, a progressive expansion of the marrow space during the first 3 weeks after infection suggests that endosteal osteoclasts are functional (Schmidt et al., 1981). Electron microscopy studies do not demonstrate the presence of virus particles associated with osteoclasts in osteopetrotic bone (Franklin & Martin, 1980; Frank & Franklin, 1982). In the rare instances that virus has been associated with osteoclasts, virus particles were localized to vacuoles in the peripheral cytoplasm and appeared to have been phagocytized (Frank & Franklin, 1982).

Viral genes affecting osteopetrosis induction. The ability of a virus to induce osteopetrosis is determined by multiple regions of the viral genome. The presence of a particular sequence does not guarantee a high osteopetrotic potential, indicating that the contribution of viral sequence to the osteopetrotic potential is highly context dependent (Brown et al., 1988; Aurigemma et al., 1991).

Several regions of the genome have been specifically implicated in affecting the osteopetrotic potential of a virus. The long terminal repeat (LTR) sequences, which are important in regulating the transcriptional activity of a virus, play a role in determining osteopetrotic potential. Viruses bearing the LTRs of exogenous viruses can induce osteopetrosis. In contrast, the LTR of an endogenous virus, Rous associated virus-0 (RAV-0), is very inefficient at inducing disease (Shank et al., 1985; Brown et al., 1988). This could reflect the fact that endogenous LTRs are 10 fold less transcriptionally active than LTRs of exogenous viruses (Cullen et al., 1983). The difference in transcriptional

activity correlates with the absence of a strong enhancer element in the LTRs of endogenous viruses (Cullen et al., 1985; Habel et al., 1993). These studies suggest that the LTR sequences influence the osteopetrotic potential by determining the level of virus expression.

Osteopetrotic potential is also influenced by the subgroup specificity of the *env* gene. It has been shown that SU, the surface envelope glycoprotein, contains the determinants for receptor interaction (Dorner & Coffin, 1986). Viruses of subgroups B and E induce a higher incidence of osteopetrosis than subgroup A viruses (Brown et al., 1988; Aurigemma et al., 1991). This is interesting given that genes encoding the receptors for subgroup B and E viruses are thought to be allelic (Crittenden & Motta, 1975). The link between the subgroup specificity of an ALV and osteopetrotic potential is also intriguing given that *env* sequences have been shown to contribute to tissue-specific patterns of virus replication (Brown & Robinson, 1988). As such, osteopetrotic potential may be influenced by the distribution of ALV receptors on bone cells.

Osteopetrotic potential is also potentiated by a short region near the 5'LTR (Robinson et al., 1986; 1992). This has been localized to a 375 base pair region between positions 255 and 630, relative to the sequence of the Prague strain of Rous sarcoma virus C (Schwartz et al., 1983). Multiple functions are contained in this region including the translational start region and start site, the splice donor site, signals for virus packaging and RNA dimerization and a partial coding region for the Gag protein, MA (Schwartz et al., 1983; Bizub et al., 1984; Darlix, 1986). It is unclear which, if any, of these functions is important in affecting osteopetrotic potential. Comparison of the nucleotide sequence

in this region with other ALVs reveals a codon change unique to the osteopetrosis-inducing virus, Br21 (Robinson et al., 1992). This change in sequence causes a nonconservative amino acid change (Glu -> Lys) in the Gag protein, MA. This is potentially interesting, given the predicted role for MA in virus assembly (Wills & Craven, 1991).

Br21: an ALV of high osteopetrotic potential. In the course of investigating viral determinants affecting osteopetrotic potential, a recombinant subgroup E virus was generated which possessed an unusually high osteopetrotic potential. This virus, Br21, reproducibly induces a high incidence (70-90%) of severe osteopetrosis with a rapid onset (<2 months) in chickens inoculated at one day post hatch (Shank et al., 1985). Br21 replicates to high titers, both in tissue culture and in chickens (Shank et al., 1985; Robinson et al., 1986). In Br21 infected chickens, high viremias have been observed as early as 2 weeks postinfection (unpublished observations). As is typical for subgroup E viruses, Br21 is a noncytopathic ALV which induces little cytopathic effect in infected fibroblast cultures. Given its strong osteopetrotic potential and its ability to grow well in culture and in chickens, Br21 was chosen for use in this study.

Virus-host interactions important in the induction of osteopetrosis. The establishment of a persistent viremia is required for osteopetrosis development (Robinson & Miles, 1985). Infected birds which develop neutralizing antibodies and clear virus are protected from osteopetrosis. Administration of either antisera or lymphocytes from virus-immune chickens to infected chickens also prevents the development of osteopetrosis (Smith & Ivanyi, 1980; Smith & Morgan, 1984). Interestingly, subgroup A viruses, which

are less efficient at inducing osteopetrosis than subgroup B viruses (see above), elicit stronger immune responses in chickens than subgroup B viruses (Maas et al., 1982).

Chickens are most susceptible to osteopetrosis induction when infected at an age at which their immune systems are not fully competent. The most rapid cases of osteopetrosis are induced in chicks infected during embryonic development (between days 10-15 of incubation) (Smith & Moscovici, 1969; Franklin & Martin, 1980). The time of onset of disease is slightly delayed in chicks infected after hatch as compared to infection during embryonic development (Hirota et al., 1980). By 10 days post hatch, infection is rapidly cleared and no longer induces osteopetrosis (Paterson & Smith, 1978). Bursectomized chickens, which cannot raise an antibody response, are susceptible to osteopetrosis for a prolonged period of time (Price & Smith, 1981). As such, infection of bursectomized chickens as late as at 6 weeks of age results in the development of osteopetrosis (Smith & Morgan, 1984).

The persistence of unintegrated viral DNA in ALV-induced osteopetrosis. Osteopetrotic lesions represent polyclonal outgrowths of infected cells (Robinson & Miles, 1985; Aurigemma et al., 1989). Cells of the diseased bone sustain multiple infections as evidenced by an average of 2-20 copies of integrated and unintegrated viral DNA per cell. The severity of osteopetrosis correlates with the amount of viral DNA in the bone. That is, cases of severe osteopetrosis contain an average of 10-20 copies of viral DNA/cell, while milder cases of osteopetrosis contain, on average, 3-4 copies of viral DNA/cell (Robinson & Miles, 1985). The actual distribution of viral DNA in the cells of the bone is unknown.

ALV-induced osteopetrosis is associated with the persistence of unintegrated viral DNA (Robinson & Miles, 1985). Unintegrated viral DNA, which is predominantly in the linear form, accounts for approximately 50% of the total viral DNA in diseased bone. As such, severe cases of osteopetrosis contain, on average, 5-10 copies of unintegrated viral DNA per cell (Robinson & Miles, 1985; Robinson et al., 1986). Unintegrated viral DNA is not detected in normal bone from infected chickens and appears to be a characteristic of the osteopetrotic lesion. It is unclear whether the persistence of unintegrated viral DNA plays a direct or indirect role in the pathogenesis of ALV-induced osteopetrosis.

The synthesis of unintegrated viral DNA represents an early stage in the virus life cycle. Following entry and partial uncoating, the virion RNA is reverse transcribed into a linear, duplex DNA copy (for review, see Varmus & Swanstrom, 1984). The unintegrated viral DNA typically does not persist, but is transported to the nucleus and integrated into the host chromosome. Once in the nucleus, some of the linear DNA becomes circularized by covalently joining the ends of the molecule. Covalently closed circular DNA is not believed to be integrated (Brown et al., 1989). Integration is required for the productive infection of most, if not all, retroviruses (Schwartzenberg et al., 1984; Stevenson et al., 1990b), although integration defective mutants of spleen necrosis virus and human immunodeficiency virus (HIV) have been reported to express viral antigens (Panganiban & Temin, 1980; Stevenson et al., 1990a). Superinfection of productively infected cells is prevented by the establishment of interference. By this process, newly synthesized envelope glycoprotein interacts with the cell receptor, blocking further

interaction with infectious virions (for review, see Weiss, 1984). Delays in establishing interference have been associated with high levels of superinfection (Temin, 1988).

The mechanism by which unintegrated viral DNA persists in osteopetrotic bone is unclear. It likely results from continuing infection or reinfection of cells in the bone. Southern analyses of *Eco R*1 digested DNA have revealed a novel fragment in samples from osteopetrotic bones. This atypical DNA species was also detected in recently infected fibroblast cultures, suggesting that it may represent nascent viral DNA (Robinson & Miles, 1985). Alternatively, the persistence of unintegrated viral DNA could arise from an atypical virus life cycle in cells of the diseased bone. It has been suggested that the unintegrated viral DNA could arise by intracellular reverse transcription of newly synthesized viral RNA. Reverse transcriptase is normally processed to its active form during virus budding (Dickson et al., 1984) and is therefore not active intracellularly. The synthesis of viral DNA from newly synthesized viral RNA within an infected cell has not been described for C-type retroviruses, although it has been described for B-type viruses and hepadna virus (Ringold et al., 1978; Summers & Mason, 1982).

Association of unintegrated viral DNA with other retroviral diseases. The persistence of unintegrated viral DNA has been implicated in the pathogenesis of several retroviral-induced diseases. Cells infected with avian reticuloendothelial and leukosis viruses contain a transient accumulation of 50-400 copies of unintegrated viral DNA/cell which correlates with cell killing (Keshet & Temin, 1979; Weller et al., 1980). The dead cells contain 5-6 times more unintegrated viral DNA than live cells (Weller & Temin, 1981). Whether the high levels of unintegrated viral DNA are involved in cytopathic

effect is unclear. In ALV infections, cytopathicity correlates with envelope subgroup (Weller et al., 1980). The accumulation of unintegrated viral DNA appears to result from a massive second round of infection, which can be blocked by the presence of neutralizing antibody in the medium (Temin et al., 1980; Weller et al., 1980). The high levels of superinfection are likely to result from the failure of infected cells to rapidly establish interference.

The accumulation of high copy numbers of unintegrated viral DNA in the bone marrow of FeLV-FAIDS infected cats correlates with onset of disease symptoms (Mullins et al., 1986). The unintegrated viral DNA primarily represents a replication defective variant which carries mutations in the *env* gene (Overbaugh et al., 1988; 1992). FeLV-FAIDS isolates which induce immunodeficiency in cats generally cause cytopathicity in T cells in culture (Donahue et al., 1991). T cell killing correlates with the accumulation of 250-400 copies of viral DNA/cell, with dead cells containing 7-9 times more unintegrated viral DNA than live cells. The presence of neutralizing antibody in the medium prevents the accumulation of unintegrated viral DNA, suggesting that the unintegrated viral DNA accumulates as a consequence of superinfection (Donahue et al., 1991). Pathogenic variants of FeLV-FAIDS are characterized by the delayed processing of envelope glycoproteins (Poss et al., 1990). It has been proposed that a delay in the maturation of Env proteins slows the establishment of interference, thus permitting high levels of superinfection.

The accumulation of unintegrated viral DNA has also been reported in several lentivirus infections. The accumulation of unintegrated viral DNA is found in diseased

tissue resulting from infection with visna or equine infectious anemia virus (Rice et al., 1989; Staskus et al., 1991). In vitro infection with HIV or visna virus is also associated with the accumulation of unintegrated viral DNA (Shaw et al., 1984; Haase et al., 1982). The accumulation of high copy numbers of unintegrated HIV DNA in culture appears to result from multiple infections, since the presence of antibody which blocks infection also prevents the accumulation of unintegrated viral DNA (Robinson & Zinkus, 1990).

Infected quiescent cells can harbor unintegrated viral DNA. Quiescent T lymphocytes infected with HIV contain unintegrated viral DNA (Stevenson et al., 1990b; Bukrinsky et al., 1991). Integration of the viral DNA in these cells is thought to require cell activation.

Comparison to other forms of osteopetrosis. ALV-induced osteopetrosis differs from most other forms of osteopetrosis in the primary cell type it affects and in its etiology. Whereas ALV-induced osteopetrosis is characterized by the hyperplasia of osteoblasts and excessive bone formation, mammalian osteopetrosis results from diminished numbers and/or function of osteoclasts, resulting in a reduction in bone resorption (for review, see Brown & Dent, 1971; Marks, 1987). Interestingly, aberrant gene expression in osteoblasts has been identified in several osteopetrotic mutations in the rat (Shalhoub et al., 1991). It has been suggested that the abnormal functioning of osteoblasts could influence skeletal modeling, perhaps by affecting the development and mineralization of the extracellular matrix or by affecting the maturation or activation of osteoclasts. The etiology of avian osteopetrosis is viral, whereas mammalian osteopetrosis arises from heritable genetic mutations. Osteopetrotic mutations affect osteoclast function

by a variety of mechanisms. Some mutations cause intrinsic defects in the osteoclasts and these forms of osteopetrosis can be cured by bone marrow transplants (Walker, 1975). Other mutations cause deficiencies to the microenvironment within the bone which can affect osteoclast proliferation, differentiation or activation. For example, the mouse mutation osteopetrosis (*op*) causes a premature termination in the coding region for macrophage colony-stimulating factor (M-CSF), thereby creating the phenotypic abnormality via the deficiency in a specific cytokine (Yoshida et al., 1990).

Comparison to other retroviral-induced bone disorders. Several retroviral infections induce abnormal bone formation, however, the mechanisms of pathogenesis generally differ from ALV-induced osteopetrosis. Infection with feline leukemia virus has been reported to cause medullary osteosclerosis (Hoover et al., 1974). In this disorder, the decreased numbers of osteoclasts in the diseased bone suggest a deficiency in bone resorption. Murine sarcoma virus strains FBJ and FBR induce osteosarcomas (for review, see Bishop & Varmus, 1985). Both viruses, which are replication defective, contain the oncogene, v-*fos*. Interestingly, the unregulated expression of several proto-oncogenes, such as c-*src* and c-*fos* have been associated with abnormalities in bone formation (Ruther et al., 1987; Soriano et al., 1991).

Several replication competent murine leukemia viruses (MuLVs) induce benign bone tumors and hyperostosis. OA MuLV is an MuLV strain which when injected into newborn mice induces a high incidence of bone disease with similarities to ALV-induced osteopetrosis (Schmidt et al., 1984). The lesions occur preferentially on the femurs and in the lower sections of the vertebral column. Electron microscopy studies reveal virus

particles budding from and accumulating near osteoblasts and osteocytes in the infected bone, similar to findings in ALV-induced osteopetrosis (Murray et al., 1986). In culture, bone cells derived from OA MuLV infected mice express higher levels of osteoblast markers than control (Schmidt et al., 1987).

In vitro osteoblast cultures. Bone cell cultures have provided relatively simple systems for studying complex cellular activities such as osteoblast function (for review, see Sodek and Berkman, 1987). For this study, osteoblasts derived from embryonic chick calvaria were cultured and induced to differentiate, using a protocol based on that described by Gerstenfeld et al., 1987. These cultures are enriched for osteoblasts as evidenced by the response to osteotropic hormones and the selective production of type I collagen and bone associated proteins (Bellows et al., 1986; Gerstenfeld et al., 1987). As the osteoblasts mature in culture, they exhibit a temporal expression of markers associated with a differentiated osteoblast phenotype (Stein et al., 1990). These include osteocalcin, alkaline phosphatase and osteopontin.

A defining aspect of the differentiation of osteoblasts in culture is the formation of a mineralized matrix. To induce differentiation, cultures are grown in a specialized medium, BGJ_b (Biggers et al., 1961), which is supplemented with ascorbic acid and ß-glycerophosphate. Ascorbic acid upregulates collagen synthesis and promotes the formation of an extracellular matrix (Barnes, 1975; Aronow et al., 1990). ß-glycerophosphate promotes mineralization of the matrix (Tenenbaum & Heersche, 1982; Bellows et al., 1986). Maturation and mineralization of the extracellular matrix are dependent on the downregulation of genes associated with cell growth and the induction

of genes associated with differentiated osteoblast function and bone formation (Shalhoub et al., 1989; Stein et al., 1990). Matrix mineralization in culture exhibits chemical and ultrastructural properties similar to woven bone (Gerstenfeld et al., 1988; Escarot-Charrier et al., 1988).

Goals of the thesis. This thesis was undertaken to identify virus-host interactions in the development of ALV-induced osteopetrosis. Previous studies have concluded that the primary change in osteopetrotic bone resulted from the increased proliferation of osteoblasts. However, very little was known regarding how ALV infection caused osteopetrosis. Reports that unintegrated viral DNA persisted in osteopetrotic bone suggested that the virus might exhibit an atypical life cycle in the infected cells of the bone. The availability of an in vitro osteoblast culture system, which showed similarities to the maturation to osteoblasts in the bone, provided a means for studying virus infection in the cell type that is the target cell for disease. Our hypothesis was that some aspect of the life cycle of an osteopetrosis-inducing virus would be atypical in infected osteoblasts and the atypical virus life cycle would induce the abnormal growth and differentiation of osteoblasts.

To examine aspects of the virus life cycle, studies in Chapter III compare the patterns of viral DNA, protein and virus production in osteopetrotic bone, osteoblast cultures and fibroblast cultures (used as a control). Studies in Chapter IV focus on determining whether the high levels of infection in osteopetrotic bone reflected an inherent failure of osteoblasts to establish superinfection interference. The studies presented in Chapter V address the question of whether infection alone is sufficient to

induce the abnormal growth and differentiation of osteoblasts and how osteoblast activity is altered in infected cultures and in infected chickens.

CHAPTER II

MATERIALS AND METHODS

Cell culture. Chicken embryo fibroblasts were derived from 10 to 12 day old embryos of chicken line K28 or line 0. Line K28 is random bred line which is susceptible to subgroups A through E ALVs (C/O) (Robinson & Lamoreux, 1976) and contains a single endogenous virus (residing at *ev* 1) (Astrin & Robinson, 1979). Line 0 chickens are susceptible to subgroup A and B viruses but not to subgroup E viruses (C/E) and do not contain endogenous viruses. Subgroup E viruses, including Br21, were grown in line K28 fibroblasts. Subgroup A and B virus stocks were grown on line 0 fibroblasts, which as they do not contain endogenous viruses, prevent viral recombinations between exogenous and endogenous viruses that could occur in line K28 fibroblasts.

Fibroblasts were grown in D(10,4,1): Dulbecco's modified Eagle medium supplemented with 10% tryptose phosphate broth, 4% calf serum and 1% chicken serum plus penicillin and streptomycin. Cultures were grown at 37°C in 95% air / 5% CO_2. Fibroblasts were used between the second and tenth passages.

The protocol used for infection included polybrene to increase the rate of virus adsorption (Toyoshima & Vogt, 1969). In all cases, polybrene was used at a final concentration of 10 μg/ml. Recently replated fibroblasts (4 to 20 hours post-seeding) were rinsed once with medium containing polybrene. Fibroblasts were exposed to cell-free virus stock or dilutions of virus in D(10,4,1) supplemented with polybrene. Mock infections

used conditioned media containing polybrene. Typically, 1 ml of virus stock was used per 100mm plate, and the cultures were infected for 60 minutes at 37°C.

Chick osteoblast culturing was performed in collaboration with the laboratories of Dr. Gary Stein and Dr. Jane B. Lian, Department of Cell Biology, University of Massachusetts, Worcester. Calvarial chick osteoblasts were isolated and cultured based on the method described by Gerstenfeld et al., 1987. The culturing scheme is presented in Figure 1. Calvaria were isolated aseptically from 16 day old line K28 chick embryos. The calvaria were removed from the embryos and split along the sagittal and frontal suture lines. Adherent suture tissue and periosteum were removed by gently scraping away the fibrous tissue at the edges of the calvaria with a scalpel. Osteoblasts were isolated by enzymatic digest of the calvaria, using a modification of the technique originally described by Wong and Cohn (1975). The calvaria were digested using a mixture of trypsin and collagenase (0.125% trypsin (GIBCO, Grand Island, NY) and 0.1% collagenase (Type P; Boehringer Mannheim, Indianapolis, IN) for periods of 20, 20 and 90 minutes at 37°C. This process allows the timed release of cells from the bone surface. Between each digest, the bone chips were separated from the released cells by centrifugation and washed once in phosphate buffered saline (PBS: 0.15 M NaCl, 1 mM KH_2PO_4, 1 mM K_2HPO_4, pH 7.2). The cells released from the first two digests were discarded. Prior to the third digest, the calvaria were minced into small fragments and then resuspended in the trypsin/collagenase mixture and digested for 90 minutes. Following this period, the entire contents of the digest were filtered successively through

a Sweeney metal mesh filter (Millipore Corp., Bedford, MA) and then through a 10μm nitex membrane. Cells were collected by low speed centrifugation and resuspended in primary medium (minimal essential medium supplemented with 10% fetal calf serum). The cell number was determined using a hemocytometer. Cells were seeded at low density (0.5 x 10^6 per 100mm plate) and cultured for 21 days in primary medium. Medium was changed every third day.

Following a primary culturing phase of 21 days, osteoblast cultures were trypsinized for 10 minutes using trypsin/EDTA (0.125% trypsin, 5mM EDTA). Cells were counted, rinsed once in primary medium containing polybrene and infected by replating (7 x 10^5 cells per 100 mm plate or 8 x 10^4 cells per 35 mm plate) in virus stock supplemented with polybrene. Control cultures were mock infected by plating in conditioned medium supplemented with polybrene. The day of infection was referred to as day 0 (Figure 1). Medium was changed 4 hours after plating, at which time the cells had become adherent. The cultures were grown to confluence and the medium was changed every other day. At confluence (4-6 days into the secondary phase), differentiation was induced by changing to a specialized medium, BGJ_b (GIBCO, Grand Island, NY), supplemented with 10% fetal calf serum (HyClone, Logan, UT) plus penicillin and streptomycin. As different lots of fetal bovine serum have been found to affect the extent of mineralization in the culture (Aronow et al., 1990), lots of fetal bovine serum were screened to identify one which best supported an ordered pattern of cellular differentiation (unpublished observations). BGJ_b medium was supplemented with 10mM

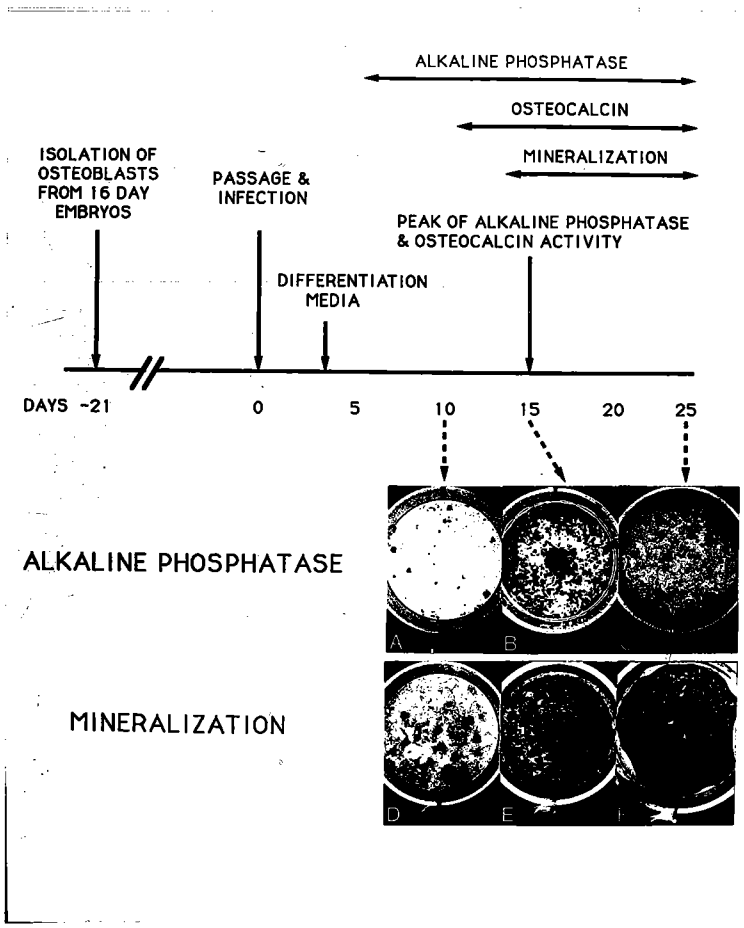

Figure 1. Protocol for the infection and differentiation of chick osteoblast cultures. Osteoblasts, isolated from embryonic chick calvaria, were grown as primary cultures for a period of 3 weeks. Following trypsinization, cells were infected by replating in virus-containing media (day 0). At confluence (approximately day 4-6), cultures were induced to mineralize by the addition of BGJ_b media supplemented with ascorbic acid and ß-glycerophosphate (differentiation media). Osteoblast differentiation was monitored by histochemical staining. A temporal pattern of alkaline phosphatase activity (A-C) and mineralization (D-F) was observed. The typical periods of expression of alkaline phosphatase, osteocalcin and mineralization, all indicative of a maturing osteoblast phenotype, are also indicated.

ß-glycerophosphate, a source of organic phosphate, which promotes mineralization of the extracellular matrix (Tenenbaum & Heersche, 1982). Graded increases in concentration of L-ascorbic acid were added (25 µg/ml at the first medium change, 50 µg/ml thereafter) to enhance synthesis of the collagen matrix (Barnes, 1975). Cultures were maintained for 20-30 days following infection and monitored for osteoblast differentiation by histochemical staining (see below).

Osteoblast cultures that were passaged at confluence were cultured in primary medium and replated every 3-4 days. After a brief rinse in PBS, 1-2 mls of trypsin/EDTA were added to confluent cultures and incubated at 37°C for 2-5 minutes. Trypsinized cells were resuspended in primary medium, cell numbers were determined and cells were replated at 7 x 10^5 cells per 100 mm plate.

Neutralizing antibody derived from immune chickens (see below) was added to osteoblast cultures 4 days after infection at a 1:100 dilution. Normal chicken serum (GIBCO, Grand Island, NY) was added to control cultures also at a 1:100 dilution. Chicken serum was heat inactivated prior to use.

Viruses. Construction and description of virus Br21 was previously described (Shank et al. 1985). Br21 is a recombinant subgroup E avian leukosis virus which induces a high incidence of severe, rapid-onset osteopetrosis. Br21 was derived from parent viruses NY203RAV60 and NTRE-2, which themselves induce different degrees of severity of osteopetrosis (Leamnson & Shank, 1986). Br21 contains long terminal repeat sequences (LTRs), sequences encoding the envelope transmembrane protein (gp37) and portions of

both *gag* and *pol* genes derived from exogenous viruses. The 5' region of the *gag* gene and the surface envelope glycoprotein coding region are derived from the endogenous virus, Rous-associated virus type 0 (RAV-0).

Virus pseudotypes containing a ß-galactosidase-expressing vector, pRDlac1 (Reddy et al.,1991) were created using a combination of infection and transfection strategies. The resulting virus pseudotypes were designated RDlac(helper virus). (1) Transfection and infection of a quail cell line. A ß-galactosidase-expressing clone was derived by transfecting QT6 quail cells (Moscovici et al., 1977) with pRDlac1 and pSV_2neo (which contains a selectable marker, neomycin), at a 10:1 molar ratio. Cells were transfected using the calcium phosphate method (Graham and van der Eb, 1973). Four hours after transfection, the cells were incubated for 3-5 minutes with 15% glycerol (a glycerol shock). Cotransfected cells were selected for growth in G418 (10 µg/ml) (GIBCO, Grand Island, NY). G418 resistant colonies were individually trypsinized and expanded. pRDlac1 expression was ascertained by histochemically staining fixed cells for ß-galactosidase activity (see below). RDlac1+ clones frequently lost expression of ß-galactosidase while under G418 selection, but one clone, designated 2C, maintained a constant expression of ~10% ß-galactosidase positive cells over 10 cell passages (unpublished observations). Subgroup E pseudotypes, such as RDlac(Br21), were created by infecting the 2C clone with a high titer stock ($\geq 10^6$ infectious units/ml). Infected cells were passaged 2-3 times prior to recovery of stocks. Pseudotypes of a subgroup A virus, Rous-associated virus type 1 (RAV-1) were generated by using the calcium phosphate method to transfect the 2C

clone with pRAV10R, a plasmid containing a full length copy of RAV-1. Stocks generated by use of the 2C clone yielded titers of 10^4-10^6 ß-galactosidase units/ml. Stocks used for infections had titers of 10^6 units/ml. (2) Cotransfection of fibroblast cultures. Subgroup A virus pseudotypes were also created by cotransfecting line 0 fibroblasts with pRDlac1 and pRAV10R at a 10:1 molar ratio. Stocks generated by this method yielded titers of 10^4-10^6 ß-galactosidase units/ml. Stocks used for infections had titers of 10^6 units/ml.

Virus pseudotypes were titered on line K28 fibroblasts by infecting at several dilutions of virus and quantitating the number of ß-galactosidase-expressing colonies 2 days after infection (see below). In general, the stocks were very stable and could be titered over several orders of magnitude.

Detection and quantitation of virus production. Relative levels of virus particles were determined using an antigen capture enzyme linked immunosorbent assay (ELISA) directed against the Gag protein, p27 (CA) (Brown & Robinson, 1988). The assay employed a polyclonal rabbit anti-p27 IgG (SPAFAS, Norwich, CT) for antigen capture. Samples were diluted in blocking buffer (1% bovine serum albumin in PBS) and analyzed in 96 well microtiter plates. The signal was detected by incubation with a biotinylated polyclonal anti-p27 antibody. The plates were washed with 0.1% Tween-20 in PBS and incubated with a streptavidin-alkaline phosphatase conjugate (GIBCO, Grand Island, NY). All incubations were done at room temperature for 60 minutes. The signal was visualized by addition of an alkaline phosphatase substrate, nitrophenol phosphate disodium substrate

(Sigma Chemical Co., St. Louis, MO) dissolved in 1M Tris pH 8.5. The reaction was terminated by addition of 13% K_2HPO_4. Samples were read using a Bio-Tek EIA reader, EL-307 (BioTek Instruments Inc., Winooski, VT) set at 405 nm. Titers were quantitated relative to a Rous-associated virus type 2 (RAV-2) stock used as a standard.

Cells expressing viral CA protein were detected using a modification of the immunocytochemical assay described by Stoker and Bissell (1987). Infected osteoblasts or fibroblasts which had been plated at low densities (10^5 cells/22 mm plate) were fixed by incubating in 2% paraformaldehyde. The fixed cultures were incubated with CVI-ALVp27-9Q, a mouse monoclonal against the Gag protein, CA (DeBoer and Osterhaus, 1985) at a concentration of 10 μg/ml. After washing, the signal was amplified by incubating with a biotinylated goat anti-rabbit IgG (Cappel, Malvern, PA). The cultures were washed and incubated with a streptavidin-alkaline phosphatase conjugate (GIBCO, Grand Island, NY). All incubations were done for 2 hours at room temperature with constant rocking. The signal was visualized by addition of a detection solution (5-bromo-4-chloro-3-indolyl phosphate (100 μg/ml), nitroblue tetrazolium (1 mg/ml) in 100 mM NaCl, 5mM $MgCl_2$ and 100mM Tris, pH 8.8). The reaction was terminated by flooding the plates with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

The extent of infection in the cultures were determined by immunocytochemical staining. The expression of the viral antigen, CA, served as a marker for virus infection. The extent of infection was determined by counting the number of CA positive cells under low magnification and was expressed as the proportion of CA-expressing cells in

the total cell number in a microscope field. Five microscope fields were counted per culture dish and the mean value was calculated.

Retroviral Interference Assay. The extent of interference established in infected osteoblast and fibroblast cultures was determined as a function of the susceptibility to superinfection by virus pseudotypes that express a detectable marker (ß-galactosidase activity). The generation of the pseudotyped stocks was described above. Infected and uninfected cultures were seeded at 1.5 x 10^5 cells/ 22 mm well and infected with the pseudotype stocks using a series of dilutions of the virus stock. All infections were done in duplicate. Infections were performed for 60 minutes at 37°C in the presence of polybrene (10µg/ml). Following infection, the virus stock was removed and the cultures were grown for 2 days. Two days following pseudotype challenge, the cultures were fixed in 2% paraformaldehyde, washed three times with PBS and incubated overnight at 37°C with a ß-galactosidase staining solution (10mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, supplemented to 1 mg/ml 5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (X-gal) in N, N-dimethyl formamide) (Maniatis et al., 1982). ß-galactosidase-expressing cells stain blue.

Histochemical analyses. All histochemical assays were performed in collaboration with the laboratories of Dr. G. Stein and Dr. J. Lian, Department of Cell Biology, University of Massachusetts, Worcester. Alkaline phosphatase expression and mineralization, two markers of osteoblast differentiation were monitored by histochemical staining. Replicate cultures were fixed with 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, rinsed

in 0.1 M cacodylate and stained as follows. Alkaline phosphatase activity was visualized by incubation with AS-MX phosphate and Fast Red TR salts (in N,N-dimethyl formamide and 0.1 M Tris acid Maleate, pH 8.4) at 37°C for 30 minutes. The reaction was terminated by removal of the staining solution and addition of distilled H_20. Mineralization was detected by von Kossa silver staining. Fixed cultures were incubated with 3% silver nitrate for 30-60 minutes under bright light.

Biochemical analyses. All biochemical analyses were performed in collaboration with the laboratories of Dr. G. Stein and Dr. J. Lian, Department of Cell Biology, University of Massachusetts, Worcester. DNA content in cell layers was determined fluorometrically using the method of Vytasek (1982). Samples were prepared by extraction in 1N perchloric acid and hydrolysis at 70°C in 0.5 N HCl for 30 minutes. Equal volumes of sample and dye reagent (5% m-diaminobenzoic acid dihyrochloride in 7.5 mM Na_2CO_3 prepared in 1M NaOH) were incubated at 37°C for 60 minutes in the dark. The reaction was stopped by the addition of cold 1N HCl. Samples were read in a Perkin-Elmer LS-50 Fluorimeter, excitation set at 408 nm and emission at 500 nm. Amounts of DNA were determined relative to dilutions of ultrapure calf thymus DNA (Hoeffer, San Francisco, CA).

The level of osteocalcin in culture medium or in chicken serum was determined by radioimmunoassay, as described in detail previously (Gundberg et al., 1984). For this assay, a species-specific polyclonal rabbit anti-chicken osteocalcin antiserum was used for

the standard and purified chicken osteocalcin (isolated in the laboratories of Dr. G. Stein and Dr. J. Lian) was used for the tracer.

Alkaline phosphatase activity was determined using a technique developed by Lowry et al., 1954. Scraped cell layers or samples of chicken sera were disrupted using multiple freeze-thaw cycles and sonication. Activity was assayed by incubation of the sample in glycine buffer (0.1% Triton X-100, 1mM $MgCl_2$, 100mM glycine, pH 10.5) containing 2 mg nitrophenol phosphate disodium substrate (Sigma Chemical Co., St.Louis, MO). The reaction was terminated by addition of 0.2 M NaOH. The samples were quantitated spectrophotometrically at 410 nm and the level of activity determined relative to concentrations of a p-nitrophenol standard (Sigma Chemical Co., St. Louis, MO).

Western analysis. Protein samples were prepared by incubating cell pellets or ground bone in 2x Laemmli buffer (1% SDS, 10% glycerol, 5% ß-mercaptoethanol, 8 M urea, 10 µg/ml leupeptin and 62.5 mM Tris, pH 6.8) and boiling for 5 minutes (Laemmli, 1970). Particulates were removed by centrifugation and the samples were aliquotted and stored at -80°C. Protein concentrations were determined by the BioRad assay (BioRad, Richmond, CA) using a lysozyme standard. Equal amounts of total protein (typically 5-20 µg) were electrophoretically separated on either 8% (for analyses of Env proteins) or 12% (for analyses of Gag proteins) SDS-polyacrylamide gels run at constant current. Proteins were electro-transferred to Immobilon-P membranes (Millipore, Bedford, MA), or nitrocellulose (Schleicher and Schuell, Keene, NH) at 20 volts for 16-18 hours in transfer buffer (380 mM glycine, 0.1% SDS, 20 % methanol and 50mM Tris, pH 6.8) (Ausubel

et al., 1989). While wet, filters were UV-crosslinked using a Stratalinker (Stratagene, LaJolla, CA) set at 1200 μJoules x 100/cm^2. Incubation buffers contained 0.1% Tween-20 in PBS and contained different blocking agents, depending on the viral proteins to be detected. For detection of Gag proteins, 3% bovine serum albumin was used as a nonspecific blocking agent; buffers used for detection of Env proteins contained 5% nonfat dried milk. Blots were preincubated for 1 hour at room temperature. Incubations with antibodies were carried out either overnight at 4°C or for 4 hours at room temperature. Gag proteins were detected using a polyclonal rabbit anti-CA antibody (SPAFAS, Norwich, CT) at a concentration of 10 μg/ml. Detection of Env proteins was carried out using a 1:25 dilution of a polyclonal rabbit anti-gp85 antiserum (kindly provided by C.M. Stoltzfus). Blots were washed, and then incubated for 2 hours at room temperature with ^{125}I-labelled Protein A (Amersham, Arlington Heights, IL) diluted 1:2000 in incubation buffer. Blots were washed at least 5 times in 0.1% Tween-20 in PBS, dried and exposed to X-ray film at -80°C in the presence of an intensifying screen.

Southern analyses. DNA was extracted from cell pellets and from chicken bone using a modified version of the protocol previously described (Robinson & Miles, 1985). Bones from normal or osteopetrotic birds were pulverized in the presence of liquid nitrogen by grinding samples into a fine powder using a mortar and pestle. DNA was harvested from ground bone or from cell pellets by incubating samples for 2 hours at 37°C in TEN buffer (10 mM EDTA, 100 mM NaCl, 10mM Tris, pH 8.0) supplemented with 1% SDS and 0.2 mg/ml Proteinase K. The digests were extracted twice with an equal volume of phenol,

then once in chloroform-isoamyl alcohol (40 volumes of chloroform to 1 volume of isoamyl alcohol) and precipitated with 2 volumes of cold ethanol. Precipitated DNA was recovered by centrifugation. Samples were washed briefly in 70% ethanol and then dried under a vacuum. The samples were resuspended in TEN buffer and treated for 2 hours at 37°C with RNase A (10 μg/ml) to remove contaminating RNA. The samples were again extracted twice with phenol, once with chloroform/isoamyl alcohol, reprecipitated using two volumes of ethanol and recovered by centrifugation. The samples were resuspended in TE (10mM Tris, 1mM EDTA, pH 8.0). DNA concentrations were determined and relative purity was ascertained spectrophotometrically.

Isolated DNA was examined by Southern analysis either undigested or digested by restriction endonucleases. Southern analysis of DNA digested with *Eco*R1 or *Kpn*1 were used to estimate the forms and amounts of viral DNA (Robinson et al., 1993). 5-20 μg of DNA was loaded onto 0.8% agarose gels and electrophoresed at 36 volts for 16-24 hours using a high-salt buffer (40 mM Tris, 50 mM sodium acetate, 10 mM EDTA, pH 7.9). Equivalent loading was ascertained by ethidium bromide staining of the gel following electrophoresis. Following alkaline denaturation and neutralization, the DNA was transferred to nitrocellulose (Schliecher & Schuell, Keene, NH) by capillary action, using 10x standard saline citrate (SSC) (1.5 M NaCl, 0.15 M sodium citrate). The transferred DNA was UV-crosslinked to nitrocellulose using a Stratalinker (Stratagene, La Jolla, CA). Filters were prehybridized by incubating at 67°C for 2-4 hours in 0.125% dried milk and 6x SSC. Radiolabelled probes were prepared by nick translation of

pRAV-1, a full length copy of RAV-1 cloned into pBR322. The probes were generated using ^{32}P-dATP and ^{32}P-dCTP (Amersham, Arlington Heights, IL) to a specific activity of $\geq 10^8$ cpm/μg. The probe was added directly to the prehybridization mix and hybridization was carried out overnight (16-20 hours) at 67°C. Following hybridization, the filters were washed with at least 4 changes of 0.2x SSC and 0.1% SDS at 67°C over a period of 90 minutes. Filters were dried, and exposed at -80°C to X-ray film using an intensifying screen.

Generation of Neutralizing Antibody. Six month old line K28 chickens were inoculated intramuscularly with 1 ml of clarified subgroup E virus and intravenously with 5 x 10^6 fibroblasts infected with a subgroup E virus. Chickens were boosted at 8 weeks with 1 ml of virus given intramuscularly. Chickens were bled weekly and the sera were tested for virus neutralization activity. Neutralization activity was identified by the ability of the chicken serum to restrict or eliminate infection by a subgroup E viral pseudotype following preincubation of the serum with the virus. Neutralizing antisera was generated which at a 1:500 dilution would neutralize 2 x 10^4 infectious units of an RDlac(Br21) stock. This antiserum was used at a 1:100 dilution in osteoblast cultures to restrict virus infection. Antiserum was cleared of complement by heat inactivation (56°C for 30 minutes) prior to use.

Pathogenicity trials. Approximately 0.2 ml of undiluted virus was injected into the leg vein of day old line K28 chicks. Infected birds were housed separately from uninfected controls. Birds were observed twice weekly for signs of onset/progression of osteopetrosis

and/or any discernible pathology. Sera samples were collected at biweekly intervals and stored at -80°C. At 11 weeks postinfection, the birds were sacrificed and samples of bone from the tibia and femur were collected. The bones were broken open and cleared of marrow by flushing with saline. Bone samples were quick frozen in liquid nitrogen for later analysis.

Statistical analysis. For tests in chickens, levels of significance (P) were calculated by the Welch-Aspin two tailed *t* test using the Minitab statistical analysis software program (Minitab Inc., State College, PA). In all cases, P values refer to differences between normal and osteopetrotic groups of the same age.

Densitometric analysis. Autoradiograms of Western blot and Southern blot analyses were scanned by densitometry to quantitate relative levels of viral proteins and viral DNA, respectively. Quantitation was done by computer-assisted image analysis, using the software program Image Measure IM1100 for densitometric analysis (Microscience, Seattle, WA).

CHAPTER III

REPLICATION OF AN OSTEOPETROSIS-INDUCING VIRUS IN OSTEOBLASTS IN VITRO AND IN VIVO

Abstract

The replication of an osteopetrosis-inducing avian leukosis virus (ALV) was compared in cultures of chick fibroblasts, calvarial osteoblasts and in osteopetrotic bone. Diseased bone contained 10 times more integrated and unintegrated viral DNA than either osteoblast or fibroblast cultures. Moreover, the expression of viral Gag proteins was 10 times higher in osteopetrotic bone than in the infected cultures. No atypical forms of Gag or Env proteins were observed either in infected osteoblast cultures or in osteopetrotic bone. Osteoblast cultures replicated virus and expressed viral proteins at levels comparable to those found in infected fibroblasts. These results indicate that osteopetrotic bone sustains substantially higher levels of infection and accompanying viral protein expression than osteoblasts in culture. The high virus load in the bone could be important in disease pathogenesis. Osteopetrotic bone also contained 30 times the level of mature CA protein found in infected cultures. The high levels of mature CA in osteopetrotic bone suggest an accumulation of mature virus particles in the bone. A high local concentration of virus could facilitate the high level of infection characteristic of ALV-induced osteopetrosis.

Introduction

Avian leukosis virus (ALV)-induced osteopetrosis is a polyclonal disorder that is characterized by the increased proliferation of osteoblasts in the bone (for reviews see Simpson & Sanger, 1968; Smith, 1982). As osteoblast function is primarily in bone formation, the disease results in substantial enlargement of affected bones, increasing the diameters by 2-6 fold (Banes & Smith, 1977). Although ALV infection results in osteopetrosis, it is unclear what role the virus plays in perturbing osteoblast growth. Previous studies have demonstrated a correlation between the level of viral DNA in the bone and the severity of osteopetrosis (Robinson & Miles, 1985). Moreover, ALV-induced osteopetrosis is associated with the unusual accumulation of unintegrated viral DNA in the bone, suggesting an atypical virus life cycle in infected osteoblasts.

To examine aspects of the virus life cycle in the cell type principally affected in ALV-induced osteopetrosis, ALV infections were studied in cultures of chick osteoblasts. Osteoblast cultures isolated from embryonic calvaria display a temporal pattern of expression of phenotypic markers of the osteoblast which are consistent with osteoblast differentiation during bone development (Gerstenfeld et al., 1987, 1988; Stein et al., 1990). The availability of an isolated osteoblast culture system also permits examination of virus replication in osteoblasts in the absence of other cell types found in the bone.

The pattern of in vitro expression of an osteopetrosis-inducing virus (Br21) was examined in cultures of chick osteoblasts and compared to infections in chick embryo fibroblasts. The pattern of virus spread, virus production and expression of viral DNA and

proteins were determined. Virus expression was also compared between infected cultures and Br21-infected osteopetrotic bone. The results reveal that substantially higher levels of viral infection and viral protein production occur in osteopetrotic bone than in infected osteoblast cultures.

Results

Examination of Br21 replication in culture. The availability of an osteoblast culture system permitted the study of virus replication in the cell type whose function is distorted in ALV-induced osteopetrosis. Osteoblast culturing was carried out in collaboration with the laboratories of Dr. Gary Stein and Dr. Jane Lian, Department of Cell Biology, University of Massachusetts, Worcester. Cell isolation and culturing were based on the method of Gerstenfeld and coworkers (1987). Primary cultures were isolated from embryonic chick calvaria by enzymatic digestion and grown for a primary period of 21 days (for details, see Chapter II). Following the primary culturing period, osteoblast cultures were infected by replating trypsinized cells in Br21-containing media (day 0). Mock infected cultures were replated in conditioned media. To compare patterns of Br21 replication in culture, infections in chick osteoblast cultures were compared to infections in chick embryo fibroblasts. Chick fibroblasts are a cell type commonly used to study retroviral replication. Further, chick fibroblast and osteoblasts have similar doubling times in culture, prior to the induction of differentiation in osteoblast cultures (data not shown).

To characterize Br21 replication in culture, the pattern of virus production was compared in fibroblast and osteoblast cultures. Infections were initiated in both osteoblast and fibroblast cultures with equivalent levels of virus particles. To quantitate viral replication, the level of virus released into the culture medium was determined using an antigen capture ELISA directed against the viral CA protein (Brown & Robinson, 1988a). The results demonstrate that the patterns of virus production were very similar in infected

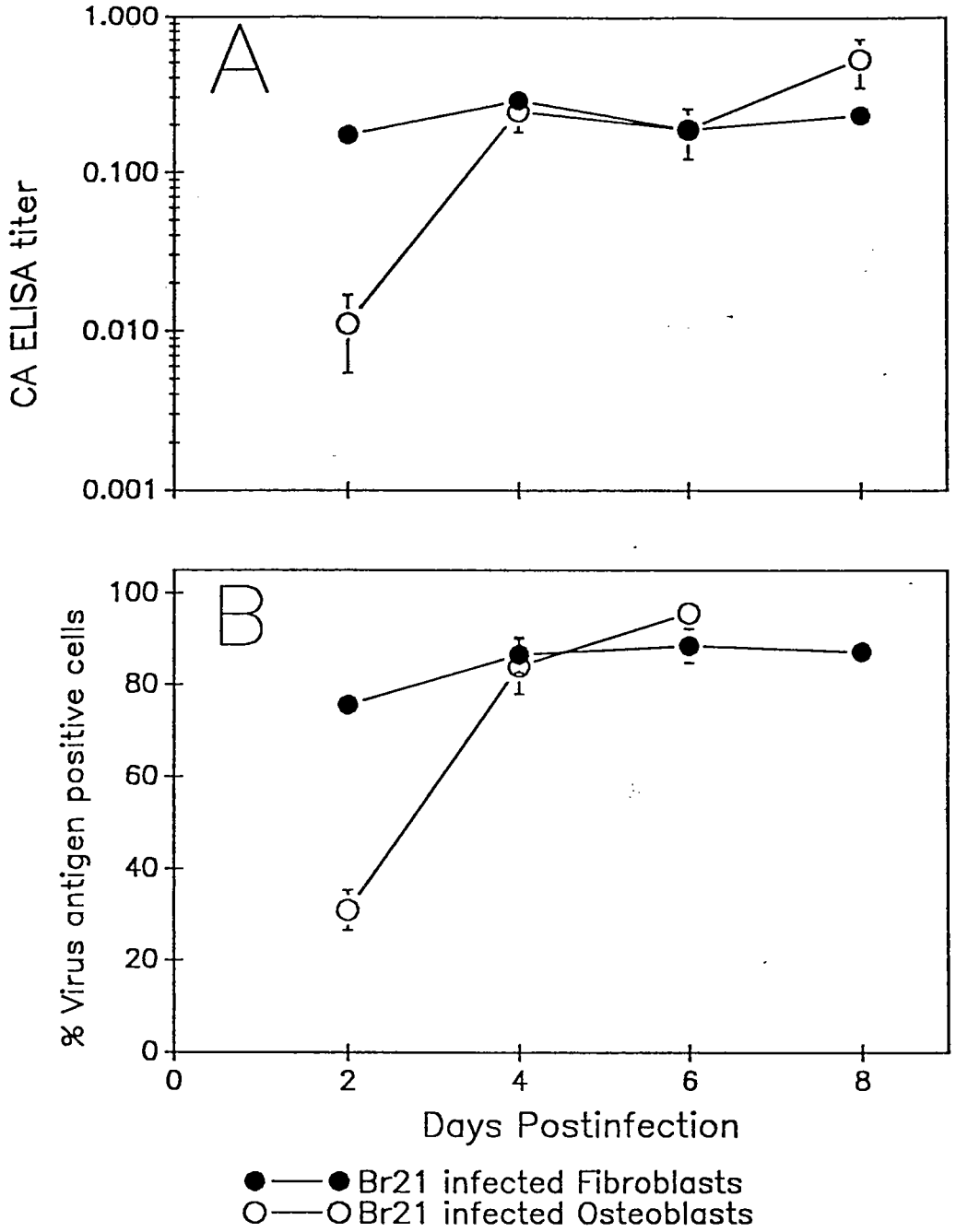

Figure 1. Virus production and viral spread in Br21 infected fibroblast and osteoblast cultures. Values represent the mean ± SD; n = 3-5 samples. Some error margins are very small and the error bars are not evident on the graph. (A) Relative level of virus replication. The level of virus in 48 hour cultured medium was determined using an antigen capture ELISA against the Gag protein, CA. ELISA titers are quantitated relative to a Rous-associated virus type 2 (RAV-2) standard. (B) Extent of virus infection as determined by % virus antigen positive cells in the culture. Virus positive cells were detected by immunocytochemical staining for CA protein.

osteoblast and fibroblast cultures at all but the earliest times after infection (Figure 1A). At two days after infection, virus in the medium had already reached plateau levels in fibroblast cultures. By contrast, virus in the medium of osteoblast cultures did not reach plateau levels of virus expression until 4 days after infection. The patterns of virus accumulation in the medium of fibroblasts and osteoblasts were consistent in 4 independent experiments. Further, the pattern of intracellular expression of viral antigens was evaluated in cell lysates of osteoblast and fibroblast cultures. As determined by ELISA, the level of viral antigen expression in infected osteoblasts was similar in level to that in fibroblasts at all time points with the exception of 2 days after infection. At 2 days after infection, the expression of Gag protein was 4-6 fold lower in osteoblasts than in fibroblasts. Similar patterns of expression were observed in two independent experiments. Thus, the patterns of viral antigen expression in osteoblast and fibroblast cultures were similar to the patterns of virus released into the medium. This indicates that virus production in the medium was a reflection of virus replication in the cell.

To determine the extent of infection in osteoblast and fibroblast cultures over time, immunocytochemical staining was used to estimate what proportion of the culture was infected (Stoker & Bissell, 1987). Productively infected cells were detected using a monoclonal antiserum against the virus capsid protein, p27 (CA). Two days after infection, approximately 30% of the cells in the osteoblast culture were virus antigen positive as compared to 75% of the fibroblast culture (Figure 1B). As infections were initiated with comparable levels of virus, these results would suggest that osteoblast

TABLE 1

VIRUS PRODUCTION PER INFECTED CELL
(ELISA units/Virus antigen positive cell)[1]

	Days following infection					
Cell type	day2	day4	day6	day8	day10	day11
Osteoblast	0.049	0.150	0.087	0.204	0.145	ND
Fibroblast	0.012	0.059	0.067	0.063	ND	0.070
Ratio	4.1	2.5	1.3	3.2		

1. Values were calculated as the relative level of virus production in the medium (as determined by ELISA) divided by the number of virus antigen-expressing cells in the culture (as determined by immunocytochemical staining). Values represent the mean of n = 3 individual samples analyzed per time point. Values are x 10^6. The results are representative of 2 independent analyses. ND, not done.

cultures are less susceptible to initial infection. By 4 days after infection, the proportion of infected cells in the osteoblast culture was comparable to that in fibroblast cultures, suggesting that virtually all of the cells in the osteoblast culture could be productively infected.

To examine how the level of virus production per cell compared in osteoblast and fibroblast cultures, the amount of virus in the medium relative to the number of virus-antigen positive cells in the culture was calculated (Table 1). The level of virus in the medium per infected cell was overall similar in osteoblast and fibroblast cultures. Osteoblasts consistently produced 2-4 fold higher levels of virus per cell than fibroblasts. These results indicate that osteoblast cultures infected with an osteopetrosis-inducing virus produce similar or slightly higher amounts of virus than infected fibroblasts.

Immunocytochemical staining also revealed differences in the intracellular distribution of viral CA protein in infected osteoblasts and fibroblasts (Figure 2). Although the majority of the osteoblast population contained a uniform distribution of viral CA protein, approximately 20-50% of the virus antigen positive cells in osteoblast culture displayed a localized distribution of CA within the cell (Figure 2B). The localization of viral protein was manifest as an area of intense staining restricted to a region of the cell, with the remainder of the cell appearing devoid of signal. The localized distribution in osteoblasts was apparent at all times postinfection and occurred irrespective of whether the cells were actively proliferating or differentiating. In contrast, immunocytochemical staining of infected fibroblasts typically revealed a uniform

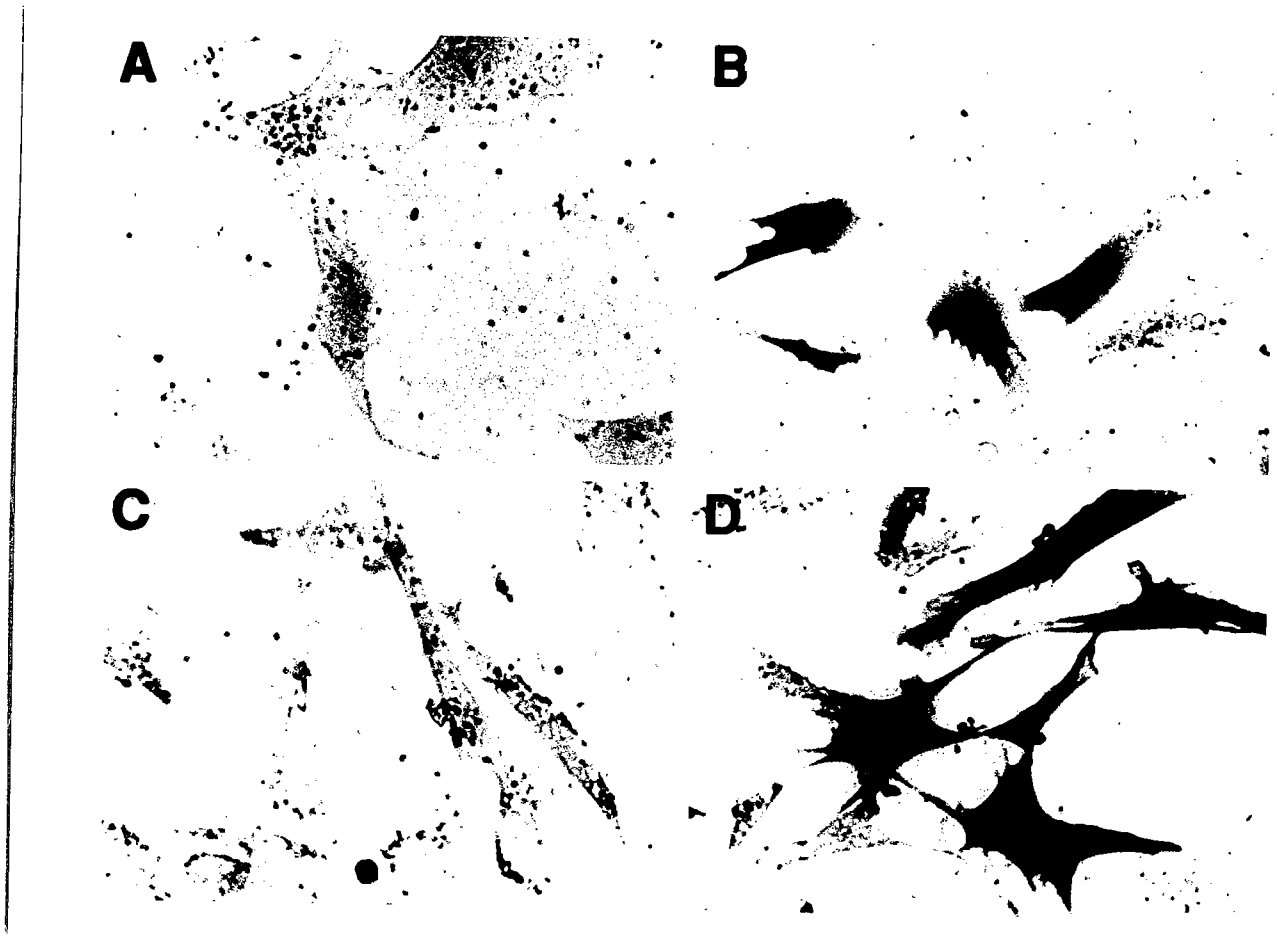

Figure 2. Photomicrographs of infected osteoblasts and fibroblasts immunocytochemically stained for viral CA protein. (A) Uninfected osteoblasts. (B) Br21 infected osteoblasts, bearing the localized pattern of staining. (C) Uninfected fibroblasts. (D) Br21 infected fibroblasts.

distribution of viral antigen throughout the cell (Figure 2D). Uninfected osteoblasts (Figure 2A) and fibroblasts (Figure 2C) did not demonstrate a consistent signal above background. As such, the alkaline phosphatase signal used for the immunocytochemical detection did not appear to reflect endogenous alkaline phosphatase expression by osteoblasts. It is unclear whether the intracellular distribution of viral protein represents an active localization or a specific exclusion of virus within the infected osteoblast.

Analysis of viral DNA in osteoblast cultures and in osteopetrotic bone. ALV-induced osteopetrosis has been associated with the persistence of unintegrated viral DNA (Robinson & Miles, 1985; Aurigemma et al., 1989). To determine whether unintegrated viral DNA persisted in osteoblasts infected in vitro, Southern analysis was used to compare forms and amounts of viral DNA. DNA was isolated from osteoblasts and fibroblast cultures at several points postinfection and undigested DNA samples were compared by Southern analyses. Several forms of viral DNA were observed: HMW, or high molecular weight (representing proviral DNA), linear duplex DNA and covalently closed circular DNA (Figure 3).

A transient accumulation of unintegrated viral DNA was observed in fibroblasts, which was no longer detectable at 9 days after infection (Figure 3A). By contrast, a low but detectable level of unintegrated viral DNA persisted in infected osteoblast cultures up to 8 days after infection (Figure 3B). Unintegrated viral DNA was detected as late as 30 days after infection in osteoblast cultures but was not detected in infected fibroblasts later than 6 days after infection. The majority of the unintegrated viral DNA was in the form

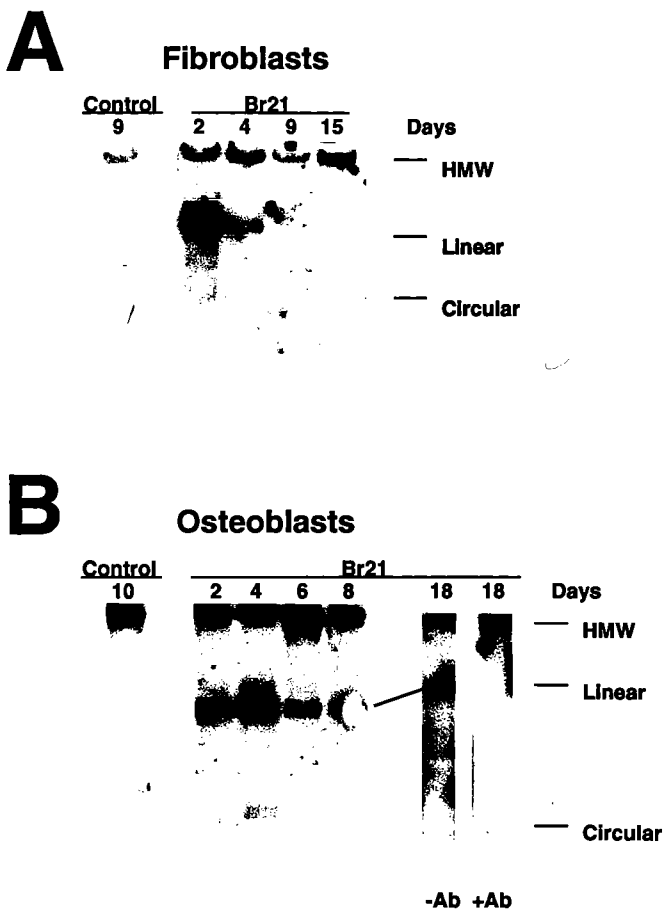

Figure 3. Southern blot analysis of undigested DNA from Br21-infected fibroblast and osteoblast cultures. (A) Viral DNA in fibroblasts at several time points after infection with Br21. (B) Viral DNA in osteoblast cultures at several points after infection with Br21. Br21 infected osteoblasts which were grown in the presence (+ Ab) or absence (- Ab) of neutralizing antibody 4 days after infection are as indicated. Forms of viral DNA are indicated to the right: HMW, high molecular weight DNA; Linear, linear duplex DNA; Circular, closed circular DNA. Blots were hybridized with a radiolabelled probe prepared by nick translating a full length genomic copy of RAV-1. The signal in control lanes reflects cross hybridization with an endogenous virus (*ev* 1) found in line K28 chickens.

of linear duplex DNA. The level of unintegrated viral DNA was highest in fibroblasts 2 days after infection but was delayed in osteoblast cultures until 4 days after infection (Figure 3A, B). This delay correlates with the slower time course of infection in osteoblast cultures (Figure 1B).

In several C-type retroviral infections, the persistence of unintegrated viral DNA results from a spreading infection or from superinfection (Weller et al., 1980; Donahue et al., 1991). To test whether a continuing infection could result in the persistence of unintegrated viral DNA in osteoblast cultures, neutralizing antibody was added to infected cultures to inhibit virus spread and reinfection. Osteoblast cultures were infected with Br21 and 4 days after infection were exposed either to neutralizing chicken antiserum (directed against subgroup E viruses) or to normal chicken serum. Two weeks after the addition of antiserum, DNA was isolated from both cultures and the levels of unintegrated viral DNA were compared by Southern analysis. Infected osteoblast cultures maintained in the presence of control chicken serum contained unintegrated linear viral DNA (Figure 3B; lane -Ab). In contrast, infected osteoblasts cultured in the presence of neutralizing antibody did not contain detectable unintegrated linear viral DNA (Figure 3B; lane +Ab). These results suggest that a continuing infection was required for the persistence of unintegrated viral DNA in osteoblast cultures.

To quantitate the relative levels of unintegrated viral DNA in osteoblast cultures and osteopetrotic bone, Southern analysis was used to compare DNA amounts in samples from infected osteoblasts with serial dilutions of DNA isolated from cases of severely

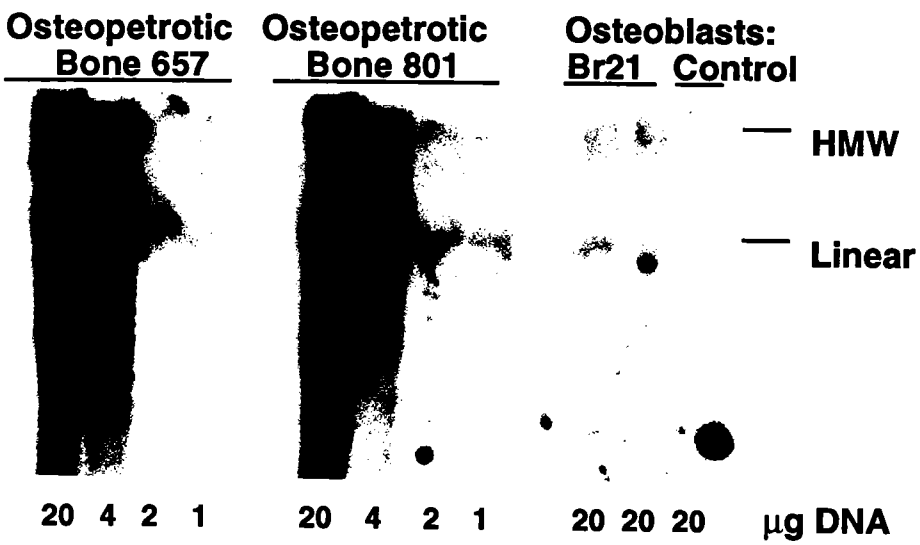

Figure 4. Southern analysis of viral DNA in Br21 infected osteoblast cultures and Br21 infected osteopetrotic bone. Osteoblast samples were derived from cultures 18 days after infection. Bone samples originate from Br21 infected chickens 657 and 801 which developed severe osteopetrosis. Forms of viral DNA are indicated to the right: HMW, high molecular weight DNA, Linear, linear duplex DNA. Amounts of analyzed cellular DNA are indicated at the bottom. The blot was probed using a nick translated copy of pRAV-1, containing a full length copy of the ALV, RAV-1.

TABLE 2
RELATIVE AMOUNTS OF UNINTEGRATED LINEAR VIRAL DNA[1]

Sample	Amount of Cellular DNA (μg)	Relative Amount of Unintegrated Linear Viral DNA[2]
Severely osteopetrotic bone[3]	20	Off scale[4]
	4	6.7 ± 0.8
	2	2.2 ± 0.2
	1	1.0 ± 0.1
Osteoblasts	20	1.0 ± 0.1

1. The relative amount of linear unintegrated viral DNA was determined by densitometric analysis of autoradiograms from Southern blot analyses, as in Figure 4. Values reflect the mean ± SD of n = 3-4 analyses.
2. Densitometric units were standardized relative to levels in Br21 infected osteoblasts.
3. Samples of osteopetrotic bone were obtained from Br21 infected chickens that developed severe osteopetrosis. In each case, the mean of 2-3 severely osteopetrotic bone samples were analyzed on the same blot.
4. Off scale, densitometric readings that exceeded the range of detection.

osteopetrotic bone. As linear duplex DNA represents the predominant form of unintegrated viral DNA, both in infected osteoblast cultures (Figure 3) and in osteopetrotic bone (Robinson & Miles, 1985), the relative level of unintegrated linear viral DNA was compared. As demonstrated in Figure 4 and confirmed by densitometric analysis (Table 2), the amount of unintegrated linear viral DNA in osteopetrotic bone was 20 times higher than in infected osteoblast cultures. Thus, osteopetrotic bone contained significantly more unintegrated linear viral DNA than the infected osteoblast cultures. Southern analysis was also used to estimate the total level of viral DNA in infected cultures and in the diseased bone. The copy number was estimated by normalizing the level of Br21 DNA to the level of an endogenous virus, *ev* 1, present at 1 copy per haploid genome in line K28 chickens (Astrin & Robinson, 1979). *Eco*RI digestion of Br21 DNA cuts the viral genome at four sites and cleaves *ev* 1 sequences twice (Robinson & Miles, 1985). A 2.4 kilobase (kb) *EcoR*I fragment representing an internal fragment generated from both integrated and unintegrated forms of Br21 sequences contains the same length of viral sequences as a 8.5 kb *EcoR*I fragment representing an *ev* 1 junction fragment (Robinson & Miles, 1985). Autoradiograms of Southern blots were densitometrically scanned and the relative level of total viral DNA per cell was estimated by normalizing the signal of the 2.4 kb Br21 band to that of the 8.5 kb *ev* 1 band.

Using this strategy, the total viral DNA copy number in infected osteoblast and fibroblast cultures was determined at several points after infection (Table 3). Bone samples from Br21 infected chickens which developed moderate or severe degrees of

TABLE 3

COPIES OF VIRAL DNA IN BR21 INFECTED FIBROBLASTS, OSTEOBLASTS AND OSTEOPETROTIC BONE[1]
(Total Viral DNA/Cell)

	Days After Infection		
Sample[2]	day 2	day 4	day 6
Fibroblast	2.2	3.0	ND
Osteoblast	1.2	1.7	1.8
Osteopetrotic bone[3]:			
Bone 657: Severe Osteopetrosis			17.6
Bone 693: Moderate Osteopetrosis			5.9

1. Estimates of viral DNA copy number per cell were determined by normalization of levels of Br21 DNA to levels of endogenous virus *ev* 1 DNA, present at 1 copy/haploid genome in line K28 chickens. Quantitation was done by densitometric analysis of audioradiograms of *Eco*RI digested DNA from infected fibroblasts, osteoblasts and osteopetrotic bone analyzed by Southern blots. ND, not done.
2. All cultures and chickens used for this study were from chicken line K28.
3. Bone samples were collected from Br21 infected chickens which had developed differing extents of osteopetrosis. The extent of osteopetrosis was determined by the relative distortion of the long bones of the legs of the chickens at 11 weeks postinfection (see Chapter V for details). Bone 657 was derived from a Br21 infected chicken which developed severe osteopetrosis; bone sample 693 was derived from an infected chicken which developed moderate osteopetrosis.

osteopetrosis were also included in the analysis. Total levels of viral DNA averaged ~2-3 copies/cell in both osteoblast and fibroblast cultures (Table 3). The level of viral DNA remained constant in Br21 infected osteoblasts as late as 30 days after infection. In comparison, a case of moderate osteopetrosis contained ~6 total viral DNA copies/cell and a case of severe osteopetrosis contained ~18 copies/cell. Higher levels of viral DNA in the bone have been shown to correlate with more severe forms of osteopetrosis (Robinson & Miles, 1985). These results indicate that the total level of viral DNA in infected osteoblasts was comparable to the level in infected fibroblasts and 10-15 times lower than the level in severely osteopetrotic bone.

Expression of viral proteins in culture and in bone. To determine whether the higher levels of viral DNA in osteopetrotic bone resulted in increased levels of viral protein synthesis, Western analysis was performed on lysates from infected cultures and osteopetrotic bone. Comparable amounts of cellular protein from osteoblast and fibroblast cultures and serial dilutions of protein from osteopetrotic bone were separated by SDS-polyacrylamide gel electrophoresis and analyzed for steady state levels of viral Gag (using a polyclonal antisera against CA) or Env protein (using a polyclonal anti-gp85 antisera).

No qualitative differences in viral proteins were discernible between infected cultures and osteopetrotic bone (Figure 5A, B). Infected osteoblasts, fibroblasts and osteopetrotic bone expressed expected forms of Gag precursor (Pr76, Pr66, Pr60) and mature (p27) proteins as well as precursor and mature forms of Env proteins (Pr95 and

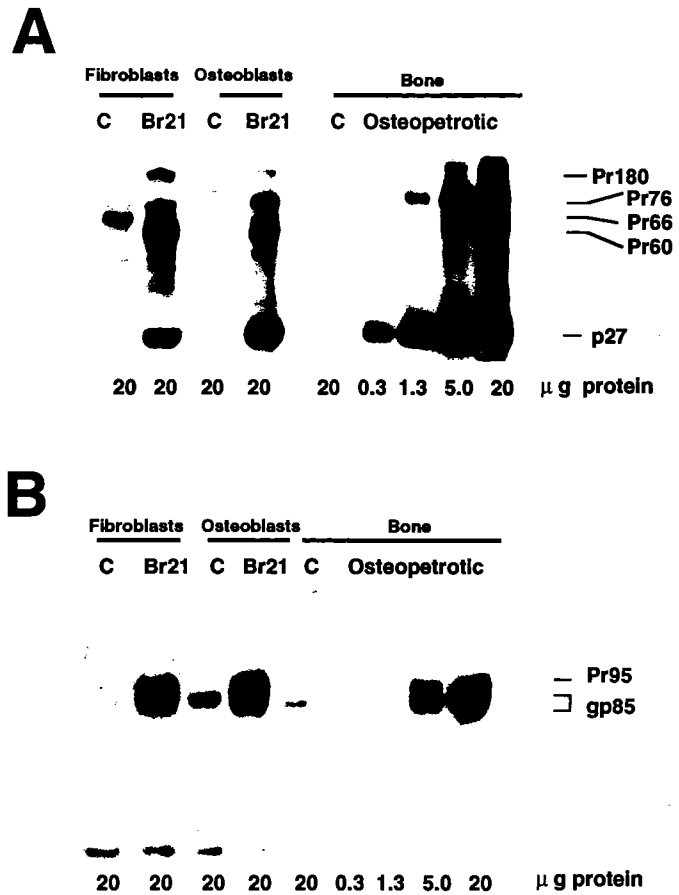

Figure 5. Western analysis of viral Gag and Env proteins in Br21 infected fibroblasts, osteoblasts and osteopetrotic bone. (A) Analysis of Gag proteins. Pr76, Pr66 and Pr60 represent Gag precursor forms; p27 represents the mature Gag protein. Pr180 is a Gag-Pol fusion protein which is a precursor to Pol proteins and contains Gag protein sequences also recognized by the antiserum. (B) Analysis of Env proteins. Pr95 represents the precursor Env protein; gp85 represents the mature envelope glycoprotein. Control (C) and Br21 infected samples are as indicated. Amounts of cellular protein analyzed are indicated at the bottom.

TABLE 4

RELATIVE LEVELS OF VIRAL PROTEINS (GAG AND ENV) IN FIBROBLASTS, OSTEOBLASTS AND OSTEOPETROTIC BONE[1]

	Relative Pr76[2]	Relative p27 (CA)[3]	Relative Env[4]
Fibroblast	1.0	1.0	1.0
Osteoblast	1.2 ± 0.3	1.6 ± 0.7	1.2 ± 0.1
Osteopetrotic Bone	7.4 ± 3.1	36.2 ± 4.1	2.3 ± 0.3

1. Levels of viral proteins were determined by densitometry. Autoradiograms of Western blots were scanned and the levels of viral proteins were standardized relative to the levels in fibroblast cultures. Values reflect the mean ± SD of at least three independent analyses using different fibroblast, osteoblast and osteopetrotic bone samples.
2. Pr76 represents the major Gag precursor protein.
3. p27 represents the mature Gag protein.
4. Env represents both precursor (Pr95) and mature (gp85) Env proteins.

gp85, respectively). No atypical forms of viral proteins were detected in either infected culture or in osteopetrotic bone.

Osteopetrotic bone contained higher levels of precursor Gag proteins and Env proteins than infected fibroblast or osteoblast cultures. Densitometric analysis indicated that levels of the major precursor form of Gag, Pr76, were ~7 times higher in severe osteopetrosis than in either osteoblast or fibroblast cultures (Table 4). In comparison, levels of Env protein [analyzed as combined precursor (Pr95) and mature (gp85) forms] were only slightly increased (2 fold) in osteopetrotic bone. Increased levels of viral protein were consistent in analyses using three different samples of severely osteopetrotic bone. In comparison, infected osteoblast and fibroblast cultures contained very similar levels of Gag and Env proteins. These results indicate that severe osteopetrosis is associated with high levels of viral protein expression.

In osteopetrotic bone, the level of mature Gag protein (p27 or CA) was increased more than 30 fold over the levels in either osteoblast or fibroblast cultures (Figure 5A; Table 4). Since processing of the Gag precursor to its mature forms occurs during budding and maturation of virus particles, the high levels of CA could represent the accumulation of mature virus in the diseased bone. This indicates that, in comparison with infected cultures, osteopetrotic bone not only contains higher levels of viral DNA and proteins, it contains much higher levels of mature virus particles.

Discussion

Virus replication in osteoblasts in culture. Surprisingly, the replication of Br21 in cultured osteoblasts was both quantitatively and qualitatively very similar to that observed in fibroblasts. Patterns of virus production and levels of virus replication per infected cell were very similar in osteoblast and fibroblast cultures (Figure 1; Table 1). Western analysis indicated that infected osteoblasts produced precursor and mature Gag and Env proteins of the expected molecular weight and in comparable amounts to infected fibroblasts (Figure 5A, B). Taken together, these data indicate that in culture, the infection of osteoblasts, the cell type associated with dysfunction in ALV-induced osteopetrosis, does not result in a novel pattern of virus replication.

Our studies indicate that the time course for infection was slower in osteoblast than fibroblast cultures. Two days after infection, osteoblast cultures contained lower proportions of infected cells than fibroblasts (Figure 1B). The decreased numbers of infected cells correlated with lower levels of virus production (Figure 1A). Osteoblast cultures also demonstrated a temporal delay in the appearance of maximal levels of unintegrated viral DNA in culture, relative to fibroblasts. In osteoblast cultures the maximal levels of unintegrated viral DNA were observed at 4 days after infection, whereas this occurred at 2 days after infection in fibroblast cultures (Figure 3A, B). These differences suggest that the osteoblast cultures have a slower time course for infection and may be less susceptible to initial infection than fibroblasts.

Intracellular localization of virus antigen in infected osteoblasts. Immunocytochemical staining revealed differences in the intracellular distribution of viral Gag protein in infected osteoblasts and fibroblasts (Figure 2). In infected fibroblasts, viral antigen was uniformly distributed throughout the cell, whereas infected osteoblasts often displayed a distinct localization of antigen within the cell. This localized distribution of viral Gag protein was also observed in osteoblasts infected with RAV-1 or Br131 (data not shown). The antiserum used for immunocytochemical detection recognizes mature as well as precursor forms of Gag proteins, indicating that the process of Gag protein production, transport and/or virus assembly could be affected by, or involved in, the localization of Gag proteins in infected osteoblasts. It is unclear whether the localization of viral proteins occurs at specific cellular organelle(s) or domains of the cell membrane.

Osteoblasts in the bone display a functional polarity in that they secrete collagen matrix and bone-associated proteins from only one face of the cell (for review, see Rodan & Rodan, 1984). It has been shown in polarized epithelial cells that enveloped viruses mature by budding at specific plasma membrane domains (Rodriguez-Boulan & Sabitini, 1978). Influenza and paramyxoviruses mature exclusively at the apical membrane whereas vesticular stomatitis virus and C-type retroviruses assemble only at the basolateral membrane (Roth et al., 1983; Stephens et al., 1986). The envelope glycoproteins of several viruses exhibit polarized transport in the absence of other viral proteins (Roth et al., 1983). Indeed, expression of viral glycoproteins appears to direct the polarized transport and release of virus particles in epithelial cells (Owens et al., 1991). It is not

clear whether osteoblast cultures, like epithelial cells, possess similar mechanisms for sorting membrane proteins. It would be useful to determine whether the polarity exhibited in trafficking cellular proteins in osteoblasts was also utilized in the transport of viral proteins.

ALV-induced osteopetrosis is associated with high levels of infection and viral protein production. Comparison of the levels of viral DNA in osteoblast cultures and in osteopetrotic bone revealed a higher level of infection of cells in the diseased bone than in osteoblast cultures. Severe osteopetrosis contained 10-15 times higher levels of total viral DNA per cell, as compared to infected osteoblast cultures (Table 2). The higher levels of viral DNA corresponded to higher levels of viral protein expression. Osteopetrotic bone contained 7 times the level of precursor Gag proteins than either osteoblast or fibroblast cultures (Figure 3A, Table 4). In infected fibroblasts, viral protein synthesis accounts for 1% of the total protein expressed in the cell (Vogt & Eisenman, 1973). It is possible that the higher levels of viral synthesis in osteopetrotic bone could be responsible for altering normal osteoblast function by placing an unusually high demand on the synthetic capacity of the infected cell. For example, the high level of viral transcription, translation and protein processing could act to divert cellular factors that are important in cellular gene expression, thereby affecting cellular processes and response to physiological stimuli. In ALV-induced osteopetrosis, the higher level of viral biosynthesis (viral load) could alter the normal activity of more complex processes in the

infected cell, such as regulation of cell proliferation or progression to a differentiated cell type (for more detail, see Chapter VI).

High levels of mature virus in the diseased bone. Western analysis demonstrated that high levels of mature CA protein were expressed in the diseased bone. As compared with infected osteoblasts or fibroblasts, osteopetrotic bone contained over 30 times more mature capsid protein (Figure 5A, Table 3). Mature capsid is not typically found intracellularly but is generated by the processing of precursor forms of Gag proteins during virus budding and maturation (for review, see Dickson et al., 1984). Thus, the high levels of CA protein suggest the presence of high levels of virus particles in osteopetrotic bone.

It is possible that the accumulation of virus in the bone could result from trapping of virus produced by infected osteoblasts. The presence of mature virus in the diseased bone is consistent with electron microscopy studies which reveal virus particles budding from and accumulating near osteoblasts and osteocytes in osteopetrotic bone (Simpson & Sanger, 1966; Boyde et al., 1978). Moreover, the level of virus particles increases in the bone as the disease progresses (Frank & Franklin, 1982; Powers et al., 1987). Indeed, periosteocytic spaces often become crowded with virus particles.

This accumulation of mature virus in the bone could, over time, create a high local concentration of virus. If so, the high levels of mature virus in the bone suggest a mechanism through which osteoblasts may become multiply infected. The accumulation

of virus in the diseased bone could expose cells to a high multiplicity of infection, facilitating multiple infection events per cell.

Persistence of unintegrated viral DNA in osteoblast cultures. Differences in the temporal pattern of unintegrated viral DNA in osteoblast and fibroblast cultures indicated differences in the pattern of infection in culture. The accumulation of unintegrated forms of viral DNA in infected fibroblasts was short lived and was not detected at 9 days after infection (Figure 3A). In contrast, cultured osteoblasts maintained low but persistent levels of unintegrated viral DNA well after the first few rounds of virus replication (Figure 3B). The persistence of unintegrated viral DNA in osteoblast cultures appeared to result from continuing infection or reinfection. When infected osteoblast cultures were grown in the presence of neutralizing antibody, the persistence of unintegrated viral DNA was blocked (Figure 3B).

In productively infected cells, superinfection is prevented by the establishment of interference (for review, see Weiss, 1984). Since infected osteoblast cultures contain persistent, albeit low, levels of unintegrated viral DNA, it is possible that the continuing infection in culture represents the superinfection of already infected osteoblasts that are unable to establish interference. This could be relevant to the pathogenesis of osteopetrosis since the mechanism behind the persistence of unintegrated viral DNA in osteopetrotic bone is not understood. The research in Chapter IV focuses on this question by examining the ability of cultured osteoblasts to establish interference to superinfection.

CHAPTER IV

ESTABLISHMENT OF INTERFERENCE IN INFECTED OSTEOBLASTS

Abstract

Avian leukosis virus (ALV)-induced osteopetrosis is a proliferative bone disorder associated with the persistence of unintegrated viral DNA. In several retroviral infections, the accumulation of unintegrated viral DNA has been associated with a delay or failure to establish interference to superinfection. To learn more about the mechanism behind the accumulation of unintegrated viral DNA in osteopetrosis, cultures of chick osteoblasts, infected with an osteopetrosis-inducing virus, Br21, were examined for the ability to establish interference. Infected chick osteoblast cultures were examined for their ability to block superinfection by virus pseudotypes expressing ß-galactosidase. When cultures were multiply trypsinized and passaged, Br21 infected osteoblasts were able to establish interference. Interference was maintained when passaged cultures were induced to differentiate. The ability of cultured osteoblasts to establish interference suggests that productively infected osteoblasts in the bone may be able to prevent reinfection by establishing interference. This suggests that the persistence of unintegrated viral DNA in osteopetrotic bone may not result from superinfection of already infected osteoblasts. Other mechanisms to explain the persistence of unintegrated viral DNA in osteopetrosis are discussed.

Introduction

Interference is a process by which retrovirus infected cells are rendered resistant to superinfection by viruses with the same receptor specificity. Interference occurs via the interaction of the envelope glycoprotein of the interfering virus and the cellular protein acting as the cell receptor (Steck & Rubin, 1966a; Vogt & Ishizaki, 1966). The envelope glycoprotein is believed to act by competitively binding to the cell receptor, blocking receptor activity in virus infection (Steck & Rubin, 1966a, 1966b). The establishment of interference depends solely on the expression of envelope glycoproteins. Interference has been reported in cell lines expressing only the *env* gene (Stevenson et al., 1988; Delwart & Panganiban, 1989; Federspiel et al., 1989) and in cell lines expressing replication-defective endogenous viruses (Robinson et al., 1981). Further, interference can be disrupted by treating cultures with glycosylation inhibitors that prevent the maturation of the envelope glycoproteins (Rein et al., 1982; Kai et al., 1986).

Interference occurs only between viruses containing envelope glycoproteins with the same receptor specificity (for review, see Weiss, 1984). As such, patterns of viral interference, along with host range and antibody cross reactivity, have been used to classify avian retroviruses into envelope subgroups (Ishizaki & Vogt, 1966; Vogt & Ishizaki, 1965, 1966). Avian retroviruses have been divided into five glycoprotein subgroups, denoted A through E. Viruses of subgroups A, B and C recognize independent receptors (Rubin, 1965; Payne & Biggs, 1966; Crittenden et al., 1967). Susceptibility to infection by subgroups A, B and C viruses is conferred by the dominant loci *tv-a, tv-b*

and *tv-c*, respectively. Viruses of subgroups B, D and E appear to recognize closely related receptors. Reciprocal interference is observed between viruses of subgroups B and D, although cells resistant to subgroup B viruses have decreased susceptibility to subgroup D viruses (Duff & Vogt, 1969). Subgroup B viruses exhibit nonreciprocal interference with subgroup E viruses. That is, cells infected with subgroup B viruses can block infection by subgroup E viruses, but cells infected with subgroup E viruses can be infected by subgroup B viruses. Susceptibility to subgroup E viruses appears to be encoded by *tv-b* and is allelic to subgroup B susceptibility (Crittenden & Motta, 1975).

It had been proposed that in the establishment of interference, the interaction of the envelope glycoprotein with the cell receptor occurs either intracellularly, during the transport of both proteins to the cell surface, or at the cell surface following the transport of both proteins. Early studies demonstrated that "early" interference to infection could be induced by preincubating cells with high levels of virus (Steck & Rubin, 1966a). However, in productively infected cells or in cells expressing the *env* gene, the interaction between glycoprotein and cell receptor appears to occur intracellularly, most likely in the endoplasmic reticulum (ER) (Delwart & Panganiban, 1989; Crise et al., 1990; Jabar & Nayak, 1990; Yoshimura et al., 1990). Interestingly, when the envelope glycoprotein of the reticuloendotheliosis virus (REV) was rendered defective in its ability to be transported to the cell surface, it still retained the ability to interact intracellularly with the cell receptor and render the cell resistant to REV infection (Delwart & Panganiban,

1989). This suggests that cell surface expression of viral glycoproteins was not required for the establishment of interference.

Several retroviral infections demonstrate a downregulation of the cell receptor on the surface of infected cells. Recent studies with spleen focus forming virus (SFFV) and human immunodeficiency virus (HIV) have indicated that expression of the viral glycoprotein is sufficient to reduce the level of receptor expression on the cell surface (Stevenson et al., 1988; Kawamura et al., 1989; Yoshimura et al., 1990). The intracellular interaction of the receptor with the unprocessed form of the viral glycoprotein causes a prolonged retention of the receptor in the ER (Yoshimura et al., 1990; Crise et al., 1990). The retention of the receptor in the ER probably results from its interaction with the viral glycoprotein, which is itself inefficiently transported to the cell surface. Only an estimated 5-10% of the envelope glycoprotein of SFFV or HIV is actually expressed on the cell surface (Ruta & Kabat, 1980; Willey et al., 1988). Further, it has been shown that two auxiliary proteins of HIV, Vpu and Nef, act independently to induce the downregulation of the cell receptor, CD4 (Willey et al., 1992; Garcia et al., 1993). Vpu is believed to induce the rapid degradation of CD4 by destabilizing the interaction of the glycoprotein and CD4 (Willey et al., 1992). Thus, the establishment of interference is associated with complex processes regulating the transport and stability of the viral glycoprotein and the host cell receptor in the infected cell.

The inability to rapidly establish interference to retroviral infection can result in high levels of superinfection and cytopathic effect (Keshet & Temin, 1979; Weller et al.,

1980; Haase et al., 1982; Mullins et al., 1986). Infections by cytopathic ALVs and the feline leukemia virus strain which induces feline AIDS (FeLV-FAIDS) are associated with the accumulation of high levels of linear unintegrated viral DNA that result from high levels of superinfection (Weller et al., 1980; Mullins et al., 1986). In cytopathic ALV infections, the high level of superinfection is believed to result from rapid reinfection of the cell prior to the establishment of interference (Weller et al., 1980; Temin et al., 1980). In both FeLV-FAIDS and cytopathic ALV infections, the high levels of unintegrated viral DNA can be prevented by growing infected cells in the presence of neutralizing antisera. In FeLV-FAIDS infections, the delayed processing of viral glycoproteins appears to cause a delay in the establishment of interference (Poss et al., 1990).

ALV-induced osteopetrosis is also associated with the persistence of unintegrated viral DNA (Robinson & Miles, 1985; Aurigemma et al., 1989). However, unlike cytopathic infections which accumulate high levels of unintegrated viral DNA, infection with osteopetrosis-inducing viruses does not cause cytopathicity either in culture or in the bone (Schmidt & Smith, 1981; Powers et al., 1987). Moreover, osteopetrotic bone is associated with 10-20 fold lower levels of unintegrated viral DNA accumulation as compared with cytopathic infections. The mechanism behind the persistence of unintegrated DNA in ALV-induced osteopetrosis is unclear, but could involve superinfection. On average, osteopetrotic bone contains multiple copies of viral DNA per cell, suggesting that each cell sustains multiple infections (Robinson & Miles, 1985).

Results from Chapter III indicated that when osteoblast cultures were infected with Br21, an osteopetrosis-inducing ALV, a low but persistent level of unintegrated viral DNA was observed in culture. When infected cultures were grown in the presence of neutralizing antisera, the persistence of unintegrated viral DNA was blocked, suggesting that viral spread or reinfection is required for the persistence of unintegrated viral DNA in osteoblast cultures. It is possible that the continuing infection in culture reflects the failure of infected osteoblasts to establish interference.

The studies in Chapter IV focus on examining the ability of infected osteoblasts to establish interference by determining the susceptibility of infected osteoblast cultures to superinfection. To detect superinfected cells, virus pseudotypes containing a ß-galactosidase expressing vector, pRDlac1 (Reddy et al., 1991) were used. The effect of virus spread, virus subgroup and differentiation state of the osteoblast on the establishment of interference in osteoblasts were also examined.

Results

Examination of the establishment of interference in culture. To examine the ability of infected osteoblast cultures to establish interference to superinfection, an assay was designed to measure the level of superinfection (Figure 1). Retroviral pseudotypes were generated which expressed the bacterial *lacZ* gene from a replication defective retroviral vector (pRDlac1) (Reddy et al., 1991). Cells that had been (super)infected by a virus pseudotype could be identified by histochemical staining for ß-galactosidase activity. As such, the level of interference established was inversely related to the relative susceptibility of infected cultures to superinfection.

Pseudotypes of subgroup A and E viruses (RAV-1 and Br21, respectively) were created using a combination of transfection and infection strategies (see Chapter II). Virus stocks varied in titer from 10^4-10^6 blue colony forming units/ml. Subgroup E pseudotypes were generally 10 fold lower in titer than subgroup A pseudotypes (data not shown). Pseudotyped viruses were termed RDlac(helper virus).

The patterns of establishment of interference were compared in infected osteoblast and fibroblast cultures. Results from Chapter III indicated that infected osteoblasts contained a persistence of unintegrated viral DNA which suggested a continuing infection in the culture. In comparison, unintegrated viral DNA was only detected early after infection in fibroblast cultures. This indicated that differences in patterns of infection occurred between fibroblast and osteoblast cultures.

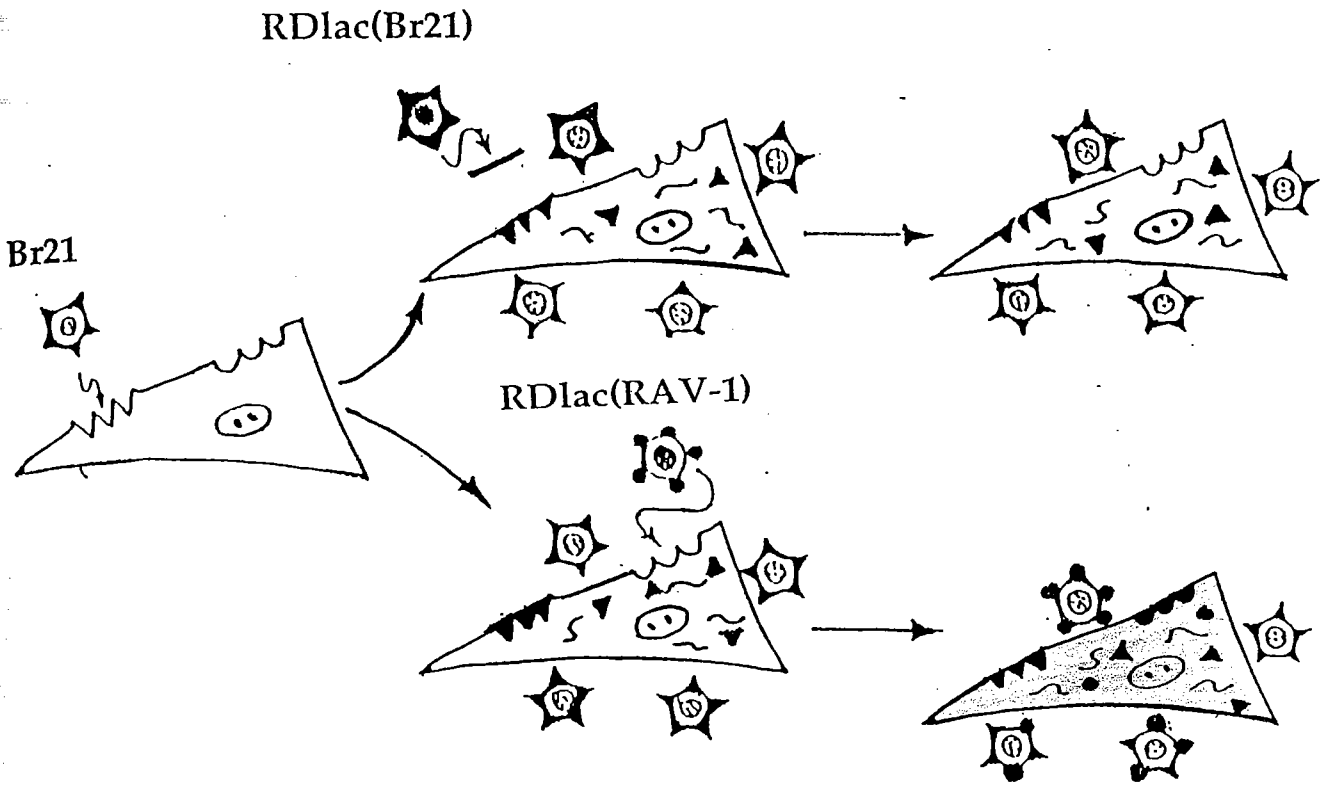

Figure 1. Schematic representation of interference and the use of virus pseudotypes expressing ß-galactosidase to detect superinfected cells (adapted from R. Weiss, 1984). When cells are infected with Br21, a subgroup E virus, newly synthesized envelope glycoproteins (represented as dark triangles) can interact with and block the activity of the cell receptor. In this way, superinfection by a pseudotype bearing the same receptor specificity (RDlac(Br21)) is blocked. However, pseudotyped viruses using a different receptor (eg. RDlac(RAV-1), a subgroup A virus) can superinfect the Br21 infected cell. Histochemical staining for ß-galactosidase activity causes cells superinfected with the virus pseudotypes to appear blue.

To determine the susceptibility to superinfection of infected osteoblasts or fibroblasts, newly seeded cultures were challenged with several dilutions of the viral pseudotypes. To determine the level of interference established, cultures infected with Br21, a subgroup E virus, were challenged with a pseudotyped virus of the same subgroup, ie. RDlac(Br21). To demonstrate that the decrease in susceptibility reflected the establishment of interference, Br21 infected cells were also challenged with a pseudotype bearing a different receptor specificity [RDlac(RAV-1), a subgroup A virus]. To control for differences in cell susceptibility at the time of infection, the pseudotype titer on uninfected cultures was also determined. Two days following pseudotype challenge, the cell monolayers were fixed and stained for ß-galactosidase activity. The pseudotype titer on either infected or uninfected cultures was determined by quantitating the number of ß-galactosidase-expressing (blue) cells and adjusting for the dilution and volume of the infecting stock. The relative susceptibility of an infected culture to pseudotype infection was calculated as the pseudotype titer on the infected culture divided by the pseudotype titer on the uninfected culture. Background levels of superinfection were defined as very low, nontitratable pseudotype titers (ie. less than 10 blue colonies in cultures infected with a 10^{-1} dilution of virus).

Using this protocol, the temporal establishment of interference was determined in osteoblast and fibroblast cultures infected with Br21 (Figure 2). Br21 infected osteoblasts remained very susceptible to superinfection by RDlac(Br21) at 10 days after infection, decreasing less than 10 fold in susceptibility to pseudotype infection (Figure 2A). In fact,

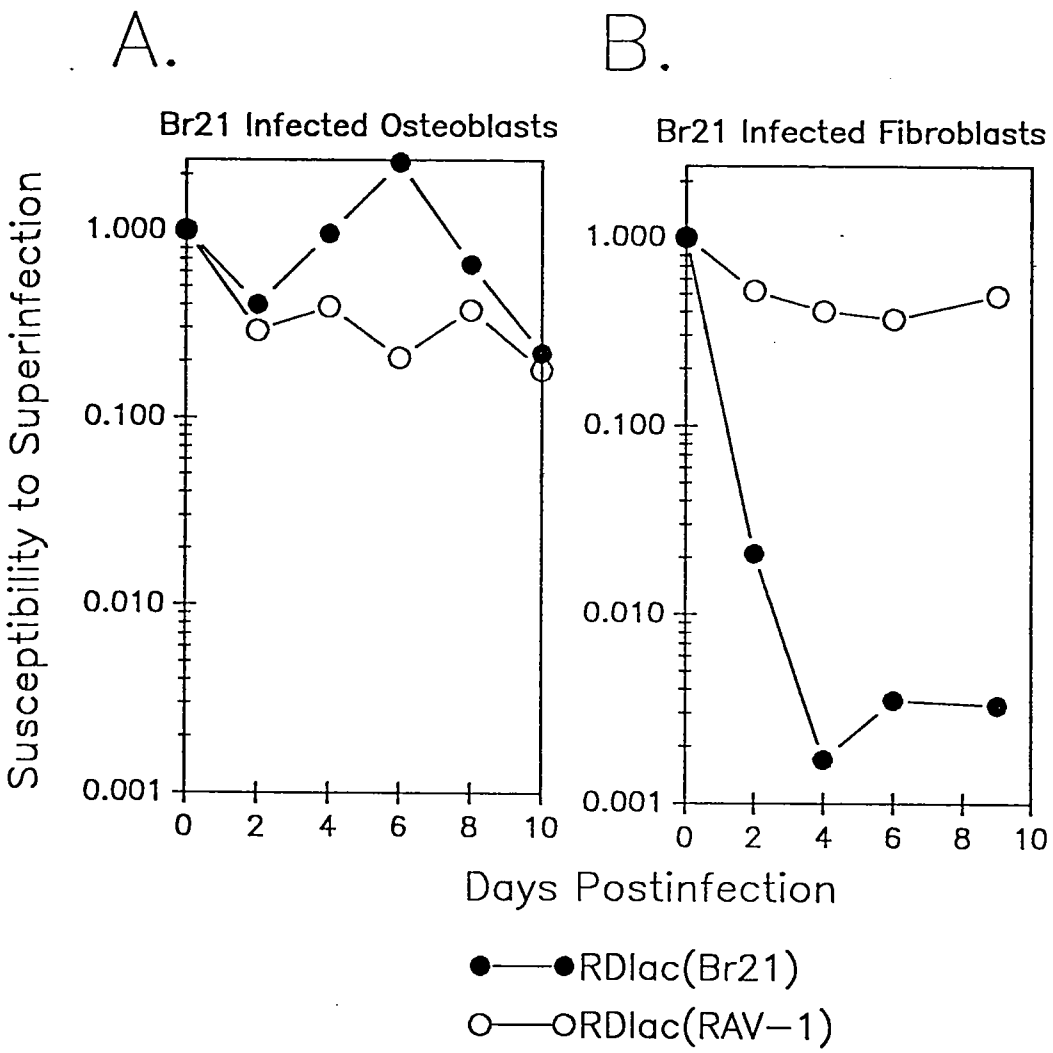

Figure 2. Temporal establishment of interference in Br21 infected osteoblast (A) and fibroblast cultures (B). Values represent the relative susceptibility of the infected culture to challenge by a viral pseudotype. Br21 infected cultures were challenged with virus of the same subgroup specificity, RDlac(Br21) or with virus of a different subgroup specificity, RDlac(RAV-1).

Br21 infected osteoblast cultures remained as susceptible to RDlac(Br21) infection as they did to infection by RDlac(RAV-1), a virus which uses a different receptor. This suggests that very little, if any, interference specific to subgroup E viruses was established in Br21 infected osteoblast cultures.

In contrast, fibroblasts infected with Br21 rapidly established interference (Figure 2B). Susceptibility to RDlac(Br21) infection decreased almost 1000 fold, to background levels, by 4 days after infection. Interference established in Br21 infected fibroblasts was receptor specific, as the susceptibility to subgroup A [RDlac(RAV-1)] infection decreased by less than 10 fold. A slight decrease in susceptibility to superinfection by a virus with a different subgroup specificity has been observed previously (Vogt & Ishizaki, 1966). The patterns of susceptibility to superinfection in fibroblasts and osteoblasts were consistent in 4 independent experiments. These results indicate that Br21 infected osteoblast and fibroblast cultures differ in their ability to establish interference.

Comparison of Env proteins expressed in infected cultures. In order to establish interference, the correct synthesis and processing of envelope glycoproteins is required. It is possible that osteoblast cultures express altered forms of Env proteins which results in the failure to establish interference. In some mutant viruses, a correlation between altered glycoprotein processing and changes in viral infectivity and virulence has been observed (Machida et al., 1985; Russell et al., 1989; Szureck et al., 1990; Poss et al., 1990, 1992; Srinivas et al., 1992).

To determine how the expression of viral glycoproteins in culture corresponds with the interference patterns, cell lysates from Br21 infected osteoblasts and fibroblasts were examined by Western analysis. Samples were compared at times post infection when fibroblasts had established high levels of interference (9 days after infection) and osteoblasts had established very poor interference (10 days after infection). Envelope glycoprotein expression was quantitatively and qualitatively very similar in infected osteoblasts and fibroblasts (Figure 3). Both cultures contained precursor (Pr95) and mature, processed (gp85) forms of Env proteins. In addition, no atypical forms of envelope glycoprotein were detected in either culture. These results indicate that the failure of infected osteoblasts to establish interference in culture was not due to a lack of expression of mature forms of envelope glycoprotein.

Role of virus spread in the establishment of interference. The failure of osteoblast cultures to establish interference could be specific to subgroup E virus infections. When osteoblast cultures were infected with either Br21 or Br131, both subgroup E viruses, the decline in susceptibility to RDlac(Br21) infection was less than 10 fold (Figure 4), suggesting that very little interference was established in these cultures. In contrast, cultures infected with Rous-associated virus type 1 (RAV-1), were able to establish interference, as evidenced by the 1000 fold decline in susceptibility to RDlac(RAV-1) challenge (compare to Figure 2). The establishment of interference in RAV-1 infected cultures occurred by 10 days after infection, slightly longer than the 4-6 days required to establish interference in fibroblast cultures. RAV-1, as a subgroup A virus, utilizes a

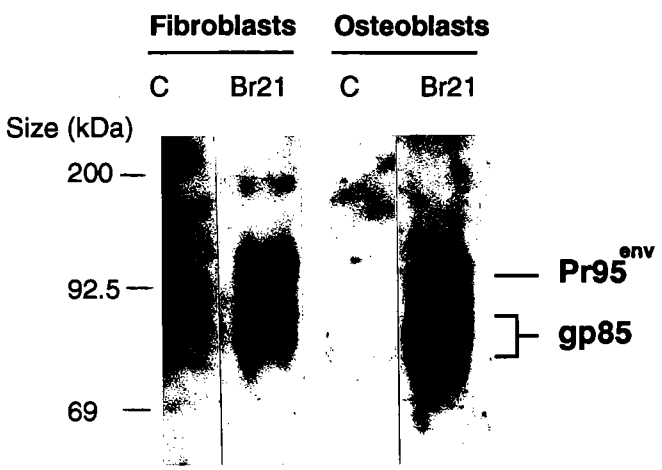

Figure 3. Western analysis of viral envelope glycoproteins in Br21 infected fibroblasts and osteoblasts. Samples reflect time points at which infected fibroblasts had established substantial levels of interference (9 days postinfection) and infected osteoblasts had established very poor interference (10 days postinfection). Reference markers, in kilodaltons, are indicated to the left. Precursor (Pr95) and mature (gp85) forms of envelope glycoproteins are indicated to the right.

different receptor than subgroup E viruses such as Br21. This suggests that the poor establishment of interference in osteoblasts might be restricted to the interactions of specific ALV subgroups with their receptors. Alternatively, subgroup A viruses could be more efficiently spread throughout the culture than viruses of other subgroups. It has been suggested that subgroup A viruses express higher proportions of infectious particles than viruses of other subgroups (Brown & Robinson, 1988b). The higher levels of infectious virus could be important in establishing a higher level of infection and virus spread in cultures infected with RAV-1 as compared to Br21. It is known that multiply trypsinizing newly infected cultures can increase the efficiency of virus spread in culture. To test whether the establishment of interference in RAV-1 infected osteoblasts was determined by the envelope subgroup of the virus or by a higher level of virus spread, the ability to establish interference was compared in RAV-1 and Br21 infected osteoblast cultures that were multiply trypsinized by passaging cultures at confluence (every 2-3 days).

The results demonstrate that interference was rapidly established in both Br21 and RAV-1 infected osteoblasts when cultures were repeatedly passaged (Figure 5A,B). Susceptibility to superinfection by virus of the same subgroup dropped 100-1000 by 4-6 days after infection. At the same time, the susceptibility to infection by a pseudotype with a different subgroup specificity decreased by less than 10 fold. These results indicate that subgroup E viruses are not inherently unable to establish interference in osteoblasts in culture. Whereas Br21 infected cultures that established very poor interference were passaged only once (Figure 2A), Br21 infected cultures that were repeatedly passaged did

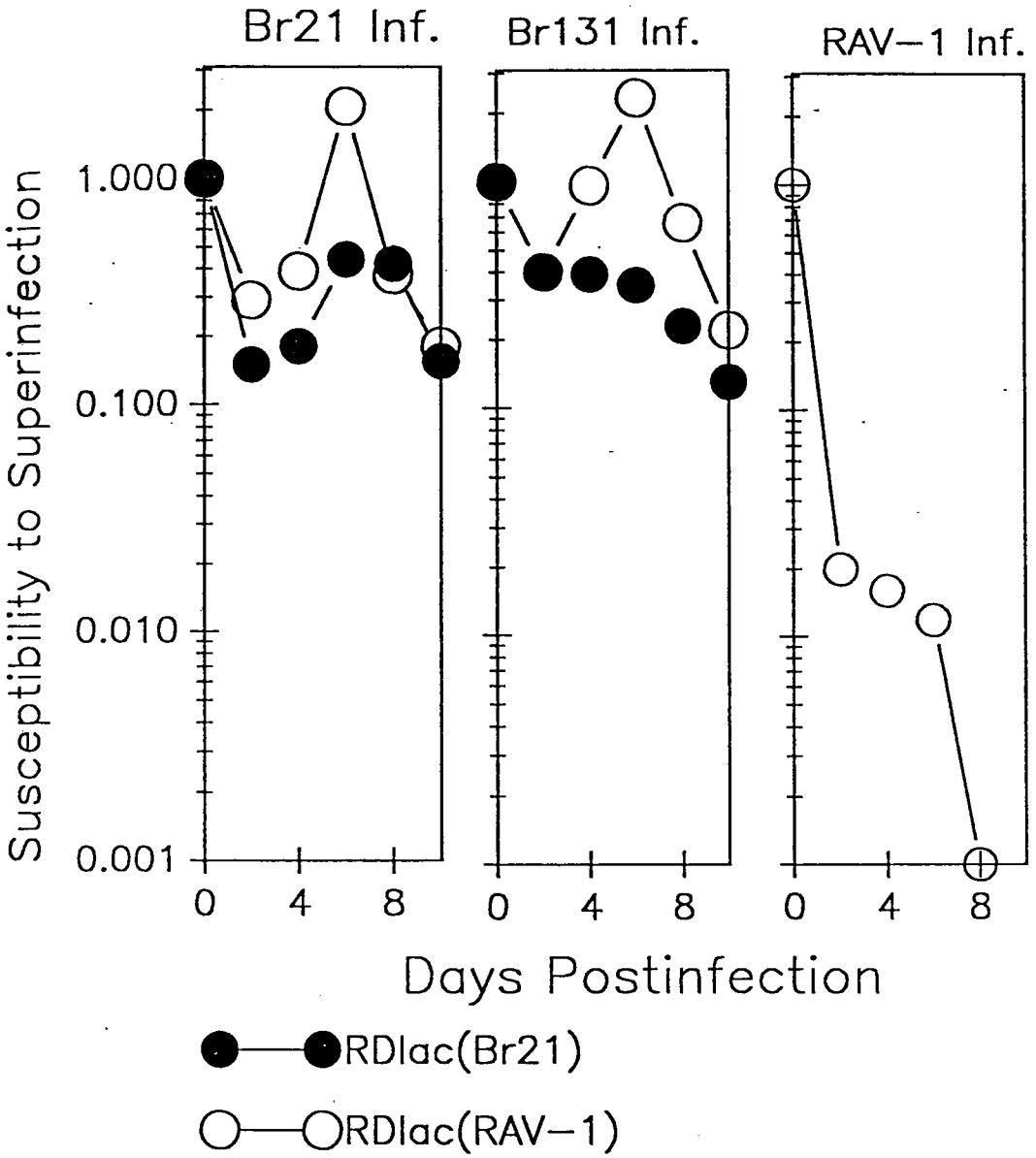

Figure 4. Temporal establishment of interference in osteoblast cultures infected with (A) Br21, (B) Br131 or (C) RAV-1. Infected cultures were challenged with virus pseudotypes: RDlac(Br21) or RDlac(RAV-1) as indicated. Susceptibility to superinfection refers to the relative ability of infected cultures to be superinfected with the virus pseudotype.

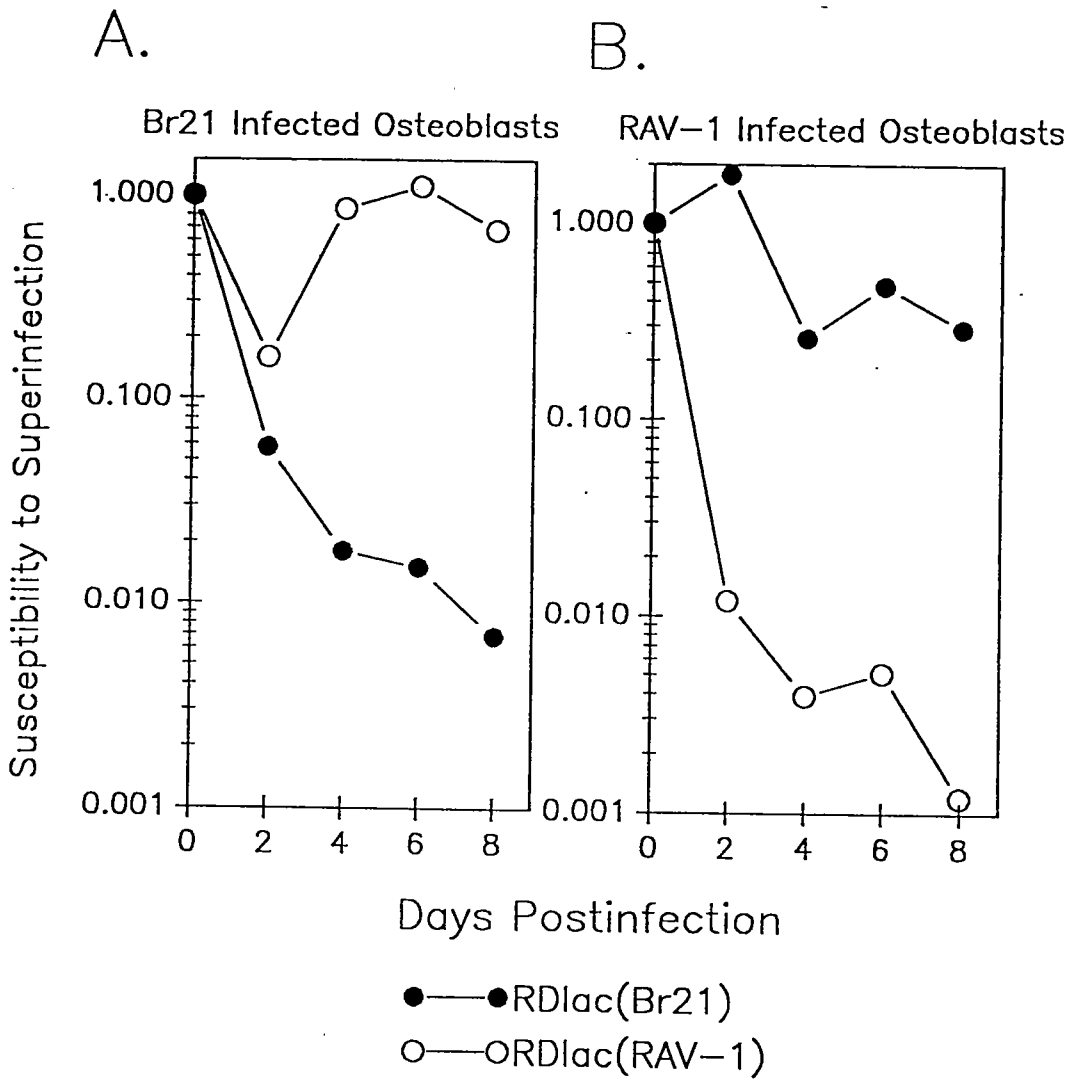

Figure 5. Temporal establishment of interference in (A) Br21 or (B) RAV-1 infected osteoblasts cultures that were passaged at confluence (every 2-3 days). Infected cultures were challenged with virus pseudotypes RDlac(RAV-1) or RDlac(Br21), as indicated. Values reflect the relative susceptibility of cultures to superinfection, as described in the text.

establish interference (Figure 5A). This suggests that the ability of Br21 infected cultures to establish interference depends on efficient viral spread in the cultures.

Role of the state of differentiation of the osteoblast in viral interference. In these studies, osteoblasts were initially examined in the context of a progressively differentiating culture. These cultures established poor viral interference (Figure 2A). Repeatedly passaged cultures, which could establish interference (Figure 5) were not induced to differentiate. It is possible that the state of differentiation of an infected osteoblast affects its ability to establish or maintain interference. To test this, the effect of osteoblast differentiation on the maintenance of interference in infected osteoblast cultures was examined.

Osteoblast cultures infected with either Br21 or RAV-1 were repeatedly passaged to establish interference. By 16 days after infection, both Br21 and RAV-1 infected cultures demonstrated background levels of susceptibility to superinfection (Table 1). At this point, cultures were divided and half of the culture was induced to differentiate (termed induced cultures) while the other half was maintained by passaging at confluence (termed passaged cultures). Sixteen days after differentiation was initiated in the induced cultures (day 32), both cultures were assayed for their susceptibility to pseudotype infection.

To verify that differentiation had been induced, both induced and passaged cultures were examined for the expression of alkaline phosphatase and the occurrence of mineralization, indications of osteoblast differentiation in culture (as reviewed by Stein

TABLE 1

MAINTENANCE OF INTERFERENCE FOLLOWING THE DIFFERENTIATION OF PASSAGED OSTEOBLASTS

Virus	Culture conditions[1]	Susceptibility to Superinfection[2] day 16	day 32	Differentiation[3], day 32 AP	Min
Br21	Passaged at confluence	.009	.010	-	-
	Induced to differentiate	.009	.010	+	+
RAV-1	Passaged at confluence	.003	.002	-	-
	Induced to differentiate	.003	.001	+	+

1. At day 16, osteoblast cultures were either maintained in MEM and passaged at confluence or cultured in BGJ_b medium and induced to differentiate.
2. The relative susceptibility to superinfection was determined at day 16, the point at which differentiation was initiated in induced cultures and at day 32, 16 days after the induction of differentiation.
3. A differentiated osteoblast phenotype was assessed by histochemical staining for alkaline phosphatase (AP) activity and matrix mineralization (Min) in the cultures at day 32. Cultures were distinguished as having negligible (-) or significant (+) levels of expression.

et al., 1990). At day 32, sixteen days after differentiation had been initiated, induced cultures expressed high levels of alkaline phosphatase activity and were highly mineralized (Table 1), consistent with a differentiated phenotype. These results also indicated that the process of continually passaging osteoblast cultures did not promote the expansion of cell types other than osteoblasts in the culture. In contrast, passaged cultures demonstrated very low expression of alkaline phosphatase and no evidence of mineralization, indicating that very little differentiation had occurred in these cultures.

At day 32, both induced and passaged cultures were tested for their susceptibility to pseudotype infection. Induced cultures demonstrated the same level of susceptibility to superinfection as was exhibited at day 16, prior to the induction of differentiation in these cultures (Table 1). This indicated that differentiation of the culture did not alter its ability to maintain interference. Passaged cultures also maintained low levels of susceptibility to superinfection (Table 1). Similar results were obtained for both Br21 and RAV-1 infections. These results indicate that the induction of osteoblast differentiation did not affect the ability of infected osteoblasts to maintain interference.

Discussion

Establishment of interference in infected osteoblast cultures. The results indicate that osteoblast cultures infected with an osteopetrosis-inducing virus, Br21, were able to establish interference to superinfection (Figure 5). Osteoblast cultures were also able to establish interference when infected with RAV-1, a subgroup A virus. RAV-1 recognizes a different receptor than Br21, a subgroup E virus, indicating that the ability to establish interference in osteoblast cultures is not envelope subgroup specific. Moreover, RAV-1 possesses a very low osteopetrotic potential (Brown et al., 1988), suggesting that the ability to establish interference in osteoblast cultures does not correlate with the osteopetrotic potential of the virus.

Initial studies in which infected osteoblast cultures were passaged once remained highly susceptible to virus infection (Figure 2). In contrast, cultures that were passaged at confluence, demonstrated a drop in susceptibility to virus challenge of 100-1000 fold (Figure 5). Trypsinization of adherent cell monolayers increases the susceptibility of cells to infection by retroviruses. This is believed to result from an "unmasking" of the receptor on the cell surface, thereby allowing a more efficient interaction of the receptor with the virus. Results from Chapter III indicate that a low proportion of the osteoblast culture initially becomes infected. If only a low number of osteoblasts in the culture are initially infected, then virus spread becomes the primary means for completely infecting the culture. By trypsinizing only once during the culturing period, virus spread may not be sufficiently encouraged, thus allowing many cells in the culture to remain uninfected.

Therefore, it is likely that in osteoblast cultures that are not repeatedly passaged, virus spread is slow and cultures do not become completely infected. As a result, the high susceptibility to pseudotype challenge may reflect the infection of uninfected cells in the culture and not a failure to prevent superinfection of already infected osteoblasts.

In contrast to infections with Br21, RAV-1 infected osteoblast cultures were able to establish interference although the culture was passaged once (Figure 4). The rate of establishment was slower than that found in infected fibroblast or osteoblast cultures, which had been multiply passaged (Figures 3, 5). Subgroup A viruses, such as RAV-1, express higher proportions of infectious to physical virus particles than viruses of other subgroups (Brown & Robinson, 1988b). The higher level of infectious particles expressed in a productive infection could encourage a more efficient spread than infection with viruses of other subgroups.

The ability of infected cultures to maintain interference was not affected by the state of differentiation of the osteoblast (Table 1). Comparison of the patterns of interference established in differentiating (Figure 2) with nondifferentiating osteoblast cultures (Figure 5) had suggested that the process of osteoblast differentiation might disrupt the establishment of interference, perhaps by affecting the expression of either the cell receptor or the viral glycoprotein. Continually passaging calvarial osteoblast cultures has been reported to alter the response to various hormones (Williams et al., 1980). However, when passaged osteoblasts that had established interference were induced to differentiate, the relative susceptibility to superinfection was not disrupted (Table 1).

Implications of the establishment of interference in culture on ALV-induced osteopetrosis. ALV-induced osteopetrosis is associated with the persistence of unintegrated viral DNA (Robinson & Miles, 1985). As unintegrated viral DNA represents an early stage in the virus life cycle, the persistence of unintegrated viral DNA suggests that continuing infection or reinfection is occurring in cells in the diseased bone. Several other retroviral infections demonstrate an accumulation of unintegrated viral DNA that results from high levels of superinfection (Keshet & Temin, 1979; Weller et al., 1980; Donahue et al., 1991). In osteopetrosis, the mechanism by which unintegrated viral DNA persists is not known.

In productively infected cells, superinfection is blocked by the establishment of interference (for review, see Weiss, 1984). The results in culture indicate that infected osteoblasts are able to establish interference, suggesting that infected osteoblasts are not intrinsically unable to establish interference. However, it is unclear how these findings apply to the establishment and progression of infection in the bone. In light of this, several mechanisms can be suggested to explain how the level of infection and the persistence of unintegrated viral DNA could be regulated in osteopetrotic bone.

Option I. Consistent with the in vitro findings, infected osteoblasts in the bone could establish interference. The ability to establish interference would indicate that infected osteoblasts are susceptible to reinfection for only a brief period of time. However, in ALV-induced osteopetrosis, cells contain, on average, multiple viral DNA copies per cell (Robinson & Miles, 1985), suggesting that bone cells become multiply

infected. It is possible that multiple infections would occur if the uninfected osteoblasts were exposed to a high multiplicity of infection in the bone. Several studies suggest that osteopetrotic bone contains high levels of infectious virus. Results from Chapter III indicate that osteopetrotic bone contains high levels of mature CA protein, which is indicative of mature virus particles. Consistent with this, electron microscopy studies reveal that osteoblasts in osteopetrotic bone are associated with high levels of virus particles (Frank & Franklin, 1982; Powers et al., 1987). Together, this suggests that virus produced by infected osteoblasts could accumulate in the bone, perhaps by becoming trapped in the bone matrix. The high levels of accumulated virus could expose susceptible osteoblasts to a high multiplicity of infection, encouraging multiple infections per cell. Later, as infected cells expressed viral antigens, further infection would be blocked by the establishment of interference. As such, in this scenario, cells of the diseased bone would represent two distinct populations: newly infected cells that contain high levels of unintegrated viral DNA; and cells that had established interference that contain primarily integrated proviral DNA.

Alternatively, high levels of infection per cell could result through the infection of quiescent bone cells, such as nonproliferating osteoblast progenitor cells. As the retroviral life cycle is blocked at a preintegration step in quiescent or stationary cells (Fritsch & Temin, 1977; Bukrinsky et al., 1991), superinfection is not prevented and multiple infections could be sustained by these cells. In the infection of resting T cells with human immunodeficiency virus (HIV), cell activation resulted in integration of the

viral DNA and a productive infection (Stevenson et al., 1990b). In much the same way, when the quiescent (multiply infected) bone cells are stimulated to proliferate, viral DNA would be integrated and expressed, permitting the establishment of interference (it is possible that high levels of viral infection could provide the mitogenic signal to activate cell proliferation). By this scenario, infected cells of the bone would comprise two distinct populations: the highly infected quiescent progenitor cell, containing high levels of unintegrated viral DNA, and the proliferating, productively infected cell, containing primarily integrated viral DNA.

Option II. Infected osteoblasts in the bone could be delayed in establishing interference. As a result, osteoblasts would remain susceptible to infection for an extended period of time. Delays in establishing interference could result from decreased levels of synthesis or processing of envelope glycoproteins, as is believed to occur for pathogenic variants of FeLV-FAIDS (Poss et al., 1989; 1990). This mechanism is unlikely to occur in ALV-induced osteopetrosis since the steady state levels of mature envelope glycoproteins not only are present in osteopetrotic bone, but the levels are actually increased over those in infected cultures (Chapter III).

Option III. Infected osteoblasts in the bone could fail to establish interference and thus remain susceptible to superinfection. Infections in which cells remain susceptible to superinfection are characterized by the accumulation of exceedingly high levels of unintegrated viral DNA (100-400 copies per cell) and cytopathic effect (Keshet & Temin, 1979; Weller & Temin, 1981; Haase et al., 1982; Donahue et al., 1991). In comparison,

the level of infection in osteopetrotic bone, is 10-20 fold less than in cytopathic infections, averaging 10-20 copies of viral DNA per cell (Robinson & Miles, 1985).

It is possible that in the diseased bone, every osteoblast sustains the same level of infection. If osteoblasts were continually susceptible to infection, this would suggest that infection events occur infrequently or inefficiently. One possibility is that a low susceptibility to repeated infection could be effected at the level of viral entry. This could occur if osteoblasts contained a low density of receptors or had receptors with a decreased affinity for virus interaction. Consistent with this, are the results from Chapter III which indicate that osteoblast cultures are less susceptible to initial infection than fibroblasts. Alternatively, the rate of infection could be slowed at a post-receptor stage.

It is also possible that in the bone, osteoblasts sustain a wide variation in the level of infection. As a result, some cells could be infected to extremely high levels, whereas most other cells in the bone could be infected at a lower frequency or not at all. However, this possibility appears unlikely to occur in osteopetrosis since extremely high levels of infection are typically associated with cytopathicity (Temin, 1988) and no significant necrosis has been reported in osteopetrotic bone. Moreover, electron microscopy studies on osteopetrotic bone do not support the idea that only a minority of cells in the bone become infected. Instead, many osteoblasts in the diseased bone appear to replicate virus, as evidenced by virus budding from numerous osteoblasts in the infected bone (Frank & Franklin, 1980; Powers et al., 1987).

Significance of the persistence of unintegrated viral DNA to ALV-induced osteopetrosis. It is not clear what role the persistence of unintegrated viral DNA plays in the pathogenesis of ALV-induced osteopetrosis. It seems likely that the accumulation of unintegrated viral DNA in osteopetrotic bone results from a continuing infection of cells in the bone. The results in culture would indicate that once osteoblasts become productively infected, superinfection is blocked (Figure 5). Regardless of whether the establishment of interference prevents superinfection in infected osteoblasts of the bone, it is obvious that other mechanisms could be utilized to permit multiple infections per cell.

It is unclear whether multiple infections are a cause or an effect of the disease. However, it is interesting that the viral DNA copy number per cell correlates with the severity of osteopetrosis (Robinson & Miles, 1985). Given that a correlation between high levels of infection and cell dysfunction and death has been clearly shown in other retroviral infections, it is likely that continuing infection in the bone, as implied by the accumulation of unintegrated viral DNA, is important in inducing dysfunction of the target cell for osteopetrosis, the osteoblast.

CHAPTER V

EFFECT OF VIRUS INFECTION ON OSTEOBLAST FUNCTION

Abstract

Avian leukosis virus (ALV)-induced osteopetrosis results in the abnormal growth and differentiation of osteoblasts in diseased bone. The effect of infection with an ALV of high osteopetrotic potential (Br21) on osteoblast growth and differentiated function was examined in chickens and in osteoblast cultures derived from embryonic chick calvaria. Serum expression of the osteoblast markers alkaline phosphatase and osteocalcin was increased in Br21 infected chickens which developed severe osteopetrosis. In contrast, virus infection of osteoblast cultures did not alter either the extent of cell growth or the expression of alkaline phosphatase or osteocalcin.

Introduction

While the majority of examples of retroviral-associated hyperplasia are neoplastic in nature, the abnormal proliferation of osteoblasts associated with avian leukosis virus (ALV)-induced osteopetrosis arises from a polyclonal outgrowth of infected osteoblasts in the bone (Robinson & Miles, 1985; Aurigemma et al., 1989). Morphometric analysis of osteopetrotic bone indicates a 9 fold increase in osteoblast number (Schmidt et al., 1981). The increased osteoblast activity results in significant increases in bone formation, as evidenced by a 2-6 fold increase in the diameter of the diseased bone (Banes & Smith, 1977), most noticeable in the long bones of the legs. Osteoblast hyperplasia occurs in the periosteum, where it has been proposed that infection of osteoblast progenitors may alter their subsequent development, leading to increased growth and activity (Powers et al., 1987).

It has been proposed that osteoblasts are derived from stromal stem cells. The stroma forms a scaffolding for the hematopoietic cells and provides a microenvironment for their development. In the bone, committed progenitors from the stroma are multipotent and give rise to osteogenic cells as well as reticular, adipocytic and fibroblastic cells (for reviews, see Owen, 1985; Nijweide et al., 1986). One of the earliest committed osteogenic cells is the osteoprogenitor, a relatively undifferentiated mesenchymal cell (Young, 1962). Evidence suggests that osteoprogenitors can differentiate into either osteoblastic or chondrocytic cell types (Owen, 1985). More differentiated cells of the osteoblast

lineage are distinguishable by their location in the bone, their proliferative capacity, their morphology, and by their protein expression, determined histochemically and biochemically. Osteoblasts and their immediate precursor, the preosteoblast, are distinguishable by their position in the bone (at or just beyond the surface of the bone matrix) and their intense staining for the enzyme, alkaline phosphatase (Ashton, 1980). Osteoblasts line the bone matrix at sites of active matrix production. In the adult, osteoblasts are also present on the surface of bone that is not actively being remodeled. These cells, termed bone lining cells, have a flattened morphology and are considered to be resting or inactive osteoblasts. As active osteoblasts become embedded in extracellular matrix, they become less active and are distinguished as osteocytes, the terminal stage of osteoblast differentiation.

Alkaline phosphatase and osteocalcin are among a number of proteins that serve as phenotypic markers for the osteoblast. In both cases, their role in bone formation is unclear. Alkaline phosphatase is abundant in osteoblasts and chondrocytes prior to mineralization. The ability of the enzyme to hydrolyze organic phosphate could be important in providing phosphate for the process of mineralization. In vertebrate tissue, alkaline phosphatase is found in several isoforms, including the bone-liver-kidney enzyme that is produced by active osteoblasts (Goldstein et al., 1980). During periods of high osteogenesis, alkaline phosphatase is released into the bloodstream, raising its level in the circulation. As such, serum alkaline phosphatase is used as an indicator of bone formation and turnover (Wuthier, 1982). Osteocalcin is an osteoblast-specific protein that is

synthesized only in the bone (Beresford et al., 1984; Lian et al., 1985). Within the bone, the expression of osteocalcin is limited to mineralized tissues (Price et al., 1981). Newly synthesized osteocalcin that is not bound in the mineral phase of the bone is released into the circulation (Price & Nishimoto, 1980). As such, serum osteocalcin levels are believed to act as a measure of bone formation and bone turnover (Garcia-Carrasco et al., 1988).

Studies using calvarial derived osteoblast cultures have defined a coordinated temporal pattern of development associated with osteoblast differentiation (for review, see Stein et al., 1990). As such, this system has been useful in studying the effects of exogenous compounds on osteoblast growth and differentiation, including the establishment and mineralization of an extracellular matrix (Tassinari et al., 1991). The availability of such a well studied culture system provided a means for examining whether ALV infection was directly involved in distorting osteoblast function (growth and differentiation). However, results from Chapter III indicated that the level of infection and virus expression are 10 fold less in osteoblast cultures than in the diseased bone, indicating that differences exist between virus infections of osteoblasts in the bone and in the culture system.

To better understand how ALV infection perturbs osteoblast function in osteopetrosis, the effect of infection with an ALV of high osteopetrotic potential (Br21) on the growth and differentiation of osteoblasts in chickens and in culture was examined. Serum samples from chickens infected with Br21 were analyzed by biochemical and histochemical methods for the expression of osteoblast markers, alkaline phosphatase and

osteocalcin. Osteoblast cultures were analyzed histochemically and biochemically for cell growth (DNA content) and osteoblast activity (expression of alkaline phosphatase and osteocalcin).

Results

Effect of virus infection on osteoblast marker expression in chickens. Br21 is a recombinant avian leukosis virus which induces a high incidence of severe osteopetrosis in infected chickens (Shank et al., 1985; Robinson & Miles, 1985). To determine what effect Br21 had on osteoblast function in the diseased bone, levels of osteoblast markers were evaluated in the sera of Br21 infected chickens that developed differing extents of osteopetrosis. Chicks were infected at one day post-hatch and monitored for the development of osteopetrosis over an 11 week period following infection. All infected chickens became viremic and demonstrated high levels of circulating virus in their serum that persisted during the entire 11 week period of the experiment. In contrast, virus was never detected in the sera of control (uninfected) birds.

Chickens were observed twice weekly for signs of onset/progression of osteopetrosis and/or any discernible pathology. As roosters are more susceptible to osteopetrosis than hens (Robinson & Miles, 1985), only roosters were kept for the study. Uninfected chickens did not develop osteopetrosis and did not exhibit any abnormalities in bone structure. At 11 weeks after infection, all chickens were sacrificed and the degree of osteopetrosis (mild, moderate, severe) was determined based on the degree of distortion to the long bones of the legs, a common site of pathology. In general, osteopetrotic bone appeared slightly discolored and softer than normal bone. Mild osteopetrosis described bones with sheaths of abnormal bone growth along the surface of otherwise normal bone. Mild osteopetrotic lesions did not run the entire length of the bone or involve the entire

perimeter. These lesions would increase the bone diameter by up to 2-4 mm. Moderate osteopetrosis was distinguished as lesions involving the entire perimeter of the bone and running the entire length of the bone. Moderate osteopetrotic lesions increased the diameter of the bone by up to 5 mm. Severe osteopetrosis referred to bones which demonstrated lesions encompassing the entire perimeter and length of the bone and extending into the marrow space. Severe osteopetrosis increased the diameters of bones by greater than 5 mm.

Serum samples were collected twice a month from both infected and uninfected chickens and were analyzed for levels of alkaline phosphatase and osteocalcin. Serum alkaline phosphatase and osteocalcin have been used as markers of bone formation and bone turnover (Wuthier, 1982; Hauschka et al., 1989). All biochemical analyses on the chicken sera were performed in collaboration with the laboratories of Dr. Gary Stein and Dr. Jane Lian, Department of Cell Biology, University of Massachusetts, Worcester. Serum levels of both alkaline phosphatase and osteocalcin increased with time in Br21 infected chickens that developed osteopetrosis (Figure 1A, B). When compared at 11 weeks postinfection, Br21 infected chickens which developed severe osteopetrosis demonstrated the largest increases in serum marker level, increasing by 4 fold (Table 1). Infected chickens which developed lesser extents of osteopetrosis had only slightly elevated levels that were not statistically significantly. At 11 weeks postinfection, serum levels of alkaline phosphatase and osteocalcin were elevated in severely osteopetrotic chickens, but had decreased almost to control levels in chickens with milder forms of

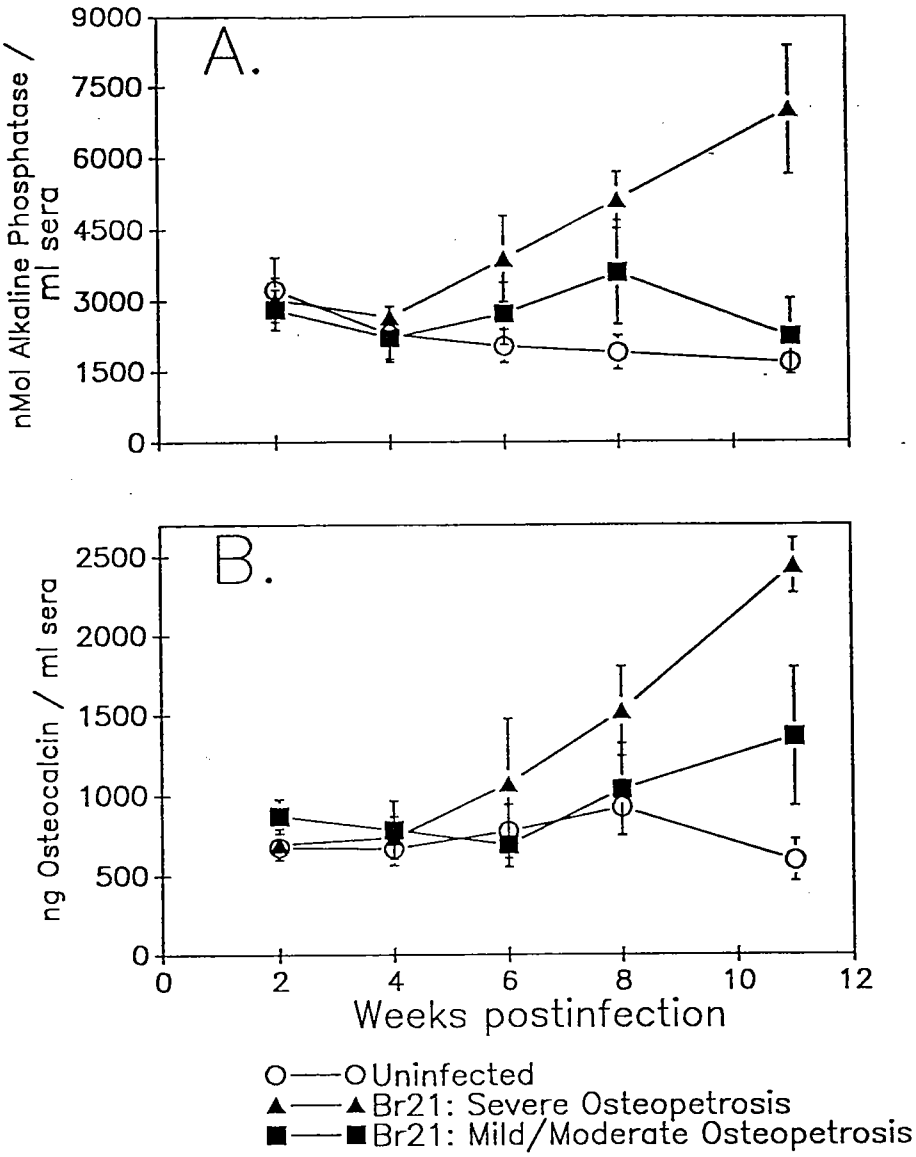

Figure 1. Serum levels of osteoblast markers in Br21 infected and uninfected chickens. Chickens were grouped by the severity of osteopetrosis observed in the long bones of the legs at 11 weeks after infection. Points represent mean values ± SD for 3-9 samples. (A) Serum levels of alkaline phosphatase. Levels were determined by enzymatic activity and were expressed as nMol p-nitrophenolphosphate conversion/ml serum. (B) Serum levels of osteocalcin. Levels were determined by radioimmunoassay and were expressed as ng osteocalcin/ml serum.

TABLE 1
SERUM LEVELS OF OSTEOBLAST MARKERS IN BR21 INFECTED AND UNINFECTED CHICKENS.[1]

Chicken group: Severity of osteopetrosis[2]		Relative Increase
Alkaline phosphatase activity (nMol p-nitrophenol conversion/ml serum)		
Uninfected	1658 ± 86	1.0
Br21 infected: Mild, Moderate	2487 ± 902[3]	1.5
Br21 infected: Severe	6990 ± 1691[4]	4.2
Osteocalcin (ng osteocalcin/ml sera)		
Uninfected	594 ± 147	1.0
Br21 infected: Mild, Moderate	1367 ± 532[3]	2.3
Br21 infected: Severe	2443 ± 244[5]	4.1

1. Measurements reflect expression levels in the sera of chickens at 11 weeks postinfection. Values represent the mean ± SD of 3-9 samples per group.
2. Severity of osteopetrosis was determined by the relative level of distortion of the long bones of the legs in chickens at 11 weeks postinfection.
3. Values were not significant by Students *t* test.
4. $P < .04$
5. $P < .07$

osteopetrosis (Table 1). This is consistent with previous reports which indicated that ALV infected chickens which developed osteopetrosis had elevated levels of serum alkaline phosphatase (Sanger et al., 1966a; Banes & Smith, 1977). In contrast, uninfected birds maintained relatively constant levels of marker expression over the 11 week period. These data indicate that the expression of osteoblast markers is increased in Br21 infected chickens which develop severe osteopetrosis.

Effect of virus infection on osteoblast growth in culture. To examine whether virus infection is directly involved in altering osteoblast function, the effect of Br21 infection on osteoblast growth and differentiation was examined in osteoblast cultures derived from embryonic chick calvaria. Osteoblast culturing and biochemical and histochemical analyses of the cultures were carried out in collaboration with the laboratories of Dr. Gary Stein and Dr. Jane Lian (Department of Cell Biology, University of Massachusetts, Worcester). Cell isolation and culturing were based on the method described by Gerstenfeld and coworkers (1987). Primary cultures were isolated from embryonic chick calvaria by enzymatic digestion (for details, see Chapter II). Cells isolated from the late (third) digest of the calvaria exhibited a mature osteoblast phenotype as determined morphologically and biochemically (Gerstenfeld, et al., 1987). Cells were infected by replating trypsinized cultures in Br21-containing media (day 0). Cells were mock infected by replating in conditioned media. In all experiments, evidence of virus replication in Br21 infected cultures was demonstrated by monitoring virus production in the medium (for details, see Chapter III).

To determine whether infection by an osteopetrosis-inducing virus could affect the growth of osteoblasts in culture, the level of total DNA was determined in infected and uninfected cultures over time. Previous results had indicated that calvarial osteoblast cultures grow exponentially for approximately the first 15 days of culture and thereafter slow as cells take on a more differentiated character (Gerstenfeld et al., 1987). The pattern of DNA accumulation and the absolute amount of DNA were very similar in Br21 infected and uninfected cultures (Figure 2A). This result was consistent over 5 independent experiments. Determination of cell numbers over the culture period also indicated little difference in the pattern of cell growth in infected and uninfected cultures (Figure 2B). Cell number data are shown for only the first 8 days postinfection, as the cultures became more resistant to trypsinization with the accumulation of extracellular matrix and mineralization and accurate cell counts became difficult. Levels of viral DNA did not contribute significantly to the total level of DNA in the cell. As described in Chapter III, the total level of viral DNA averaged only 2-3 copies/cell in Br21 infected osteoblast cultures. Moreover, viral cytopathicity did not significantly affect cell number. As determined by trypan exclusion, Br21 infected cultures displayed little cytopathic effect as compared with controls. Together, these results indicate that Br21 infection did not appreciably alter the growth of osteoblasts in culture.

Effect of Br21 infection on the expression of osteoblast markers in culture. To examine whether Br21 infection affects the differentiated function of osteoblasts in

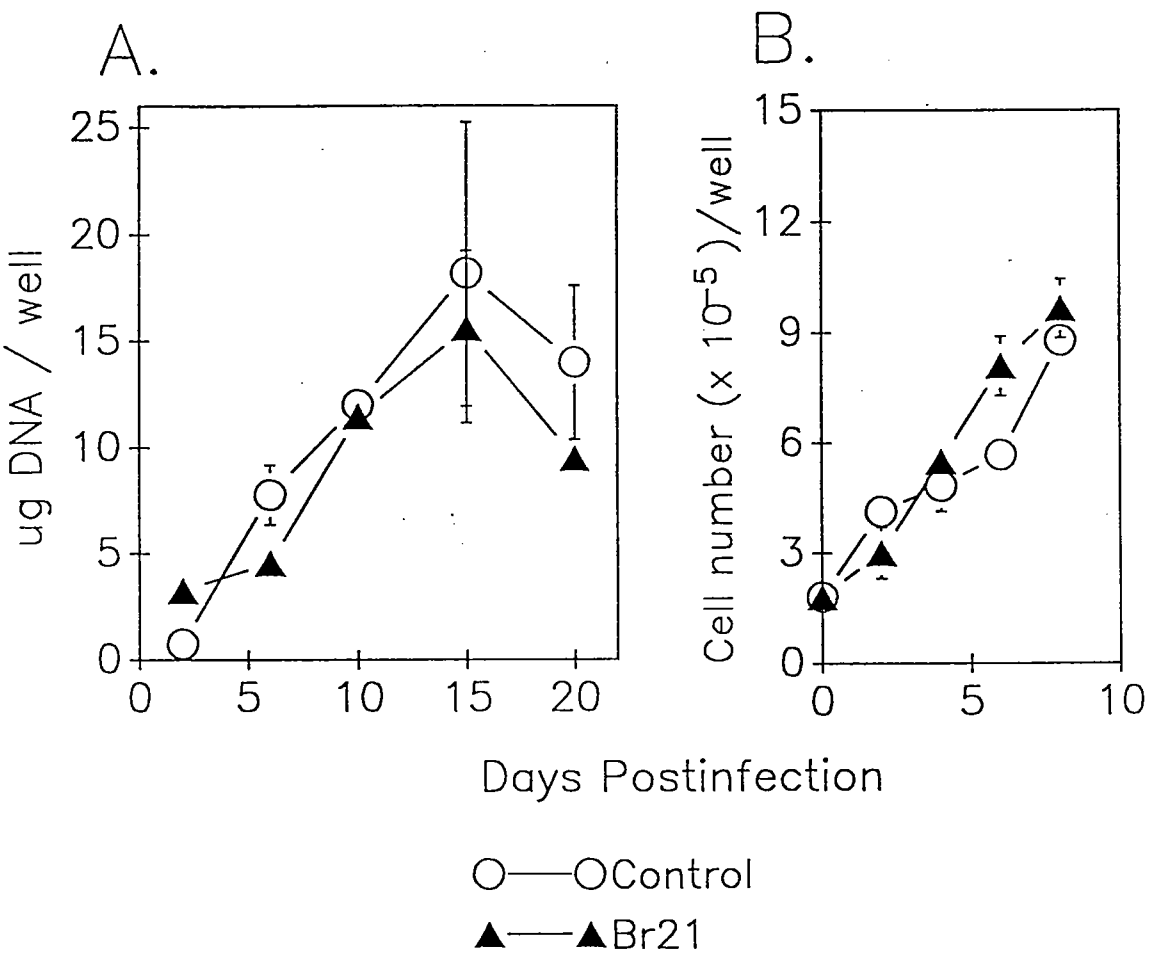

Figure 2. Total DNA content and cell number determinations in Br21 infected and uninfected chick osteoblast cultures over time. The data represent one of five independent experiments, all with consistent results. (A) Total cell DNA per 35 mm sample well. All points represent the mean ± SD for 3 wells and are expressed as μg DNA/35 mm well. (B) Cell number per 35 mm sample well as determined after trypsinization. Values represent the mean of 3-6 sample wells per time point ± SE.

culture, the expression of two phenotypic markers of osteoblast development, alkaline phosphatase and osteocalcin, were assessed in infected and uninfected cultures over time.

In culture, the expression of alkaline phosphatase coincides with the downregulation of cell proliferation and the establishment of the extracellular matrix (Owen et al., 1990; Aronow et al., 1990). Examination of alkaline phosphatase activity indicated that the pattern and magnitude of expression in infected cultures were very similar to uninfected cultures (Figure 3A). In some experiments, the expression of alkaline phosphatase was increased earlier in infected cultures than in uninfected cultures (compare 3A and 3A inset). This, however, was not a consistent finding (occurring in 2 out of 5 experiments). Fixed cultures were also examined for alkaline phosphatase expression by histochemical staining. These results were consistent with the findings of Figure 3A, indicating that the extent and timing of alkaline phosphatase expression were very similar in infected and uninfected cultures (data not shown). These results indicate that Br21 infection did not alter the expression of alkaline phosphatase in osteoblast cultures.

Osteocalcin is an osteoblast-specific protein whose expression in culture coincides with the onset of mineralization (Gerstenfeld et al., 1987; Aronow et al., 1990). Although the majority of the osteocalcin is associated with the cell layer, in vitro calvarial cultures release substantial levels of osteocalcin into the medium (Gerstenfeld et al., 1987). To examine whether infection affected the expression of osteocalcin in osteoblast cultures, the level of osteocalcin was measured in the medium of infected and uninfected cultures over time. Consistent with previous reports in chick osteoblast cultures, the increase in

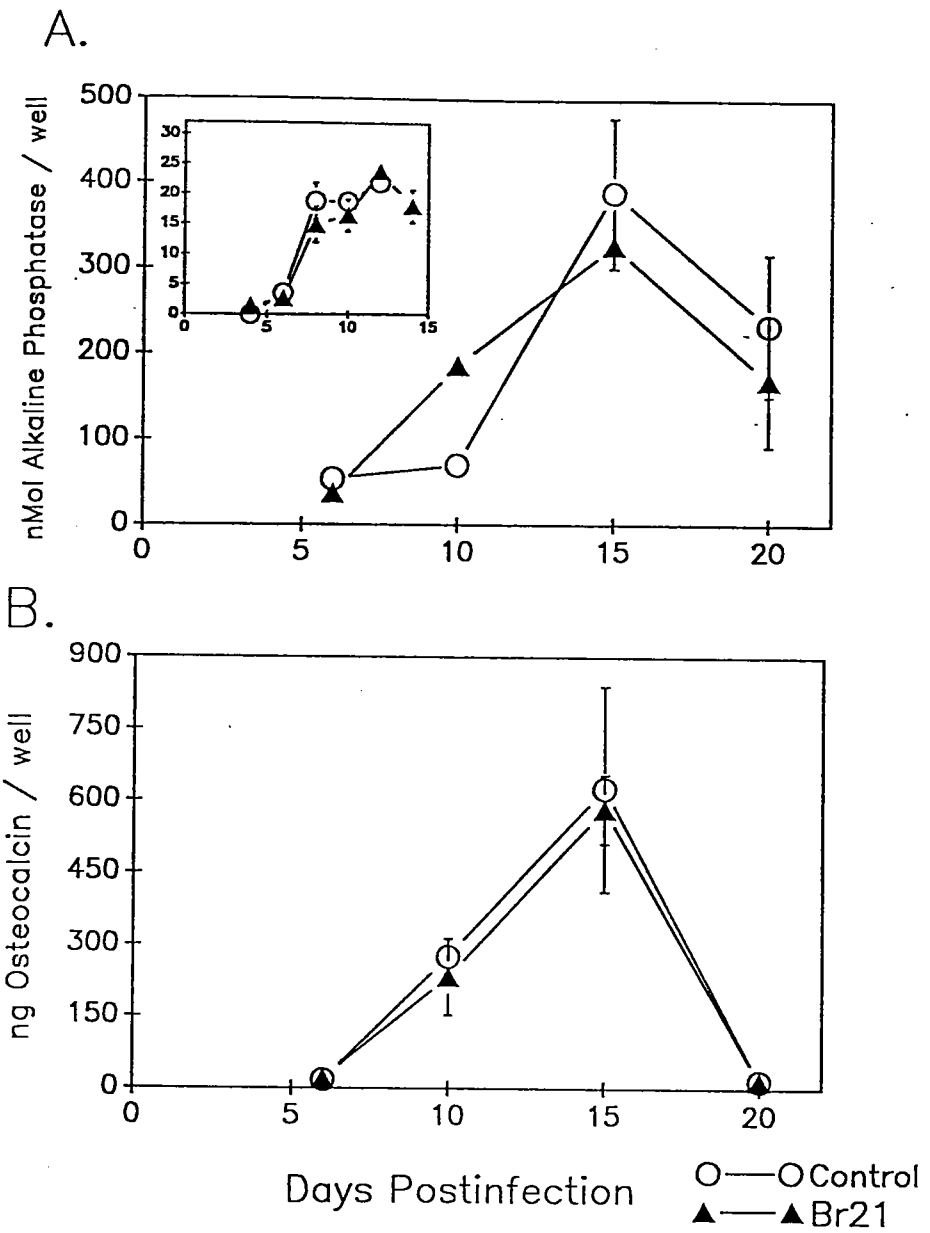

Figure 3. The expression of osteoblast phenotypic markers in Br21 infected and uninfected osteoblast cultures. The data are representative of 3-5 independent experiments, which demonstrated consistent findings. All points represent a mean $\pm$ SD of n = 3 sample wells. In some cases, the SD is very small such that error bars are not evident. (A) Alkaline phosphatase activity per 35 mm well. Activity is expressed as nMol p-nitrophenolphosphate conversion per well. An early stimulation of alkaline phosphatase activity was sometimes observed in infected cultures (day10), but was not a consistent finding (see inset). (B) Level of osteocalcin released into the medium, expressed as ng osteocalcin per 35 mm sample well.

osteocalcin expression was coincident with the rise in alkaline phosphatase activity (Aronow et al., 1990). However, the pattern and level of osteocalcin expression did not significantly vary between infected and uninfected cultures, suggesting that virus infection did not alter the expression of osteocalcin in osteoblast cultures (Figure 3B). This result was consistent in 3 separate experiments.

Discussion

Increased osteoblast activity correlates with osteopetrosis in infected chickens. Osteoblast activity, as determined by serum levels of alkaline phosphatase and osteocalcin, was elevated in Br21 infected chickens that developed severe osteopetrosis over the 11 week period following infection (Figure 1A, B). In comparison, chickens which developed milder forms of osteopetrosis had serum levels that were not significantly different from uninfected control chickens. Alkaline phosphatase and osteocalcin are phenotypic markers of the osteoblast and their expression in the circulation acts as a measure of bone formation and turnover (Wuthier, 1982; Poser et al., 1983). The increased serum osteoblast marker levels could be a reflection of the increase in osteoblast number. Indeed, morphometric analysis reveals a 9 fold increase in osteoblast number in osteopetrotic bone over uninfected bone (Schmidt et al., 1981). This would suggest that the elevated levels of osteoblast markers observed in ALV-induced osteopetrosis result primarily from stimulating osteoblast proliferation and not from increasing the level of differentiated products expressed per osteoblast. It is interesting that osteoblast proliferation and alteration to bone structure must be appreciable to affect the serum levels of markers of bone formation.

Elevated serum osteoblast marker levels could also be indicative of abnormal rates of bone formation and resorption, as has been reported in Paget's disease, a bone disorder characterized by excessive bone turnover (Deftos, et al., 1982; Gundberg, et al., 1983). It is possible that higher than normal rates of bone resorption occur in osteopetrotic bone,

however, these levels do not keep pace with the unusually high level of bone formation. Moreover, serum calcium and phosphorous levels are not elevated in osteopetrotic birds, as they might be if bone resorption was increased (Biltz & Pellgrino, 1965).

Virus infection does not affect osteoblast growth or differentiation in culture. Osteoblast cultures were infected with Br21 to determine whether virus infection was itself sufficient to alter osteoblast function as observed in the diseased bone. The results indicate that the pattern of cell growth, as determined by the pattern of total DNA content and cell number, was consistently very similar in control and infected cultures (Figure 2). This indicated that in contrast to the hyperplasia of osteoblasts observed in infected, diseased bone, virus infection did not disturb osteoblast growth in culture. Furthermore, in comparison with the pattern of expression of two osteoblast phenotypic markers, alkaline phosphatase and osteocalcin in uninfected cultures, virus infection did not alter osteoblast differentiation in culture (Figure 3A, B). Thus, virus infection did not appear to affect either the proliferation or the maturation of osteoblasts in culture.

Interestingly, the rapid decline in osteocalcin expression after day 15 in both infected and uninfected cultures differs from previous reports. In both chick and rat calvarial derived osteoblast cultures, high levels of osteocalcin expression are maintained throughout the period of active mineralization and declined late in the development of the culture, when the cultures were heavily mineralized (Aronow et al., 1990). When culture conditions induced a rapid accumulation of calcium, indicative of mineralization in the culture, a corresponding decline in expression of osteocalcin was observed (Aronow et

al., 1990). This suggests that the sudden decline in osteocalcin expression in our cultures could have resulted from a prematurely high level of mineralization in the cultures, sufficient to downregulate osteocalcin expression.

Culturing conditions are critical for the establishment of a differentiated osteoblast phenotype in culture. The inclusion of ascorbic acid and ß-glycerophosphate in the culture medium increases collagen synthesis and the capacity for mineralization, respectively (Barnes, 1975; Tenenbaum & Heersche, 1982). Interestingly, ascorbic acid has been reported to limit the replication of the avian retrovirus, Rous sarcoma virus, although the mechanism is unknown (Bissell et al., 1980; Schwarz, 1991). Experiments in which infected and uninfected osteoblast cultures were grown in the absence of ascorbic acid and/or ß-glycerophosphate indicated that virus infection could not substitute for components of the culturing protocol in inducing osteoblast differentiation. These cultures over time did not display appreciable differentiation and the continuing proliferation resulted in an overcrowding and subsequent peeling of the monolayer (data not shown).

Possible limitations presented by the culture system. In contrast to the increased proliferation of osteoblasts in osteopetrotic bones of infected chickens, virus infection did not appear to alter either their growth or differentiation of cultured osteoblasts (Figures 2, 3). This would indicate that infection is itself not sufficient to induce the abnormal function of osteoblasts observed in diseased bone. Further, this would suggest that the osteoblast culture system lacks either aspect(s) of the bone environment required to stimulate osteoblast function or the specific osteoblast population whose function is

affected by virus infection. Identification of the component(s) that are lacking in culture would be useful in understanding how virus infection affects osteoblast function in the infected bone.

Differences in viral load in osteopetrotic bone and in culture. An important component of the osteopetrotic bone environment which was lacking in the culture system was a sufficiently high virus load. The results of Chapter III revealed that osteopetrotic bone contained 10 fold more viral DNA than infected osteoblast cultures. This indicated that osteoblasts in vivo become more highly infected than osteoblasts in culture. High levels of infection could be required to induce abnormal function in osteoblasts. In retroviral infections which sustain high levels of infection, the accumulation of high levels of unintegrated viral DNA has been correlated with cell dysfunction and death (for review, see Temin, 1988). Osteopetrotic bone is characterized by the accumulation of up to 5-10 copies of unintegrated viral DNA per cell, which is 10-20 fold less than levels observed in cytopathic infections (Robinson & Miles, 1985). It is possible that the level of unintegrated viral DNA, representing the level of infection, may determine whether an infected cell malfunctions or is killed.

How the unintegrated viral DNA could cause aberrant osteoblast functioning is unclear. It is possible that the presence of unintegrated viral DNA, itself, could perturb cell function, as has been suggested in cytopathic virus infections (Temin et al., 1980). Alternatively, abnormal function could result from the expression of RNA or proteins encoded by the viral DNA or perhaps by the level of their expression. Results in Chapter

III indicate that the high levels of infection in osteopetrotic bone were associated with correspondingly high levels of viral protein synthesis (Chapter III). As compared to infected fibroblast or osteoblast cultures, osteopetrotic bone contained 7 times the level of precursor Gag protein, an indication of viral protein synthesis. The increased levels of viral protein synthesis in osteopetrotic bone suggest a higher than normal level of the total protein synthesis in the infected cell which is viral. The higher demand placed on the processes of the infected osteoblast by the high levels of viral synthesis could disrupt the normal cell functioning. For instance, high levels of viral biosynthesis could interfere with the ability of the infected osteoblast to differentiate. Viral proteins could be processed by a cellular pathway also used in the differentiation of osteoblasts. The increased demand on the pathway by viral proteins could limit or disrupt cellular processes involved in differentiation, perhaps resulting in a delay in osteoblast differentiation. As a result, these cells could continue to proliferate for a longer than normal period of time. Alternatively, high levels of viral biosynthesis could directly stimulate cellular proliferation, perhaps by providing a mitogenic signal to the infected osteoblast or by mimicking a physiological signal promoting cell proliferation (for further discussion see Chapter VI).

Differences in cell populations in culture and in the bone. The inability of virus infection to alter osteoblast function in culture could be a consequence of the stage(s) of differentiation of the isolated osteoblast population. Osteoblast cultures derived from the calvaria represent a relatively mature osteoblast phenotype in that they have a limited capability to reinitiate cell proliferation in culture but are sufficiently differentiated that

they express proteins and activities characteristic of a more differentiated osteoblast (such as expression of alkaline phosphatase and osteocalcin). Calvarial derived chick cultures contain osteoblasts that are capable of forming mineralized matrix under certain conditions in culture or when transplanted on the chorio-allantoic membrane of quail embryos (Nijweide et al., 1981; 1982).

In contrast, cultures derived from embryonic periosteum appear to contain inducible osteoprogenitors in that they can be induced to form mineralized matrix either when cultured with stripped bone rudiments or when intact periosteum is folded upon itself (Nijweide, 1975; Burger et al., 1986). Mesenchymal cultures derived from the periosteum of chick limb buds also demonstrate osteogenic capabilities when under the appropriate culture conditions (Osdoby & Caplan, 1979). Whereas plating mesenchymal cells at high densities favors the outgrowth of chondrocytic cells, intermediate cell densities encourages the growth of cultures expressing alkaline phosphatase activity and developing mineralized nodules (Osdoby & Caplan, 1979; 1986). Together, these studies would suggest that the chick periosteum contains inducible osteogenic precursors which can differentiate into osteoblasts if the surrounding environment is favorable.

Several studies have shown that periosteal derived cultures containing early osteoblast progenitors demonstrate a greater cell response to virus infection than osteoblast cultures derived from calvaria. Periosteal cell cultures derived from chick limb buds, express elevated levels of alkaline phosphatase activity when infected with MAV-2(O), an ALV of high osteopetrotic potential (Schmidt & Smith, 1981). Infection of

murine periosteal cell cultures, but not calvarial derived cultures, with OA MuLV, a murine retrovirus which induces an osteopetrosis-like condition in mice, also stimulated alkaline phosphatase activity in culture (Schmidt et al., 1987). These studies suggest that the state of differentiation of the isolated population may be important in determining whether virus infection can affect osteoblast activity.

Furthermore, it has been suggested that osteoblast progenitors could represent a target for infection in the bone. Electron microscopy studies of osteopetrotic bone indicate that in bone samples early after infection of the chicken, virus particles can be found budding from osteoprogenitors and only at later times after infection, from osteoblasts and osteocytes (Powers et al., 1987). It is unclear whether infected osteoblasts represent those infected as progenitors or as newly infected mature osteoblasts. Interestingly, experiments in culture examining OA MuLV infection have indicated that murine periosteal cultures, which contain osteoprogenitors, are more susceptible to virus infection than murine calvarial derived cultures, which contain more differentiated osteoblasts (Schmidt et al., 1987). These studies together suggest that in ALV infection in the bone, the susceptibility of osteoblasts to infection may depend on relative state of differentiation.

It is possible that the tissue from which the osteoblasts are isolated could be important in affecting the response to virus infection. The mechanism by which bone is formed in the calvaria (intramembranous) differs from that in the long bones (enchondral). A histological study comparing MAV-2(0) infections in the femur and calvarium has suggested that the more superficial vasculature of the calvarium may be responsible for

the limited presence of virus and the absence of osteopetrotic lesions in the calvaria at 19 days after infection (Powers et al., 1987). Although a predilection for periosteal osteoblast proliferation has been observed in ALV-induced osteopetrosis, previous reports do indicate that calvaria are susceptible to infection and pathogenesis. Osteopetrotic lesions have been reported in the calvaria but were not observed until 23 weeks after infection, long after lesions in the long bones were first detected (Holmes, 1961). The increased vasculature of the long bone could permit an increased exposure to virus and/or to factors that could influence osteoblast development. A difference in exposure to factors such as hormones and growth factors could create differences in osteoblast populations in the long bone versus calvarium, even if both were isolated from the same stage of development.

Consideration of indirect mechanisms affecting osteoblast function. Osteoblast cultures could lack the contributions of other cell types that regulate osteoblast activity in the bone. Regulation between bone cell types has thus far primarily been established as an activity of osteoblasts on other cell types. For instance, osteoblasts are involved in the resorptive response of osteoclasts to parathyroid hormone and vitamin D. It has been shown that the activation of osteoclasts by parathyroid hormone is dependent on the presence of osteoblasts (Braidman et al., 1983). Moreover, receptors for parathyroid hormone and vitamin D have not been found in osteoclasts but are found in osteoblasts (Silve et al., 1982). Although the exact nature of this regulation is unclear, it is thought to involve the release of factor(s) by the osteoblast that either stimulate the activity of existing osteoclasts or stimulate the development of osteoclast precursors (Rodan &

Martin, 1981; Kahn & Partridge, 1987; Chambers, 1988). Studies in culture have also suggested that mature osteoblasts may regulate the proliferation of osteoprogenitors by a mechanism that appears to require direct cell-cell contact (van der Plas & Nijweide, 1988). It is possible that virus infection could affect the normal regulation by osteoblasts or other cell types on the activity or development of osteoblasts or osteoprogenitors.

Given the sensitive balance between bone formation and resorption in bone homeostasis, deleterious effects on osteoclast activity in osteopetrotic bone could contribute to abnormal bone formation. However, osteoclasts in osteopetrotic bone appear normal histologically (Schmidt et al., 1981) and ultrastructurally (Frank & Franklin, 1982). In contrast, many forms of mammalian osteopetrosis demonstrate a decrease in total numbers or activity of osteoclasts. It is interesting that in several osteopetrotic mutations in the rat, abnormalities in osteoblast gene expression have also been noted (Shalhoub et al., 1991). Altered osteoblast function in the rat mutations could contribute to abnormalities in the development of the extracellular matrix, mineralization or perhaps to osteoclast development. In a related way, the abnormal osteoclast activity could contribute to alterations in the bone architecture observed in ALV-induced osteopetrosis.

The osteoblast cultures could lack the contribution of growth factors or hormones elicited either locally in the bone or systemically. Numerous factors have been identified which stimulate osteoblast replication and/or differentiation (for reviews see Canalis, 1985; Raisz, 1984; Mohan & Baylink, 1991). It is not known whether the observed in vitro effects are expressed in vivo. In culture, osteoblast differentiation has been affected

by different lots of added fetal bovine serum. It has been suggested that differences in concentration of growth factors or hormones in the added serum could affect the extent of matrix mineralization (Aronow et al., 1990).

Many factors have been shown to stimulate osteoblast growth in culture (including platelet-derived growth factor, epidermal growth factor, and transforming growth factor-ß) and are termed "competence factors" (Canalis, 1985). Other factors have been shown to encourage osteoblast growth and the expression of a differentiated phenotype and are termed "progression factors". Included in these are insulin-like growth factor and prostaglandins, which are both secreted by osteoblasts (Nolan et al., 1983; Raisz et al., 1979). Hormones, such as glucocorticoids have been shown to promote osteoprogenitor differentiation in vitro (Bellows et al., 1985). It is possible that in response to virus infection, factors that stimulate osteoblast growth could be elicited.

While the lack of cellular response to virus infection in culture appears to conflict with the increased activity of osteoblasts in osteopetrotic bone, the complex nature of bone homeostasis suggests that a simplified model such as the isolated osteoblast culture may be too limited to study the mechanism of perturbed osteoblast growth in ALV-induced osteopetrosis.

CHAPTER VI

SUMMARY & FUTURE DIRECTIONS

Possible mechanisms for viral induction of abnormal osteoblast activity in ALV-induced osteopetrosis. A goal of this research was to understand what role the virus played in inducing the abnormal growth of osteoblasts in osteopetrotic bone. Characterization of the pattern of replication of an osteopetrosis-inducing virus, Br21, in isolated cultures of chick embryonic osteoblasts provided a means for examining virus expression in the target cell for disease. The original hypothesis suggested that an osteopetrosis-inducing virus may exhibit atypical characteristics in its life cycle which, in turn, could cause aberrant osteoblast function. For example, infected osteoblasts could express aberrant viral species or atypical levels of viral production. However, characterization of Gag and Env protein species in osteoblast cultures, as well as in samples of osteopetrotic bone, revealed only the expression of viral proteins of the expected molecular sizes (Chapters III, IV), lacking neither precursor nor mature forms. Moreover, as compared to cultured fibroblasts, osteoblasts did not demonstrate unusually high or low levels of virus replication (Chapter III). Alternatively, it was possible that simply the process of infection or normal viral expression could be required to induce abnormal activity in infected osteoblasts. However, when cultured osteoblasts were productively infected, their pattern of cell growth and differentiation was comparable to controls (Chapter V).

High virus load in osteopetrosis. Data presented in this thesis supports the idea that ALV-induced osteopetrosis is associated with high levels of infection and viral protein

synthesis. Osteopetrotic bone contains 10-20 times more viral DNA than infected osteoblasts in culture (Chapter III). Further, diseased bone contains ~10 times the level of Gag precursor protein than do infected cultures. Thus, the high level of viral DNA in osteopetrotic bone corresponds to comparably high levels of viral protein expression (Chapter III), suggesting that the increased amount of viral DNA is actively being used as a template for viral expression. In infected chick fibroblasts, an estimated 1% of the total protein expressed in the cell is viral (Vogt & Eisenman, 1973). The high level of viral expression suggests an added burden placed on the biosynthetic capacity of infected bone cells. Cellular processes used for virus production, such as transcription, splicing, translation and protein processing pathways could limit or disrupt the use of these pathways for cellular synthesis. For example, the enhanced use by the virus of the transcriptional machinery of the cell could limit the availability of factors that are also required for basal and/or regulated transcription of cellular genes, thus disrupting cellular expression. High levels of viral translation could also limit the processing of cellular proteins requiring glycosylation, myristylation or phosphorylation.

It is possible that the demand placed on the synthetic processes of the cell by the virus could limit or disrupt more complex cellular functions such as those involved in osteoblast differentiation. For instance, high levels of viral synthesis could cause a delay in the differentiation of infected osteoblasts, permitting cell proliferation to occur for an extended period of time. This could occur if virus biosynthesis affected the regulation of genes associated with osteoblast differentiation. During osteoblast proliferation, the occupancy of AP-1 sites by the Fos-Jun protein complex is thought to be important in

suppressing the transcription of genes associated with osteoblast differentiation, such as the osteocalcin gene (Owen et al., 1990c). Following the induction of differentiation, loss of binding correlates with increases in gene transcription. Cotransfection of c-*fos* and c-*jun* into cells expressing osteocalcin has also been shown to inhibit subsequent osteocalcin expression (Schule et al., 1990). How AP-1 binding inhibits gene transcription and how the transition to a differentiated culture relieves this suppression is unclear. It is conceivable that high levels of viral biosynthesis could prevent the disruption of the Fos-Jun complex, stabilizing the association of Fos-Jun complexes on AP-1 sites and thus inhibiting the activation of genes associated with osteoblast differentiation. This could occur by viral biosynthesis preventing the expression of an inhibitor protein or by limiting the activity of cellular processes which may be required to inactivate this complex when bound, such as phosphorylation.

Alternatively, high levels of viral expression could affect the expression of cellular factors which regulate cellular proliferation. A cellular factor, HiNF-D, has been identified which interacts with the H4 histone promoter in osteoblasts that are actively proliferating, but demonstrates a selective loss of binding in differentiated osteoblasts (Owen et al., 1990b). As such, HiNF-D binding has been suggested to play a regulatory role in the expression of genes associated with cell growth. High levels of viral expression could stabilize the binding of factors such as HiNF-D to the promoter and allow for prolonged periods of osteoblast proliferation. The stabilization of HiNF-D binding could arise from the disruption of transcription or processing of an inhibitor protein or processes which normally would inactivate binding of HiNF-D to the promoter.

The accumulation of mature virus particles in the diseased bone: a mechanism for establishing high levels of infection. ALV-induced osteopetrosis is a polyclonal disease in which high levels of infection are achieved by multiple infection events per cell, as is evidenced by the 10-20 copies of viral DNA per cell in osteopetrotic bone (Robinson & Miles, 1985). Our studies indicate that osteopetrotic bone contains high levels of mature virus. This is evidenced by diseased bone containing high levels of mature CA protein, a Gag protein that is not expressed in its mature form until the maturation of budded virions (Chapter III). Electron microscopy studies of osteopetrotic bone have also revealed numerous viral particles closely associated with osteoblasts (Sanger & Simpson, 1966; Frank & Franklin, 1982). The presence of high levels of virus could reflect trapping of newly budded virus in the matrix of the bone. It is possible that as more cells in the bone become infected, virus particles accumulate to high levels. This agrees with previous observations that higher levels of virus particles are present in the infected bone as disease progresses (Frank & Franklin, 1982; Powers et al., 1987). As discussed in Chapter IV, the accumulation of virus over time could result in the establishment of a sufficiently high local concentration of virus to result in an unusually high multiplicity of infection for susceptible osteoblasts. If this were the case, bone cells infected early in the course of disease might sustain lower levels of infection than cells infected at later times, when virus produced from infected cells would have had time to accumulate. As a result, the induction of osteopetrosis may require a sufficient period following initial infection to accumulate the high levels of virus within the bone microenvironment.

The significance of unintegrated viral DNA in osteopetrosis. Osteopetrotic bone is characterized by the accumulation of unintegrated viral DNA (Robinson & Miles, 1985; Aurigemma et al., 1989). The persistence of unintegrated viral DNA suggests the continuing infection of cells in the diseased bone. In several retroviral infections, the accumulation of unintegrated viral DNA results from high levels of superinfection (Keshet & Temin, 1979; Weller et al., 1980; Donahue et al., 1991). In these infection, high levels of superinfection are believed to result from a delay or failure of cells to block further infection, a process known as interference.

Studies in Chapter IV indicate that in culture, osteoblasts can establish interference to superinfection. It is not clear how the results in culture apply to infections in the diseased bone. If infected osteoblasts did establish interference, this would indicate that once infected, osteoblasts would only be susceptible to reinfection for only a brief window of time. As discussed in Chapter IV, osteoblasts could sustain high levels of infection, perhaps by being exposed to a high concentration of virus or by being infected as a nonproliferating precursor. It is also possible that osteoblasts (or a subpopulation of osteoblasts) in the bone might be delayed or unable to establish interference. However, infections in which the ability to establish interference is delayed or diminished accumulate 100-400 viral DNA copies per cell, 10-20 times higher than the DNA copy number sustained in ALV-induced osteopetrosis (Robinson & Miles, 1985; Temin et al., 1980; Donahue et al., 1991). The infected cells in the bone could contain a wide distribution of DNA copy number, containing very low levels as well as unusually high levels of infection. However, infections resulting in extremely high levels of

superinfection are associated with cell death (for review, see Temin, 1988). If osteoblasts in the bone were continually susceptible to infection, the level of infection observed in osteopetrosis and the lack of necrosis in the bone would suggest the existence of some mechanism to prevent extremely high levels of infection from occurring. This could involve processes affecting viral entry, such as receptor density or receptor affinity.

A potential role for the cell receptor in mechanisms of osteopetrosis induction.It is conceivable that the density of the cell receptor on osteoblasts could act in the regulation of the level of infection of cells in the bone. Several reports have suggested that growth factor receptors for TGF-ß and EGF display different cell surface densities as a function of the state of differentiation of the osteoblast population (Rodan et al., 1988). It is possible that during maturation, osteoblasts modulate the expression of the viral receptor and thus act as susceptible targets for infection only at specific stage(s) of development. Although antibodies against ALV receptors are not currently available, it would be interesting to determine at what level receptors are expressed on osteoblast populations and whether the cell surface expression is developmentally regulated in a manner which corresponds to the pattern of infection in the bone. Also, given the higher osteopetrotic potential of subgroup B/E viruses, it would be useful to determine how the density of the subgroup B/E receptor on osteoblasts compares with that of other ALV receptors. The results in Chapter III indicate that in culture, osteoblasts are less susceptible to initial infection than fibroblasts. The relative level of receptor in these two cell lines could be compared to determine whether differences in susceptibility to infection reflect differences in receptor density.

Cell receptors which are recognized by viruses possess physiological function(s) in the cell. Although the cellular function of several retroviral receptors has been identified (Maddon et al., 1986; Albritton et al., 1989), the receptors for different ALV subgroups are only now becoming directly characterized. The subgroup A receptor has been recently cloned, however, its cellular function is as yet unknown (Young et al., 1993). It is possible that the association of receptor with virus particles could alter the normal cellular signalling that results from association with the cellular ligand. In fact, high levels of virus-receptor interactions on osteoblasts could result in unusual levels of physiological function of the cell receptor, perhaps causing aberrant signalling within the infected cell. If the cellular function of the viral receptor acted in cell growth, high levels of interactions with the virus could result in deregulated cell growth, akin to oncogenic transformation.

Alternatively, virus expression could diminish the physiological activity of the receptor by blocking interactions with its cellular ligand. High levels of virus could competitively block interaction at the cell surface and the expression of high levels of envelope glycoprotein could downregulate subsequent expression of the receptor on the cell surface. As a result, the cellular function of the viral receptor could be significantly limited in highly infected osteoblasts. If the receptor responsed to signals that were involved in cell differentiation, such as growth factors, virus interactions and receptor downmodulation could limit or delay the process of cell maturation.

However, several lines of evidence suggest that viral interactions at the level of the receptor cannot act as the sole determinant in osteopetrosis induction. ALVs of

multiple subgroups, which recognize independent receptors can induce osteopetrosis (Robinson & Miles, 1985). Moreover, studies using recombinant viruses suggest that the *env* gene is important but not sufficient in determining the osteopetrotic potential of a virus (Brown et al., 1988; Aurigemma et al., 1991). This suggests that virus-receptor interactions cannot alone induce osteopetrosis.

Utility of the osteoblast culture in studying osteopetrosis. The results have suggested that chick calvarial osteoblast cultures do not serve as an adequate model for studying ALV-induced osteopetrosis. Levels of infection and virus expression were 10-20 fold lower in culture than in osteopetrotic bone (Chapter III). In contrast to the massive proliferation of osteoblasts in the diseased bone, virus infection did not alter the growth or differentiation of osteoblasts in culture (Chapter V). These results suggest that osteoblast cultures lack component(s) of the bone that are required for infection to induce the hyperplasia of osteoblasts. The inability to affect osteoblast function was not due to a lack of productive infection in culture (Chapter III). However, the level of infection and virus expression was significantly lower than that observed in the diseased bone. To circumvent limitations of the level of infection in culture, infections could be carried out in vivo, infecting the chick embryo several days prior to osteoblast isolation. This intervening period would allow time for several rounds of replication in the chick, in the context of a normal infection. In the mouse retrovirus, OA MuLV, infections in vivo have been successful in demonstrating an effect on osteoblast function following explant (Schmidt et al., 1987). Alternatively, high levels of infection could be induced in osteoblast cultures by using concentrated virus stocks to infect osteoblast cultures at very

high multiplicities of infection (MOI). Cultures infected at high MOIs (eg. 40 infectious units/cell) could be compared to infections at low MOIs (4 infectious units/cell) to determine whether the level of infection affected osteoblast growth and differentiation in culture.

It is also possible that the culture lacks the contributions of other cell types (osteoclasts, fibroblasts), osteoblasts at other stages of development, or growth factors that could influence osteoblast growth (as discussed in Chapter V). It is possible that the inability of infected cultures to demonstrate altered osteoblast activity could be a function of the state of differentiation of the osteoblast population. The results presented in Chapter III indicate that osteoblast cultures derived from calvaria are not very susceptible to initial infection. This population represents a fairly mature osteoblast as evidenced by the expression of markers associated with osteoblast differentiation and the ability to become mineralized. As discussed in Chapter V, evidence suggests that early osteoblast progenitors may represent a target for retroviral infection. To examine whether infection of osteoblasts at early stages of development could result in high levels of infection and altered cell function, cultures derived from the bone periosteum, which contain osteoprogenitors (Burger et al., 1986; Wlodarski, 1990) could be infected and monitored for virus production and effect of infection on cell growth and differentiation.

Future directions in examining infection in the bone. The studies in culture demonstrate the difficulty of examining virus infection outside the complex environment of the bone. As such, valuable information could be gleaned from characterizing the pattern of infection as it occurs in the bone. In situ hybridization could be performed on

bone samples from infected chickens to determine the relative amounts and distribution of viral DNA in cells of the bone at different times after infection. For increased sensitivity, the viral DNA could be amplified in samples using the polymerase chain reaction (PCR) prior to detection. Use of PCR amplification has been reported to increase the sensitivity of in situ hybridization by more than two orders of magnitude (Haase et al., 1990) and has recently been successfully applied to fixed tissue sections (Nuovo et al., 1991). Using this technique, several questions regarding the timing and pattern of infection in the bone could be addressed:

1. What cell type is first infected in the bone?

Bone samples collected early after exposure of the chicken to virus could be examined by histological analysis combined with in situ hybridization to determine what cell types are early targets for infection (ie. contain viral DNA). This analysis could reveal at what stage of development osteoblasts become infected. In situ hybridization could also reveal whether cells infected in bone early after infection sustain as high levels of infection as compared to later in infection.

2. Do cells in the infected bone sustain different levels of infection?

It is unclear whether all cells in the bone contain the same amount of viral DNA or perhaps more likely, that the level of viral DNA varies within cells of the bone. In situ hybridization could be used as a quantitative tool to determine the relative amounts of viral DNA in cells of the infected bone. Variability in the amount of viral DNA per cell could be indicative of the level of infection being influenced by some aspect of the bone,

such as the stage of development of the cell, the location of the cell relative to the vascularization of the bone or the ability of the infected cells to establish interference.

Moreover, in situ analysis could be used to investigate the distribution of unintegrated viral DNA in the cells of the bone. Cytoplasmic (unintegrated viral forms) and nuclear (primarily integrated viral forms) localization of viral DNA could be distinguished in individual cells of the bone. The distribution of cytoplasmic viral DNA in cells of the bone could be useful in understanding how cells become multiply infected and how unintegrated viral DNA persists in the diseased bone.

In summary, an osteoblast culture system was used to examine virus-host interactions in the development of ALV-induced osteopetrosis. Although the system did not prove to be a satisfactory model for examining the mechanism behind osteoblast dysfunction in osteopetrosis, the culture system provided a means for studying the life cycle of the virus in the target cell for disease. The results indicated that infection did not result in a novel pattern of virus expression or in atypical levels of virus replication. Unlike the diseased bone, cultured osteoblasts did not accumulate high levels of viral DNA and did not undergo atypical growth or differentiation. Thus, the osteoblast cultures provided information about the replication of a virus in its target for disease but did not mimic the conditions that occur in osteopetrotic bone.

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