Canine Distemper Virus and Bone Disease in Dogs

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science

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Abstract

Paget's disease of bone is a chronic, focal, human disorder characterised by excessive osteoclastic resorption and secondary new bone formation, which affects approximately 5% of the UK population over 55 years of age. Previous evidence obtained from electron microscopic, immunocytochemical and molecular studies, has led to the suggestion that it might be the result of a slow virus infection by a member of the Paramyxovirus family. Recently, it has been suggested that canine distemper virus (CDV) might be involved in the aetiology of this disease. This was based on epidemiologic evidence, and on the demonstration of CDV mRNA in bone biopsies of these patients by *in situ* hybridisation and reverse transcriptase-polymerase chain reaction (RT-PCR). Further studies have shown that interleukin-6 (IL-6) and c-FOS levels are raised in pagetic osteoclasts, and that bone marrow cells from Paget's patients are hyper-responsive to vitamin D ($1,25(OH)_2D_3$).

In the light of this evidence, it was decided to examine bone samples from the natural host of CDV, the dog. For this purpose, the technique of *in situ* hybridisation was used with ^{35}S-labelled sense and antisense riboprobes (to the mRNA and genomic RNA) of the nucleocapsid (N) gene of CDV. This revealed that the virus was able to infect and actively replicate in bone cells near to the growth plates of naturally infected young dogs.

In view of this specific localisation of CDV, near to the growth plates, it was decided to examine bone from dogs with metaphyseal osteopathy (MO). This is an idiopathic bone disease which primarily affects young, large breeds of dog. Animals usually recover spontaneously, although they can die from the disease, and are sometimes euthanased, due to the extreme pain associated with the condition. Bone samples from dogs with MO were examined for the presence of the CDV-N gene using *in situ* hybridisation. Positive hybridisation was again seen, however, as in Paget's disease, only CDV transcripts were detected. The presence of distemper was confirmed in one of these cases using RT-PCR.

To further investigate the possible role of CDV in bone diseases of dogs, marrow cells from young distemper-infected and uninfected dogs were cultured in the presence of $1,25(OH)_2D_3$ and varying concentrations of CDV. This revealed that cultures from distemper-infected dogs generate more osteoclast-like cells than those from normal dogs, that distemper-infected cells are hyper-responsive to $1,25(OH)_2D_3$ and that *in vitro* infection with CDV produces a dose dependent increase in the number and size of osteoclast-like cells formed. CDV infection was also shown to induce IL-6 and c-Fos expression in the marrow cells.

These results show that CDV might be involved in the pathogenesis of canine bone disorders, and further support the hypothesis that CDV is involved in human Paget's disease of bone.

Declaration

No part of the work referred to in this thesis has been submitted in support of an application for another degree or qualification, at this or any other University or institute of learning.

Acknowledgements

Firstly, I would like to thank Professor David Anderson, who initially had the "crazy" idea that CDV might be involved in Paget's disease. It was his interest and enthusiasm that got this project off the ground and, without him, none of this work would ever have been done. David, thank you!

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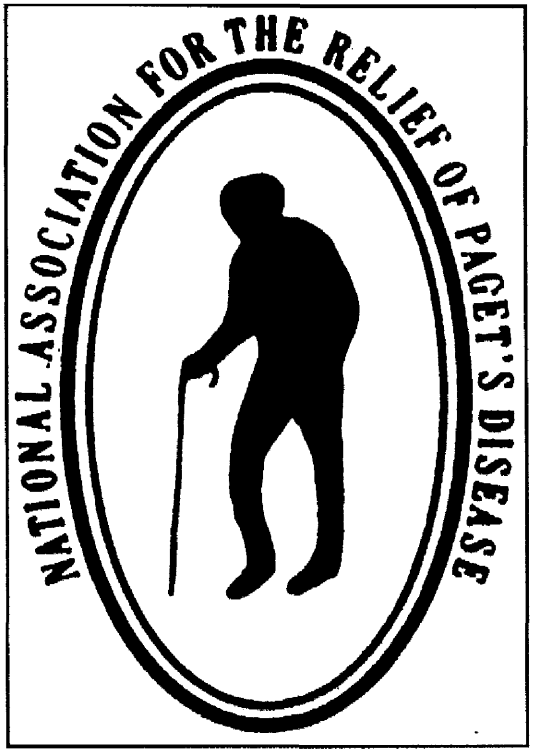

I would also like to thank both my supervisors for their support and encouragement, Dr Paul Sharpe at the University of Manchester (now in London), and Dr David Bennett at the University of Liverpool.

Thank you also to my "unofficial supervisors" who helped with the learning of techniques and discussions related to the work , Drs David Webber and Margaret Gordon at Manchester, and Drs Christopher May and Stuart Carter at Liverpool. Also Jo Price in Sheffield, for her help with the cell culture work, and Malcom Bennett at Leahurst, for his knowledge of viruses. Thank you also to Isobel Braidman, Bob Cooper and David Anderson for proof reading and comments.

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Lastly, but by no means least, I would like to thank the people who mean the most to me. To my parents for their neverending help, love and encouragement, even when it seemed I was becoming a student for life! To Ann, for her constant love, support and understanding (and for putting up with me whilst I've been writing this thesis!).

To Ann, Mum and Dad,

Thank you for everything, I love you.

Preface

I graduated from the University of Liverpool in 1990 with an honours degree in Veterinary Science. After working in practice for several months, I took up a position as House Surgeon at the Small Animal Hospital at the University of Liverpool. Research at the Small Animal Hospital had implicated canine distemper virus (CDV) as a possible aetiologic agent in canine rheumatoid-like arthritis, and a collaboration was set up with Paul Sharpe's Molecular Biology group at the University of Manchester. As part of this collaboration, I began working on a project funded by the National Association for the Relief of Paget's Disease to examine the possible role of CDV in canine bone disorders. This forms the work detailed in this thesis.

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Abbreviations

$1,25(OH)_2D_3$	1α,25 dihydroxyvitamin D_3
AICAH	Autoimmune chronic active hepatitis
ALP	Alkaline phosphatase
AMA	Ascending medullary artery
BDGF	Bone derived growth factor
BGP	Bone Gla-protein
BMP	Bone morphogenetic protein
CAII	Carbonic anhydrase
CDV	Canine distemper virus
c-fos	Murine proto-oncogene (c-*fos* = gene)
c-Fos	Human proto-oncogene (c-*Fos* = gene)
CFU	Colony forming unit
CFU-F	Fibroblast CFU
CFU-GM	Granulocyte-macrophage CFU
CFU-S	Stem cell CFU
CNS	Central nervous system
CRA	Canine rheumatoid-like arthritis
CSF	Colony stimulating factor (also, where relevant = cerebrospinal fluid)
CSF-GM	Granulocyte-macrophage CSF
CSF-M	Macrophage CSF
CT	Calcitonin
DMA	Descending medullary artery
DOPC	Determined osteoprogenitor cell
EGF	Epidermal growth factor
F	Fusion
G	Glycoprotein
Gla	Gamma-carboxyglutamic acid

H	Haemagglutinin
HA	Haemagglutination
HCMV	Human cytomegalovirus
HLA	Human leucocyte antigen
HN	Haemagglutinin-neuraminidase
HTLV-1	Human T lymphotropic virus 1
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IL-6R	Interleukin 6 receptor
IOPC	Inducible osteoprogenitor cell
L	Large
LTR	Long terminal repeat
M	Matrix
MF	Mineralisation front
MGP	Matrix Gla-protein
MIBE	Measles inclusion body encephalitis
MNC	Multinucleated cell
MO	Metaphyseal osteopathy
MS	Multiple sclerosis
MV	Measles virus
N	Nucleocapsid
NA	Neuraminidase
NCP	Noncollagenous protein
NF-IL6	Interleukin 6 nuclear factor
NF-κB	Nuclear factor κB
OA	Osteoarthritis
ODE	Old dog encephalitis
ORSA	Osteoclast resorption-stimulating activity

P	Phosphoprotein
PDV	Phocid distemper virus
PG	Prostaglandin
PTH	Parathyroid hormone
PPRV	Peste des petits ruminants virus
RA	Rheumatoid arthritis
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase-polymerase chain reaction
RV	Rinderpest virus
SSPE	Subacute sclerosing panencephalitis
SV5	Simian virus 5
SZ	Sealing zone
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRAP	Tartrate resistant acid phosphatase

Chapter 1

Bone Cell Biology

1. Bone Cell Biology

1.1 Introduction

Bone is a complex specialised connective tissue comprised of a number of cell types and extracellular matrix (Boskey and Posner 1984; Nijweide et al 1986; Marks and Popoff 1988). It serves three main functions:

1. Mechanical – sites of muscle attachment for locomotion
2. Protective – bone marrow and internal organs
3. Metabolic – reservoir of mineral ions, particularly calcium and phosphate

Despite appearing outwardly stable, bone is a dynamic, living tissue that can readily change in response to various stimuli.

1.2 Bone Structure and Composition

Based on the mechanism of their formation (endochondral and intramembranous ossification (see Section 1.4)), bones can be classified into two types (Boskey and Posner 1984); the long bones (e.g. humerus, femur) and the flat bones (e.g. scapula, ilium).

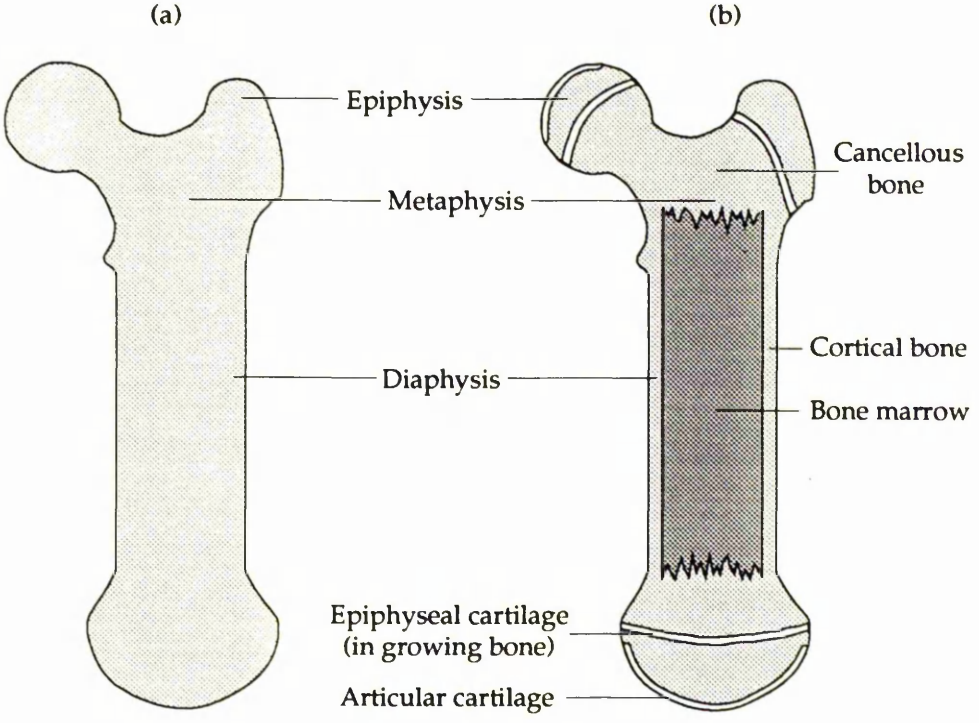
Externally, a long bone can be roughly divided into three areas (Basmajian and Slonecker 1989) (Figure 1.1(a)):

1. Epiphysis – at each extremity
2. Diaphysis – roughly cylindrical tube in the middle
3. Metaphysis – region between the diaphysis and the epiphysis

In growing bones, the epiphysis and metaphysis are separated by a layer of cartilage, the epiphyseal cartilage, or growth plate (Basmajian and Slonecker 1989) (see Section 1.4.1.2). This is involved in the longitudinal growth of the bone, and eventually calcifies at the end of the growth period.

If a long bone such as the femur is sectioned longitudinally, these areas can be further defined (Basmajian and Slonecker 1989) (Figure 1.1(b)). The external parts of the bone comprise a thick, compact layer of bone, the cortex. In the diaphysis, the cortex surrounds the medullary cavity, which contains the bone marrow. Towards the epiphysis and metaphysis, the cortex progressively thins and the extremities of the bone are filled with a network of thin, bony trabeculae, the cancellous bone. The areas enclosed within these trabeculae are continuous with the medullary cavity and are also filled with bone marrow. The external and internal surfaces of bones are lined with layers of osteogenic cells, the periosteum and endosteum, respectively. The surfaces of bone which are involved in the joint are covered by articular cartilage.

Figure 1.1 Anatomical features of a typical long bone



(a) External features (b) Features seen after longitudinal sectioning

Whilst it is likely that cortical and trabecular bone are composed of the same cells and matrix elements, there are structural and functional differences (Baron 1993). Up to 90% of the volume of cortical bone is solid bone, whereas only 15-25% of the trabecular bone volume is taken up by bone, and 75-85% by bone marrow. Cortical bone mainly provides the mechanical and supportive functions, whilst the trabecular bone is responsible for the metabolic functions.

1.2.1 The Cells

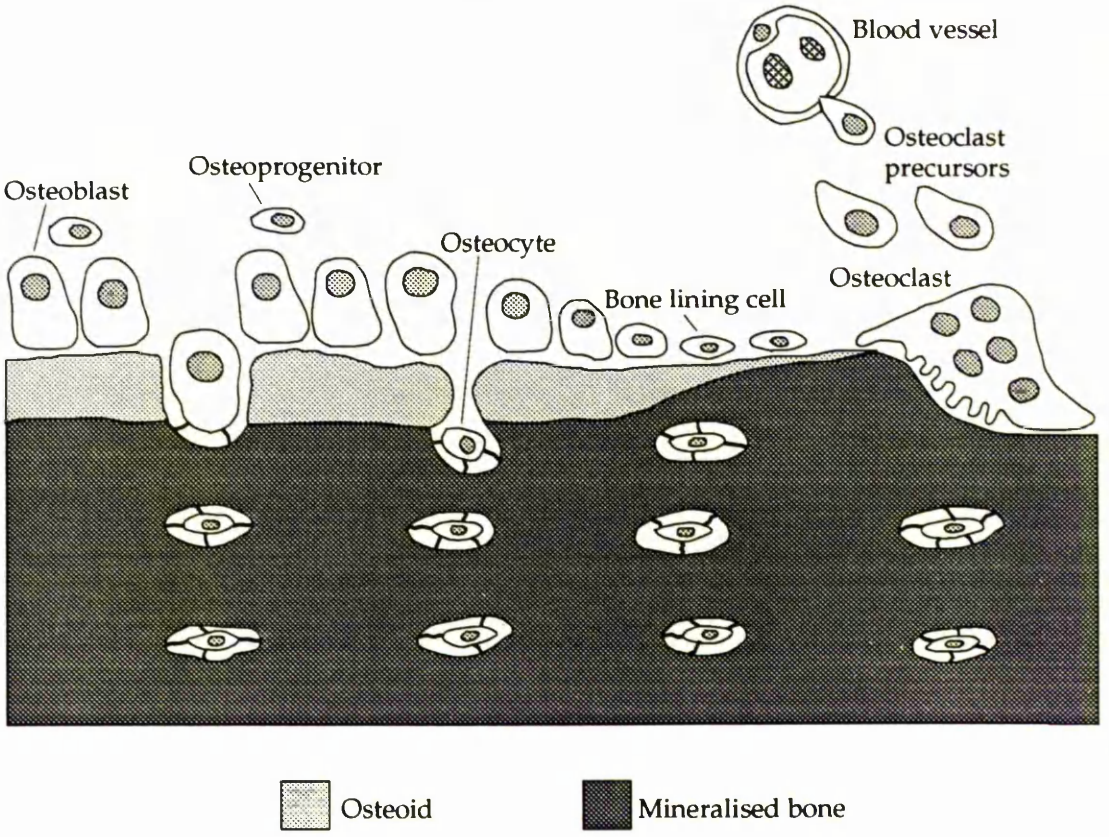
There are several morphologically distinct cell types found in bone (Figure 1.2). They can be divided into bone-forming and bone-resorbing cell families, each of which consists of a number of differentiation stages (reviewed by Boskey and Posner 1984; Nijweide et al 1986; Marks and Popoff 1988; Teitelbaum 1990). Osteoblasts, pre-osteoblasts, osteocytes and bone lining cells make up the bone-forming family, and osteoclasts and their precursors the bone-resorbing family. The exact identity of the precursors of both families is still disputed. However, it is now generally accepted that the bone-forming cells are derived from local mesenchymal stem cells (marrow stromal cells or connective tissue mesenchymal cells), and that osteoclasts are formed by fusion of mononuclear precursors which circulate in the blood (reviewed by Nijweide et al 1986; Marks and Popoff 1988; Baron 1993; Zaidi et al 1993).

The osteoblast and osteoclast lineages will be briefly described here, with reference to the composition of bone. The formation, function and regulation of these cells will be discussed in more detail in Section 1.3.

1.2.1.1 The osteoblast lineage

Pre-osteoblasts are committed osteoblast progenitor cells (Nijweide et al 1986). They are found near the surface of bone, usually a few cell layers from the active osteoblasts. They have an elongated appearance and mitotic figures are often seen.

Figure 1.2 Topographic relationships of bone cells



After Marks and Popoff (1988)

Osteoblasts are plump, cuboidal lining cells which stain strongly for alkaline phosphatase and are actively involved in matrix formation (Wergedal and Baylink 1969; Nijweide et al 1986; Wlodarski 1990). They have copious amounts of rough endoplasmic reticulum, which is separated from the nucleus by a prominent golgi apparatus (Hancox and Boothroyd 1965) (see Figure 1.4). They have surface receptors for parathyroid hormone (Silve et al 1982; Rouleau et al 1986) and secrete a number of proteins, such as osteocalcin and type I collagen (Price 1983; Von Der Mark et al 1976). Receptors for PTH have also been demonstrated on mononuclear cells in the intertrabecular spaces of metaphyseal bone (Rouleau et al 1988). The exact identity of these cells was not determined, however, it was postulated that they were preosteoblasts. The collagen is assembled extracellularly and shows increased

organisation with increased distance from the cell. The secretion of proteins is usually towards the bone surface. However, some osteoblasts surround themselves with matrix and eventually become osteocytes.

Approximately 10-20% of osteoblasts become osteocytes, which occupy lacunae in areas of calcified bone. They are connected to adjacent cells by numerous cytoplasmic projections which run in canals known as canaliculi (Basmajian and Slonecker 1989). Whilst their metabolic activity is dramatically reduced compared with osteoblasts, they are still active and may play a role in activating local bone turnover (Marks and Popoff 1988; Baron 1993). They are connected to adjacent osteoblasts by gap junctions (Doty 1981; Menton et al 1984) and it has been postulated that they may be involved in responses to bone loading (Pead et al 1988; Skerry et al 1989; El Haj et al 1990).

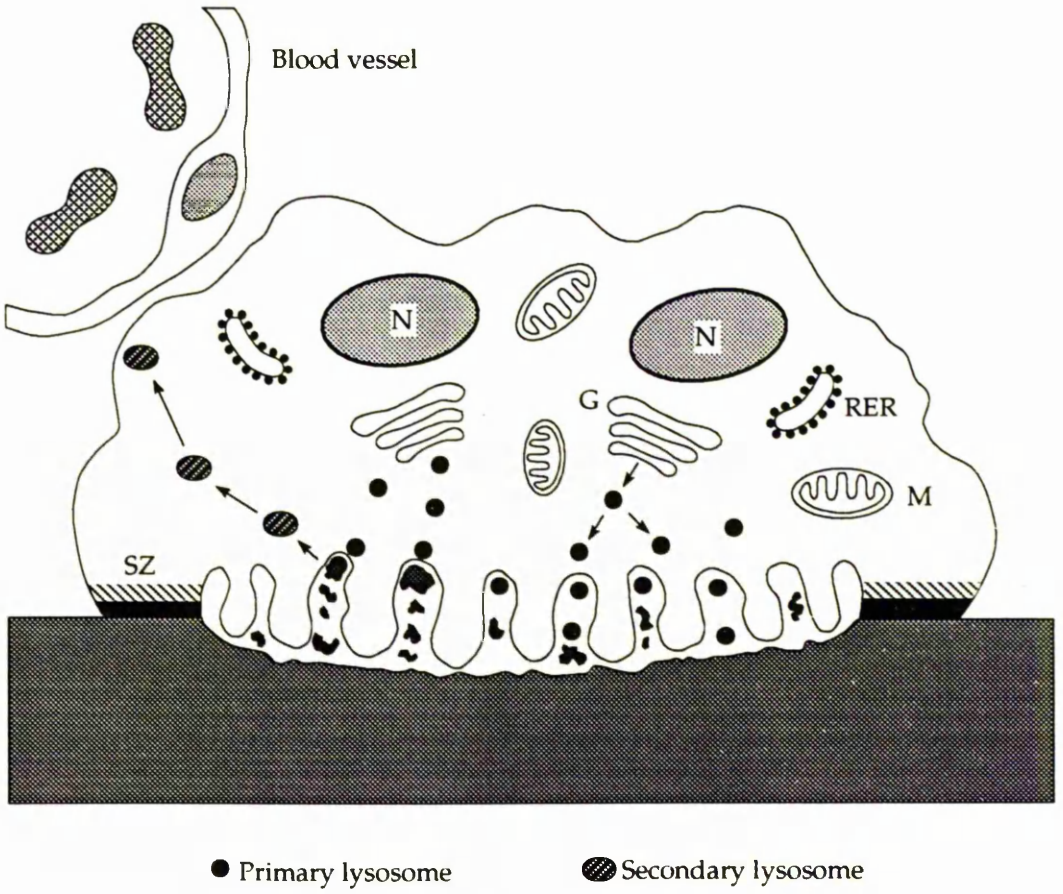
In mature bone, bone lining cells occupy the majority of bone surfaces which are not being actively remodelled (Luk et al 1974a, 1974b). They are flattened, elongated, inactive cells with few organelles, found immediately apposed to the bone surface. Their exact function is unknown, although it has been postulated that they can transform themselves into osteoblasts, and that they play a role in the functional separation of bone and extracellular fluid compartments (Miller et al 1980; Bowman and Miller 1986; Miller and Jee 1987). It has also been suggested that they serve as nutritional support for osteocytes, via joining gap junctions (Weinger and Holtrop 1974; Miller et al 1980).

1.2.1.2 The osteoclast lineage

Osteoclasts are the cells that resorb bone. They are multinucleated (on average 4-20 nuclei, although this is species dependent and can increase to several hundred nuclei in pathological circumstances (see Chapter 3)), and are usually found in contact with mineralised bone and cartilage within a resorption cavity known as a Howship's lacuna (Nijweide et al 1986; Marks and Popoff 1988; Mundy 1993) (Figure 1.3). They are usually found at sites of actively remodelling bone, such as the metaphyses of growing bones. There is now convincing evidence that osteoclasts are formed by fusion of mononuclear precursor cells that originate

in the bone marrow or other haematogenous organs and migrate to the bones via vascular routes (reviewed by Nijweide et al 1986; Marks and Popoff 1988; Mundy 1993) (described in more detail in Section 1.3).

Figure 1.3 Diagrammatic representation of a resorbing osteoclast



N – nucleus, G – golgi apparatus, RER – rough endoplasmic reticulum, M – mitochondrion, SZ – sealing zone. Enzymes are packaged in the perinuclear golgi region into primary lysosomes, which then move to the ruffled border, where they are released into the subosteoclastic space next to the mineralised tissue (the functional equivalent of a secondary lysosome). Products of resorption are taken back up into the osteoclast, further digested in secondary lysosomes and released into adjacent blood vessels.
Adapted from Marks and Popoff (1988) and Baron (1993)

The contact area of active osteoclasts is characterised by two plasma membrane specialisations, which are identifiable by electron microscopy (Holtrop et al 1974; Holtrop and Raisz 1979; Marks and Popoff 1988). The ruffled border consists of deep foldings of the

membrane and this is surrounded by a ring of contractile proteins, the sealing (or clear) zone, which attaches the osteoclast to the bone surface and seals off the subosteoclastic resorbing area (Figure 1.3).

Characteristic ultrastructural features of these cells also include an abundance of golgi apparatuses around each nucleus, mitochondria and prominent cytoplasmic vesicles (primary and secondary lysosomes) (Holtrop et al 1974; Holtrop and Raisz 1979; Marks and Popoff 1988).

1.2.2 The Extracellular Matrix

Bone is a complex tissue composed of many matrices and cell types (Boskey and Posner 1984; Triffitt 1987; Marks and Popoff 1988; Boskey 1989; Termine 1993). Many of the matrix components have yet to be identified. Some of the major matrix proteins and their proposed functions are listed in Table 1.1.

Table 1.1 Bone matrix constituents

Name(s)	Potential function(s)
Collagen type I (II, III, V, X)	Structure, mineralisation
Osteonectin	Mineralisation
Bone Gla-protein (osteocalcin)	Mineralisation, calcium homeostasis
Matrix Gla-protein	Mineralisation, calcium homeostasis
Proteoglycans I and II	Mineralisation, matrix organisation
Bone sialoproteins I (osteopontin) and II	Cell attachment, matrix organisation
Bone morphogenetic proteins and other growth factors	Morphogenesis
Thrombospondin	Cell attachment
Fibronectin	Cell attachment

Adapted from Marks and Popoff (1988), Boskey (1989) and Termine (1993)

Unlike other connective tissues, the bone matrix is mineralised with a form of calcium phosphate known as hydroxyapatite (Boskey and Posner 1984; Marks and Popoff 1988; Termine 1993). As a consequence of bone turnover, the matrix is constantly remodelled and regenerated throughout life. The organic components of the bone matrix can be divided into two groups, collagen and the noncollagenous proteins.

1.2.2.1 Collagen

The most abundant protein of the organic matrix of bone is type I collagen (85-90% of the total bone protein) (Boskey and Posner 1984; Triffitt 1987; Marks and Popoff 1988; Termine 1993). Very small amounts of types II, III, V and X collagen are also found in protein extracts from bone, but these are generally thought to be contaminants from blood vessels and cartilage (Marks and Popoff 1988). Type I collagen is a triple-helical supercoil containing two α_1(I) chains and a structurally similar α_2(I) chain (Traub and Piez 1971; Gallop et al 1972). The messenger RNA for each collagen chain codes for a precursor known as procollagen. This is modified during or immediately following secretion from the osteoblast (Fleischmajer et al 1987) to form collagen molecules which are then arranged to form collagen fibres (Figure 1.4).

Several post-translational modifications occur, including peptide cleavage, hydroxylation, addition of galactose and phosphorylation (Traub and Piez 1971; Gallop et al 1972). Following fibril formation, cross-links form between fibres and complexes are formed with noncollagenous proteins.

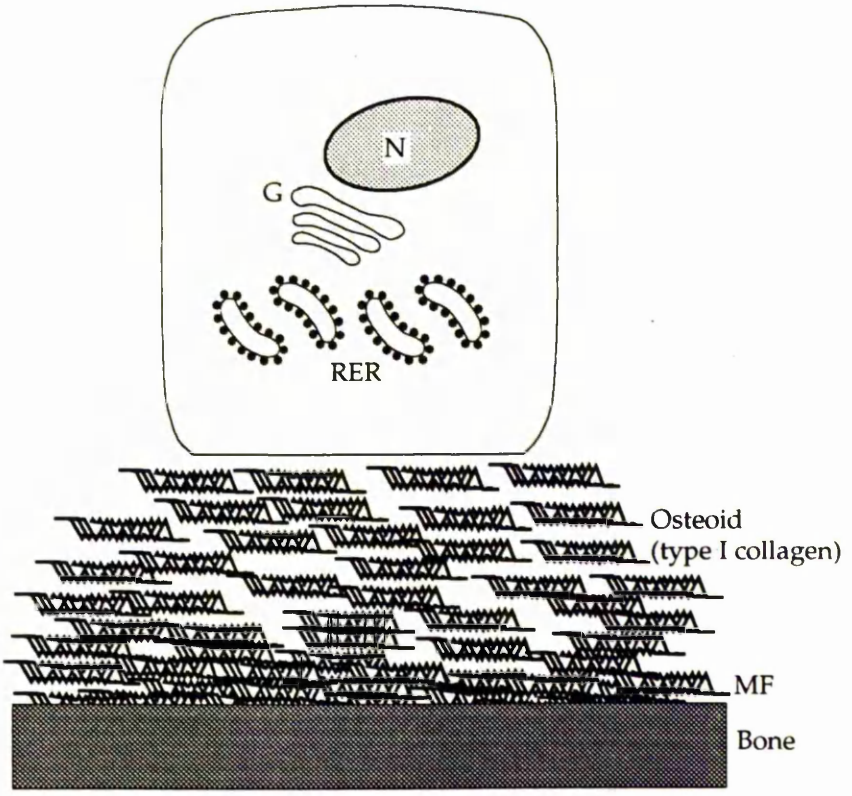
Bone collagen differs from soft tissue collagen in that there are more crosslinks and that the lysines in bone collagen are more highly hydroxylated (Bornstein and Sage 1980; Strawich and Glimcher 1983).

Following secretion, the collagen fibres must first mature, prior to mineralisation (Puzas 1993; Termine 1993). The exact mechanism of this maturation is unknown. The maturing collagen

between the cell and the edge of mineralising bone (the mineralisation front) is known as osteoid (Figure 1.4).

There are three stages of collagen synthesis and mineralisation (reviewed by Puzas 1993). Collagen is initially secreted at a rapid rate, eventually producing a wide seam of osteoid. In the second stage, the rate of mineralisation increases to match the rate of collagen synthesis, keeping the osteoid seam at a constant thickness. This is followed by a final stage, where the rate of collagen synthesis decreases, and mineralisation continues until the osteoid seam disappears.

Figure 1.4 Diagrammatic representation of an active osteoblast



N – nucleus, G – golgi apparatus, RER – rough endoplasmic reticulum, MF – mineralisation front.
Adapted from Puzas (1993)

1.2.2.2 Noncollagenous proteins

Noncollagenous proteins (NCPs) make up 10-15% of the total bone protein content (reviewed by Termine 1993). However, on a mole to mole basis, osteoblasts secrete approximately the same number of NCPs as collagen. Approximately 25% of NCPs are derived from tissues other than bone, usually from serum (Marks and Popoff 1988).

The most abundant NCP in bone is osteonectin (Termine et al 1981). It is a phosphorylated glycoprotein with numerous disulphide bonds, and has high affinity for collagen, ionic calcium and hydroxyapatite (Termine et al 1981; Marks and Popoff 1988). Despite its name, it is not bone specific, being found in platelets (Stenner et al 1986), fibroblasts (Wasi et al 1984) and other non-bony tissues undergoing changes in activity (Holland et al 1987; Wewer et al 1988). Osteonectin is thought to be involved in osteoblast development and in mineralisation (Boskey 1989; Termine 1993).

Bone Gla-protein – BGP (osteocalcin) is an abundant NCP in bone that has three γ-carboxyglutamic acid (Gla) residues per molecule (Hauschka et al 1975; Price et al 1976). It is also found in dentine (Bronckers et al 1985) and its synthesis is vitamin K dependent and stimulated by 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2$ D_3) (Price 1985; Lian et al 1985, 1987). Osteocalcin is also found in plasma, where its levels are maintained by new synthesis, rather than removal from bone matrix (Price et al 1981). Plasma levels of osteocalcin have been used as a marker of bone turnover (Price et al 1980). Osteocalcin synthesis is greatly reduced by the vitamin K antagonist, warfarin. Long term warfarin treatment in rats impairs endochondral ossification and causes premature closure of the growth plates (Price et al 1982). It has also been suggested that osteocalcin might play a role in osteoclast recruitment (Lian et al 1984). This was based on the observation that when demineralised bone matrix from osteocalcin-deficient animals was implanted subcutaneously, it was not resorbed, whereas bone matrix from normal animals was resorbed.

Another Gla-containing protein, matrix Gla-protein – MGP, is found in bone and cartilage (Price et al 1983; Price 1987). This molecule contains five Gla residues but has little homology

with BGP, except with respect to three of its Gla residues (Marks and Popoff 1988; Boskey 1989; Termine 1993). MGP appears early in skeletal development (when BGP levels are low), and is also stimulated by $1,25(OH)_2$ D_3 (Marks and Popoff 1988; Boskey 1989). This, coupled with the fact that MGP co-precipitates in bone extracts with bone morphogenetic protein (see below), has led to the suggestion that MGP may play a role in bone development (Price and Williamson 1985; Price et al 1985).

Bone contains two proteoglycans (I and II), which have smaller core proteins than cartilage proteoglycans and have fewer glycosaminoglycan side chains (Fisher 1985). They are concentrated near to the mineralisation front, where the protein core is degraded, leaving the side chains in the mineralised bone (Fisher 1985). Their exact role is unknown; however, it has been suggested that they might be responsible for orientation of collagen fibres (Scott and Haigh 1985).

One of the first NCPs to be isolated from bone was a sialic acid containing protein, sialoprotein (Herring and Kent 1963). It was further characterised (Fisher et al 1983) and shown to consist of two similar molecules, sialoproteins I and II (Franzen and Heinegard 1985). Sialoprotein I is found only in bone and has been shown to facilitate attachment and spreading of cloned rat osteosarcoma cells (Marks and Popoff 1988). It has been named osteopontin, due to its bridging potential between cells and bone mineral (Oldberg et al 1986). Sialoprotein II has been less well characterised. Biochemical analyses suggest that, in rabbit bone, it is a keratan sulphate proteoglycan (Kinne and Fisher 1987).

Bone morphogenetic protein – BMP, is a glycoprotein which can induce new bone formation *in vivo* (Urist et al 1984). It has also been shown to promote DNA synthesis and cell proliferation in bone organ cultures (Canalis et al 1985), and can cause *de novo* bone formation, even when implanted in non-bony sites (Sampath et al 1992). Further work has shown that there are several BMPs, each of which can individually cause morphogenesis (Wang et al 1988; Wozney et al 1988; Celeste et al 1990; Asahina et al 1993; Forell and Straw 1993). Characterisation of the BMPs has revealed that they are structurally closely related to transforming growth factor-ß (TGF-ß) (Wozney et al 1988; Celeste et al 1990).

Other bone derived growth factors (BDGF) have also been identified, some of which closely resemble platelet derived growth factor, fibroblast growth factor and cartilage derived growth factor (Hauschka et al 1986).

Other phosphoproteins, glycoproteins and lipids are also found in bone matrix (Marks and Popoff 1988). However, they are only present in very small amounts and their exact functions have not been determined.

There are also several proteins which are synthesised in other tissues and then attracted to the mineral phase of bone (Triffitt 1987; Marks and Popoff 1988; Boskey 1989). These include albumin, α-2HS-glycoprotein, lysozyme, transferrin and various immunoglobulins (IgG, IgE). It has been suggested that α-2HS-glycoprotein might play a role in bone resorption (Colclasure et al 1988). Albumin has been shown *in vitro* to both facilitate and to inhibit mineral deposition and IgG has been shown to have no effect on mineral deposition and growth (Boskey 1989). The fact that these proteins are not synthesised in bone would argue against a specific role for them in bone.

Other proteins derived from bone cells are found in bone extracts, including alkaline phosphatase, collagenase and lysosomal enzymes (Marks and Popoff 1988; Boskey 1989). Whether these proteins play any role in matrix development has yet to be determined.

Approximately 30% of NCPs in bone extracts cannot be identified specifically (Delmas et al 1984). Despite the characterisation of the remaining 70%, no metabolic or structural role for any of the NCPs has been proved.

1.2.3 The Vascular Supply of Bone

The blood vessels that supply bone have been classified into three groups on the basis of function, rather than their anatomic location (Rhinelander 1974a). These are:

1. Afferent – arteries and arterioles
2. Efferent – veins and venules
3. Intermediate – capillary-like vessels

1.2.3.1 The afferent vasculature

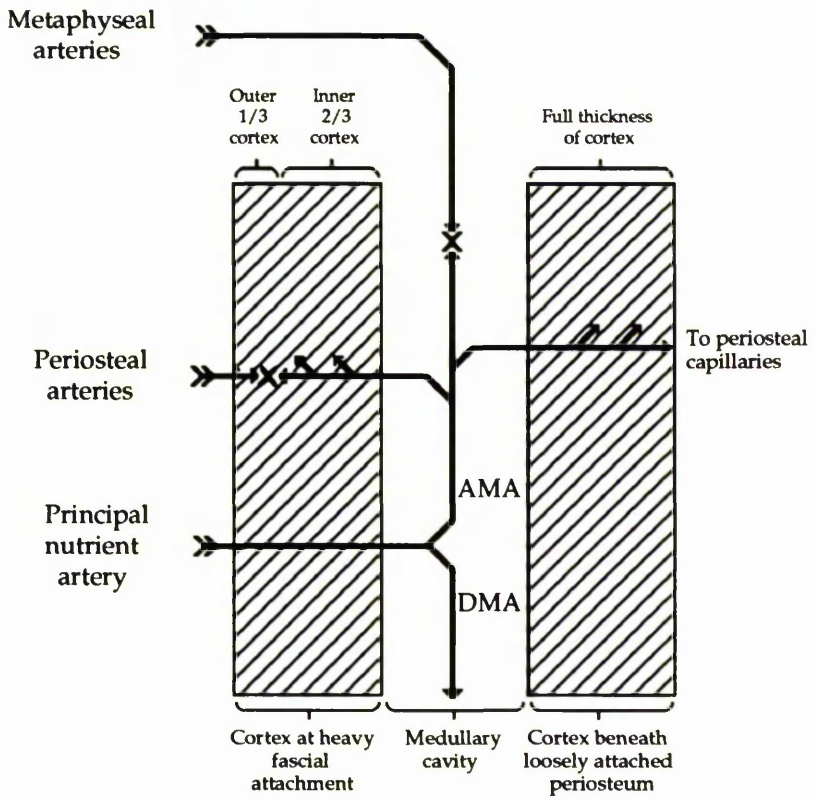
There are three main components of the afferent blood supply (Rhinelander 1974b) (summarised in Figure 1.5):

1. Principal nutrient artery – enters the cortex along a major fascial attachment. It crosses the cortex and enters the medulla without branching. It then divides into ascending and descending medullary arteries which branch further to supply the endosteal surface of the medulla.
2. Metaphyseal arteries – several metaphyseal arteries enter the metaphyses and eventually anastomose with the terminal branches of the medullary arteries. Normally, the metaphyseal arterial supply to the medullary cavity is minimal, although it can increase in pathological conditions.
3. Periosteal arteries – supply the outer quarter to one third of the cortex. Their terminal branches anastomose with those of the medullary arteries.

The intravascular pressure is higher in the medulla than in the periosteal area (Brookes 1971), hence, the flow of blood through the cortex is centrifugal, from the medulla to the periosteum (Brookes 1971; Rhinelander and Wilson 1982).

Immature bone has a more extensive blood supply than mature bone (Rhinelander and Wilson 1982). In immature bones, the growth plate acts as a barrier to blood vessels, hence the epiphyses and metaphyses have separate blood supplies (Rhinelander and Wilson 1982). In the epiphysis, many small vessels enter at the periphery of the joint cartilage. These branch and anastomose to supply the epiphysis. In the metaphyses a similar circle of larger vessels enter through numerous foramina near the growth plate. These anastomose, together with terminal branches of the nutrient artery, and travel perpendicularly towards the growth plate. At the growth plate, they form "hairpin" bends and return towards the medulla. These hairpin bends are thought to play a role in haematogenous osteomyelitis (see Section 3.2.1).

Figure 1.5 The afferent vascular supply of a mature long bone



AMA – ascending medullary artery, DMA – descending medullary artery, X – anastomosis.
Arrows indicate the direction of blood flow.
Adapted from Rhinelander (1974b) and Rhinelander and Wilson (1982)

1.2.3.2 The efferent vasculature

The efferent vascular system drains the cortical surfaces of the long bones (Rhinelander and Wilson 1982). Drainage from the diaphysis varies in different regions of the bone; the cortex supplied by medullary arteries is drained by deep venous channels and superficial periosteal capillaries, that supplied by periosteal arterioles is drained by corresponding periosteal veins. The diaphyseal medullary cavity is drained by sinusoids which empty into a large central venous sinus. This sinus is in turn drained by emissary veins and the venous counterpart of the nutrient artery. Drainage from the metaphysis is via multiple metaphyseal veins.

1.2.3.3 The intermediate vasculature

This forms the link between the afferent and efferent systems (Rhinelander and Wilson 1982). The capillary-sized vessels of the intermediate system occupy bony channels known as Haversian (vertical) and Volkmann's (horizontal) canals.

1.3 Bone Cell Formation, Function and Regulation

1.3.1 The Osteoblast

Osteoblasts are the cells that form bone (Nijweide et al 1986; Wlodarski 1990; Baron 1993; Puzas 1993). That is, they are characterised by the deposition of mineral ions (in the form of hydroxyapatite) within a collagenous framework (type I collagen). The biochemistry of osteoblasts has been mainly studied in cells grown *in vitro* and from tumour cell lines (osteosarcomas), and hence might not truly reflect the situation *in vivo*.

The structure and basic functions of osteoblasts were described in Section 1.2.1.1.

1.3.1.1 Origin of the osteoblast

The osteoblast is derived from a local connective tissue cell associated with the connective tissues of the endosteal and periosteal surfaces (Friedenstein 1976). This connective tissue, the stroma, is continuous with the marrow stroma (Basset et al 1961; McLean and Urist 1968; Burkhardt 1970). The bone marrow stroma consists of a network of reticular cells, endothelial cells and extracellular fibres, together with sinusoidal vessels and adipose cells (Weiss 1976). These stromal cells form a supporting framework for the haematopoietic cells and provide the correct microenvironment for their development and differentiation. It has therefore been suggested that the soft tissues of bone are similar to those of the marrow, in that they both consist of cells of the stromal and haematopoietic systems (Owen 1980).

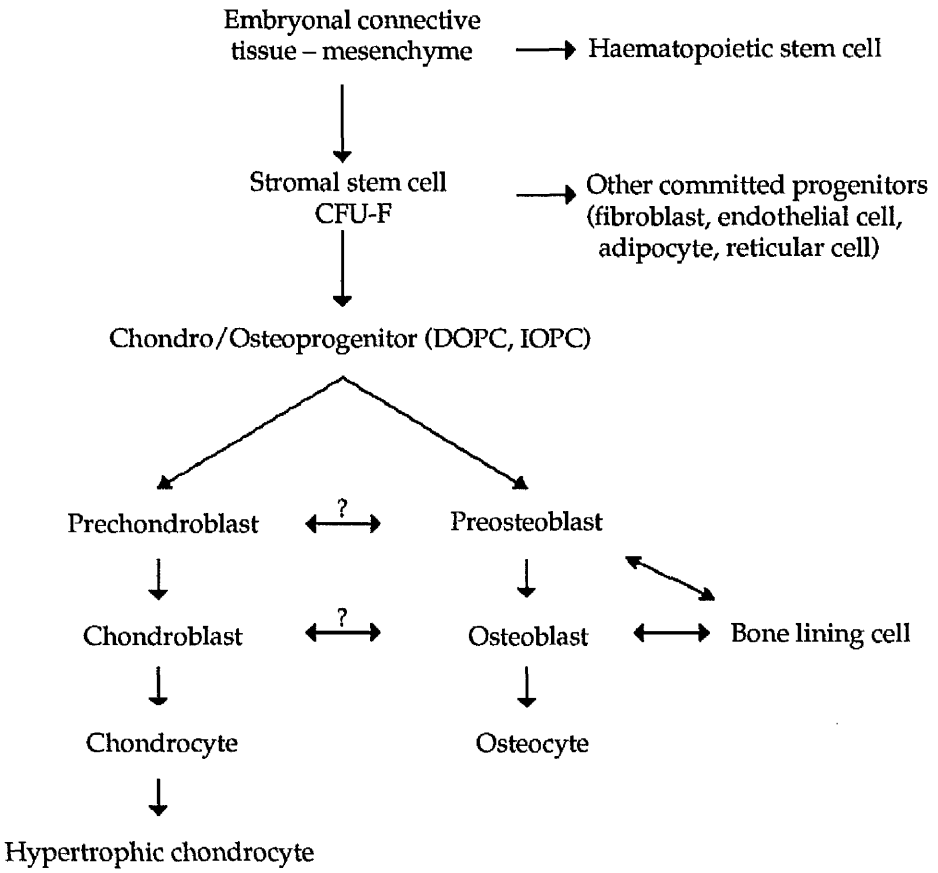
Information on the origin of osteoblastic cells has come mainly from studies on bone marrow regeneration or transplantation to ectopic sites, diffusion chamber studies and marrow

stromal cell cultures. Following mechanical depletion of the marrow cells in the medullary cavity, there is a reproducible sequence of events (Branemark et al 1964; Amsel et al 1969; Patt and Maloney 1975). Initially, a blood clot forms, which is then invaded by capillaries and proliferating connective tissue from the bone surfaces. The mesenchymal cells of this connective tissue develop into osteoblasts and form cancellous bone. Haematopoiesis then occurs and the excess bone is resorbed to form a normal marrow cavity. The sequence of events is similar following transplantation of marrow to ectopic sites (Sahebekhtiari and Tavassoli 1978). However, in this system, the transplanted haematopoietic cells die, leaving only the stromal cells. These proliferate and differentiate into osteoblasts. This is again followed by the arrival of haematopoietic cells and the resorption of excess bone. The work of Friedenstein (1973, 1976) has provided further evidence to show the osteogenic nature of marrow stromal cells. This showed that marrow cells from cultures, when placed into diffusion chambers and implanted *in vivo*, were capable of bone formation. This was later confirmed by Ashton et al (1980). Friedenstein (1973) named these cells determined osteoprogenitor cells (DOPC). Cells cultured from other haematopoietic organs such as the thymus and spleen were incapable of forming bone under the same conditions (Friedenstein 1973). However, if osteogenic inducers, such as bladder epithelium or decalcified bone matrix, were added, calcified tissue was formed. Friedenstein named these cells inducible osteoprogenitor cells (IOPC). These progenitors are also sometimes referred to as fibroblastic colony forming units (CFU-F) (Wlodarski 1990).

Based on these results, Owen (1980) proposed a stromal origin for osteogenic cells. Figure 1.6 shows an adaptation of Owen's lineage diagram.

The proposed lineage includes both osteogenic and chondrogenic (cartilage forming) components, suggesting a common progenitor for these two types of cell. This common precursor theory is supported by the switch of embryonal germinal cells from osteogenesis to chondrogenesis by the application of intermittent pressure and tension (Hall 1968), and by the switch from chondrogenesis to osteogenesis induced by immobilisation (Hall 1972a).

Figure 1.6 The osteogenic cell lineage



CFU-F – fibroblastic colony forming unit, DOPC – determined osteoprogenitor cell, IOPC – inducible osteoprogenitor cell, ? – pathway not yet determined.
Adapted from Owen (1980), Nijweide et al (1986), Marks and Popoff (1988) and Wlodarski (1990)

Other agents, such as ß-aminoproprionitril (Hall 1972b), embryonic extract (Mayne et al 1976) and high oxygen tension (Shaw and Bassett 1967; Pawelek 1969) in the culture medium, can promote osteogenesis, as can a low plating density of cells (Caplan et al 1983). Ascorbic acid (Hall 1981), corticosterone (Heersche et al 1984), reduced oxygen tension (Shaw and Bassett 1967; Pawelek 1969) and thyroxine (Pawelek 1969) all promote chondrogenesis.

1.3.1.2 The biochemical control of osteoblast function

Osteoblasts are rich in alkaline phosphatase (ALP) which has therefore been used as a histochemical and biochemical marker for their identification (Wergedal and Baylink 1969; Luben et al 1976; Schmid et al 1983; Pols et al 1986; Owen et al 1987). It is also used as a marker of osteogenesis (Nishimoto et al 1985), although the exact function of the enzyme is unknown. It has been proposed to play a possible role in several different processes, including mineralisation (Robison 1923), phosphate transport and regulation of cell proliferation (Puzas 1993). Osteocalcin is also used as a marker of osteoblastic activity (Price et al 1980; Delmas et al 1983; Ashton et al 1985).

Activation of osteoblasts by a variety of growth factors, vitamins and hormones can have various effects, including an increase in DNA , ALP and glycogen synthesis and an increase in cyclic AMP (cAMP) (Wlodarski 1990).

Normal osteoblasts and those derived from osteosarcomas have receptors for epidermal growth factor (EGF) (Ng et al 1983a, 1983b; Wlodarski 1990). EGF has been shown to cause proliferation of these cells *in vitro*.

Osteoblasts secrete various prostaglandins (PG) including PGE_2, PGF_1 and PGF_2 (Nolan et al 1983; Tatakis et al 1988). They also respond to these factors *in vitro*. PGE_1 causes differentiation of cloned osteoblastic cells via increased cAMP (Hakeda et al 1987). PGF_2 causes proliferation via the phosphatidyl inositol system (Hakeda et al 1987).

The response of osteoblasts to EGF and the secretion of PGs by osteoblasts is consistent with their proposed role in bone resorption (both EGF and PGs are potent stimulators of bone resorption (see Section 1.3.2)).

Another factor that affects osteoblasts is TGF-ß. This is found in large amounts in bone and has been shown to exhibit various effects on bone metabolism. In primary cultures, osteoblast proliferation can be either stimulated or inhibited by TGF-ß, depending on the cell density and the concentration of TGF-ß (Centrella et al 1987). In osteosarcoma cell (ROS 17/28) cultures, TGF-ß increases ALP and collagen synthesis and inhibits proliferation

(Pfeilschifter et al 1987). However, in further experiments, TGF-ß was shown to inhibit both the growth and phenotype expression (ALP and response to parathyroid hormone) of osteoblastic cell lines (Elford et al 1987; Noda and Rodan 1987). Bone resorptive factors such as $1,25(OH)_2$ D_3 and PTH increase TGF-ß activity, whilst CT decreases TGF-ß activity (Pfeilschifter and Mundy 1987).

Growth hormones can act directly on osteoblasts (mitogenic in mouse and chicken cell cultures (Slootweg et al 1988a, 1988b)), or can act indirectly via stimulation of insulin-like growth factor (IGF-1) (Schmid et al 1983; Stracke et al 1984).

Calcitonin (CT) and tri-iodothyronine (T_3) can both accelerate the *in vitro* proliferation of chondroblasts and osteoblasts, and increase osteoid production (Gaillard 1970; Weiss et al 1981; Farley et al 1988). However, the proliferative effects of calcitonin are only seen if it is administered prior to osteogenesis. If given after bone formation is initiated, calcitonin suppresses bone formation (Weiss et al 1981). The effects of T_3 are also complex. At very high doses (higher than physiological or pathological situations), T_3 inhibits replication and stimulates phenotypic expression of osteoblast cell lines (Sato et al 1987; Kasono et al 1988) and primary cultures (Egrise et al 1990; Oishi et al 1990). However, at similar doses, T_3 has also been shown to stimulate proliferation in human primary osteoblast cultures (Kassem et al 1992). Lower (though still higher than physiological) doses of T_3 stimulate osteocalcin, ALP and collagen production in cell lines and rat primary cultures (Rizzoli et al 1986; Sato et al 1987; Kasono et al 1988; Egrise et al 1990; Oishi et al 1990).

Parathyroid hormone (PTH) receptors have been found on normal and osteosarcomatous osteoblasts (Silve et al 1982; Rizzoli et al 1983; Rouleau et al 1986). Increased PTH leads to the synthesis of collagenase and plasminogen activator (Otsuka et al 1984; Cowen et al 1985; Hamilton et al 1985), both of which are thought to play a role in bone resorption (see Section 1.3.2.5). PTH also causes a decrease in type I collagen and ALP synthesis (Canalis 1983).

Osteoblastic cells also have receptors for hydrocortisone (Rodan and Rodan 1984), and hydrocortisone has been shown to increase ALP activity in stromal precursor cells (Owen et

al 1987). Dexamethasone has also been shown to cause proliferation and differentiation of osteogenic precursor cells (McCulloch and Tenenbaum 1986).

Receptors for $1,25(OH)_2$ D_3 are also found on osteoblasts and osteocytes (Boivin et al 1987), although the evidence for the presence of receptors on osteocytes is unconvincing (Boivin et al 1987). They have also been found in osteosarcoma cells (Manolagas et al 1980) and foetal bone cells (Narabitz et al 1983), although they are not found on osteoclasts (Merke et al 1986). *In vitro*, $1,25(OH)_2$ D_3 prevents de-differentiation of osteoblasts and decreases their adhesiveness (Fritsch et al 1985). The effect of $1,25(OH)_2$ D_3 on ALP and matrix formation is complex, and the available evidence is contradictory. Continuous infusions of $1,25(OH)_2$ D_3 to young mice inhibit the formation of new bone matrix, but promote the calcification of existing matrix (Reynolds et al 1976; Marie et al 1985). Conversely, chronic administration of $1,25(OH)_2$ D_3 to rats leads to increased bone formation and decreased mineralisation (Wronski et al 1986). Experiments examining the effects of $1,25(OH)_2$ D_3 on cultures of osteoblasts or osteoblast-like cells have also produced complex results. In mouse and rat cell lines, $1,25(OH)_2$ D_3 stimulates ALP activity and collagen synthesis (Manolagas et al 1981; Haneji et al 1983). A similar effect was seen in primary foetal rat cultures (Pols et al 1986). However, in primary osteoblast cultures, $1,25(OH)_2$ D_3 has also been shown to inhibit ALP activity and collagen synthesis (Wong et al 1977). Osteocalcin synthesis can also be stimulated by $1,25(OH)_2$ D_3 in rat osteoblasts (Price and Baukol 1981) and osteosarcoma cells (Price and Baukol 1980), and in human osteoblasts (Beresford et al 1984; Skjodt et al 1985). These conflicting responses of osteoblasts and osteoblast-like cells to $1,25(OH)_2$ D_3 are thought to depend on various factors, including species differences, cell density and the proliferative stage of the cells being examined (Chen and Feldman 1981; Majeska and Rodan 1982; Rodan and Rodan 1984).

Various other factors such as the BMPs and BDGF have been shown to have a proliferative effect on osteoblasts and their precursors (see Section 1.2.2.2).

1.3.1.3 Mineralisation of bone

Bone mineralisation is currently thought to be controlled by the osteoblast (Boskey and Posner 1984; Teitelbaum 1990; Puzas 1993). The process of mineralisation can be roughly divided into four stages (Boskey and Posner 1984):

1. Preparation of the matrix for mineralisation – cartilage matrix and osteoid do not mineralise immediately after their synthesis. In man, the time taken for the osteoid to mature to a state capable of undergoing calcification (the mineralisation lag time) averages 21 days (Melsen and Mosekilde 1980).

   Initially, calcium and phosphate ions are lost from the chondrocyte or osteoblast mitochondriae, producing an increase in extracellular concentration of these ions (Wuthier 1982; Anderson 1980). Also, phosphoproteins (Veis et al 1977), sialoproteins (Fisher et al 1983), glycoproteins (Wuthier 1982), and the activity of enzymes such as alkaline phosphatase (Boskey and Posner 1984), all increase.

   Membrane-enclosed extracellular mineral crystals (matrix vesicles) can be seen budding off from chondrocytes or osteoblasts (Anderson 1980, 1985). These are rich in alkaline phosphatase and are thought to play an active role in the mineralisation process, although it has been suggested that they are not essential for mineralisation to occur (Landis et al 1986).

2. Formation of initial mineral crystals (nucleation) – the majority of bone mineral forms by growth on these initial crystals, rather than by *de novo* nucleation (Glimcher 1982). While the precise structure of this initially deposited mineral is unknown, the mineral deposited on this initial mineral is known to be hydroxyapatite. Factors known to facilitate hydroxyapatite deposition *in vitro* include osteonectin, proteolipids and various phosphoproteins (Boskey and Posner 1984).

3. Growth of crystals – additional hydroxyapatite crystals are deposited on the surface of those already present. The local concentrations of calcium and phosphate ions needed for the initial deposition of crystals are much higher than those needed for the maintenance of hydroxyapatite deposition (Boskey and Posner 1984). The

orientation of mineral crystals depends on the orientation of the matrix collagen. Various NCPs may also play a role (see Section 1.2.2.2).

4. Remodelling – described in detail in Section 1.4.2. In cartilage, the calcified matrix is resorbed and replaced by bone. Osteoclasts and osteoblasts combine to remodel bone.

1.3.2 The Osteoclast

It is over 100 years since the osteoclast, the cell responsible for bone resorption, was first discovered (Kölliker 1873). However, the exact origin of the osteoclast remains a mystery to this day. Other cells, including osteocytes, monocytes and tumour cells have been implicated in bone resorption, although none of these cells exhibit the classical characteristics of osteoclastic resorption (Jones et al 1985). Monocytes and macrophages resorb devitalised bone (Mundy et al 1977; Kahn et al 1978), but no resorption pits are seen when these cells lie adjacent to living bone surfaces (Mundy 1993). Resorption pits are also absent around tumour cells (Boyde et al 1986), despite the fact that they too can resorb devitalised bone (Eilon and Mundy 1978). Osteolysis seen around osteocytes is different from that of osteoclasts and is considered by some authors to be an artefact of bone that is rapidly turning over (Boyde et al 1986).

1.3.2.1 Structure of the osteoclast

The basic structure of the osteoclast was described in Section 1.2.1.2. However, due to the importance of the osteoclast in Paget's disease and metaphyseal osteopathy (see Chapter 3), it is described again in more detail.

Osteoclasts are highly specialised multinucleated cells (MNCs) which have several unique ultrastructural features. The nuclei are usually centrally located and contain 1-2 nucleoli. Osteoclasts contain abundant lysosomes, free ribosomes, mitochondriae and golgi apparatuses (Holtrop et al 1974; Holtrop and Raisz 1979; Marks and Popoff 1988) . The plasma membrane adjacent to the bone surface is comprised of complex folds and

invaginations which allow close contact with the bone. The membrane-associated activities required for resorption are concentrated within this area, which is known as the "ruffled border". The size and complexity of the ruffled border is proportional to the resorbing activity of the osteoclast (Holtrop et al 1974; Domon and Wakita 1989; Segawa et al 1989). The ruffled border is completely surrounded by an area of cytoplasm that contains no organelles and is hence known as the "clear zone" (Miller et al 1984). The clear zone contains bundles of actin filaments and is involved in attaching the osteoclast to the bone surface undergoing resorption (hence it is also known as the "sealing zone" (see Figure 1.3)). The space thus formed between the osteoclast and the underlying bone is considered to be equivalent to a secondary extracellular lysosome (Baron et al 1985). These specialisations of the plasma membrane make up the resorption system and are unique to the osteoclast.

1.3.2.2 Origin of the osteoclast

The fact that osteoclasts are formed by fusion of precursor cells is not disputed. Yet to be determined, however, is the exact identity of the precursor cell (or cells). Despite some suggestions that the precursors are derived from local mesenchymal cells, the overwhelming evidence points towards a haematopoietic origin. Early evidence for this came from autoradiographic experiments on regenerating newt limbs (Fischman and Hay 1962). Further support for this theory has come from parabiosis experiments (Jee and Nolan 1963), the use of quail-chick nuclear markers (Kahn and Simmons 1975; Jotereau and LeDouarin 1978) and transplantation experiments with osteopetrotic mice (Göthlin and Ericsson 1973; Walker 1973, 1975; Buring 1975). Successful treatment of human osteopetrosis with infusions of bone marrow cells has also been documented (Coccia et al 1980; Sorell et al 1981).

Several ideas have been proposed as to the possible identity of the haematopoietic precursor. These include:

1. Non-committed progenitor – this theory proposes that the initial pathway is identical to that of the monocyte-macrophage lineage, and that the final pathways diverge to give two separate lineages (Loutit and Nisbet 1979; Ibbotson et al 1985; Kurihara et al 1990a, 1991; MacDonald et al 1986; Scheven et al 1986; Schneider et al 1986; Udagawa

et al 1989; Hattersley et al 1991). The osteoclast is derived from the granulocyte-macrophage progenitor cell (CFU-GM), which is derived from the multipotential haematopoietic stem cell (CFU-S). The CFU-S is the precursor of both the osteoclast and the monocyte, and a non-committed progenitor is formed which either generates a committed osteoclast progenitor, or switches to form a monocyte precursor (Burger et al 1982; Thesingh and Burger 1983; Ibbotson et al 1984).

2. Committed precursor – there is also some evidence that osteoclasts are derived from a special line of specific marrow progenitors (Loutit and Nisbet 1982; Horton et al 1985a, 1985b). The osteoclast develops from a derivative of the CFU-S that is committed to the osteoclast lineage.
3. Direct origin from immature haematopoietic precursors – evidence also exists that immature haematopoietic cells (possibly CFU-GM) can give rise directly to osteoclasts (Hakeda et al 1989; Kurihara et al 1990a, 1991). Osteoclasts have been shown to differentiate *in vitro* from haematopoietic tissue of mice and rabbits (Takahashi et al 1988a, 1988b). Cultures of chick monocytes have been induced to express "osteoclast-specific" antigens (Osdoby et al 1986), as too have a subpopulation of rabbit bone marrow cells (Fuller and Chambers 1987). In bone marrow transplantation studies, mononuclear phagocytes fail to correct the defect in osteopetrosis (Loutit and Nisbet 1982; Schneider and Byrnes 1983), and *op/op* mice are cured by transplantation of CFU-GM and granulocytes (Marks and Schneider 1982; Schneider and Relfson 1988; Felix et al 1990; Kodama et al 1991). These experiments suggest a distinct osteoclast precursor which diverges before mononuclear stem cells are formed.

Generally, it is now agreed that osteoclasts do not derive directly from cells of the mononuclear phagocyte lineage. Mononucleated and multinucleated macrophages cannot resorb bone (Chambers and Horton 1984), they do not respond to CT as osteoclasts do (Chambers and Magnus 1982), and, although they do share some antigens with osteoclasts (Athanasou et al 1986) they lack more typical osteoclast antigens (Horton et al 1985a, 1985b). However, some evidence has suggested that mature monocytes and macrophages can

differentiate into osteoclast-like cells if a suitable environment is provided. The presence of osteoblasts (Burger et al 1984; Takahashi et al 1988a; Udagawa et al 1989), marrow stromal cells (Flanagan et al 1994), macrophage colony-stimulating factor (CSF-M) (Felix et al 1990) or cytokines (Hakeda and Kumegawa 1991) can induce this differentiation.

As can be seen, the osteoclast lineage is extremely complex and still to be completely elucidated. Each *in vitro* system of investigating osteoclast cell biology has merits (discussed in detail in Section 1.5), but none correspond completely to the situation *in vivo*. Despite the recent advances in our understanding of the origin of osteoclasts, there is still more work to be done before the exact identity of the osteoclast precursor can be identified and examined in more detail.

1.3.2.3 Criteria for osteoclast identification

One of the major problems associated with *in vitro* culturing of "osteoclasts" has been the reliable identification of the cells as authentic osteoclasts. Several criteria have been used to help overcome this problem:

1. Multinuclearity – this was, until recently, the most widely used criterion. However, it is now recognised as probably the least reliable marker for osteoclast identification. The MNCs formed in bone marrow cultures are often incapable of resorbing bone, and are now generally thought to represent macrophage polykaryons (Allen et al 1981; Ali et al 1984; Burger et al 1984; Chambers and Horton 1984; Horton et al 1986). These MNCs possess macrophage surface markers and do not bind osteoclast-specific antibodies. However, there is some evidence that they can resorb ground bone powder (Kahn et al 1978; Teitelbaum et al 1979; Burger et al 1984), although this may merely reflect the phagocytic capacity of these cells. Hence, whilst multinuclearity is a reliable marker of osteoclasts in bone, the same is not true for the cells formed in bone marrow cultures. That is not to say, however, that these cells are incapable of becoming osteoclasts, as some authentic osteoclastic bone resorption has been shown to take place in these cultures (Ibbotson et al 1984; Roodman et al 1985,

1987; MacDonald et al 1986, 1987; Takahashi et al 1986a; Pharoah et al 1987; Zaidi et al 1993), and can be induced by the presence of osteoblasts and cytokines (see Section 1.3.2.2).

2. Tartrate resistant acid phosphatase (TRAP) activity – TRAP was once thought of as a specific marker for osteoclasts. However, it has now been shown that macrophages contain and develop this enzyme *in vitro* and *in vivo* (Chambers et al 1978; Efstratiadis and Moss 1985a, 1985b; Snipes et al 1986; Webber et al 1988).
3. Bone resorption and CT responsiveness – the ability to resorb bone is the major criterion for identification of osteoclasts (see Section 1.3.2). The cells in marrow cultures that behave as authentic osteoclasts are those that resorb bone and possess specific surface antigens (Fuller and Chambers 1987). Authentic mammalian osteoclasts express CT receptors on their surface and decrease resorption in response to CT (Chambers and Magnus 1982; Chambers and Moore 1983; Chambers et al 1985a; Arnett and Dempster 1987). Macrophage-like cells from bone marrow cultures have not been shown to respond to CT (Chambers and Magnus 1982; Chambers and Moore 1983; Takahashi et al 1988b).
4. Osteoclast-specific antigens – monoclonal antibodies derived from giant cell tumours (osteoclastomas) have been used as markers of osteoclasts in normal bone tissues (Horton et al 1985b). Although some of the antibodies produced cross-reacted with macrophages, several did not cross-react.

Hence, it can be seen that identification of osteoclasts in culture is not always easy. The most reliable criteria are the ability to resorb bone and the reduction in resorption in response to CT due to the expression of CT receptors. However, the latter criteria do not apply to chicken osteoclasts which do not possess CT receptors (Nicholson et al 1987) and are unresponsive to CT (Arnett and Dempster 1987; Murrils and Dempster 1990). The story is not so simple, however, as CT receptors and responsiveness can be induced in chickens fed a low-calcium diet prior to isolation of the osteoclasts (Eliam et al 1988; Hunter et al 1988).

1.3.2.4 Regulation of osteoclast formation

The average life span of human osteoclasts is approximately 2 weeks, with a half-life of about 6-10 days (Jaworski et al 1981; Loutit and Townsend 1982; Marks and Schneider 1982). The rate of bone resorption *in vivo* is proportional to the number of osteoclasts (Baron and Vignery 1981; Chambers and Hall 1991). Thus, for bone resorption to proceed, osteoclasts must be continuously replenished. Hence, the regulation of osteoclast formation is a key mechanism for the regulation of function.

The osteoclast precursor is derived from the haematopoietic tissues. Osteoclast progenitors are seeded from the circulation into bone, where they undergo further differentiation (Burger et al 1982; Thesingh and Burger 1983). Osteocalcin and type I collagen have been shown to be chemotactic for monocytes (Mundy et al 1978; Malone et al 1982; Mundy and Poser 1983). It has been suggested that these matrix components may also be chemotactic for osteoclast progenitors (Glowacki et al 1991), although this has not been convincingly demonstrated (Chambers and Hall 1991).

When the progenitor cells first arrive, they are proliferative and highly radiosensitive, and are CT-receptor- and TRAP- negative (Baron et al 1986a). They then differentiate into post-mitotic TRAP-negative cells which subsequently become TRAP- and CT-receptor- positive (Baron et al 1986a; Nijweide et al 1986; Marks and Popoff 1988; Taylor et al 1989). The precursors then become progressively more osteoclast-like. They show increased synthesis of lysosomal enzymes, pseudopodia formation, ruffling of the plasma membrane adjacent to the bone surface, and evidence of multinuclearity and ruffled border and sealing zone formation (Baron et al 1986a). Osteoblasts and osteocytes have been shown experimentally to apparently produce a soluble factor that is required for the differentiation of progenitor cells (Burger et al 1984; Oursler et al 1987).

Various agents act at different stages of this differentiation cycle:

1. Colony stimulating factors (CSFs) – MNC formation in marrow cultures has been shown to be stimulated by several CSFs, including CSF-GM and CSF-M (MacDonald

et al 1986; Roodman 1991), and interleukin-3 (IL-3) (or multipotential CSF) (Hagenaars et al 1991; Scheven et al 1986). However, as previously mentioned, these cells have not been conclusively shown to be authentic osteoclasts, although it is accepted that at least some of the cells behave as osteoclasts (see Section 1.3.2.3).

CSF-GM has recently also been shown to be involved in osteoclast formation *in vivo*. In *op/op* mice, there is impaired production of CSF-GM, leading to osteopetrosis, which can be cured with CSF-GM treatment (Felix et al 1990).

2. Cytokines and growth factors – several cytokines have been shown to alter the differentiation of osteoclast progenitors in marrow cultures. Transforming growth factor alpha (TGF-α) and EGF increase the proliferation of committed osteoclast precursors (Lorenzo et al 1986; Takahashi et al 1986a). TGF-ß inhibits osteoclast differentiation *in vitro* and blocks the effect of TGF-α (Chenu et al 1988). Interleukin-1 (IL-1) (both α and ß forms) stimulates proliferation of progenitors and differentiation of committed precursors (Gowen and Mundy 1986). IL-6 modulates differentiation and fusion of preosteoclasts (reviewed by Roodman 1992) (discussed in more detail in Section 3.3.5.1), as do tumour necrosis factors (TNFs) α and ß (Johnson et al 1989). Interferon-γ inhibits osteoclast progenitor differentiation in response to high levels of CSF-GM and IL-3, and inhibits the fusion of precursor cells at low concentrations of PTH, IL-1 and $1,25(OH)_2$ D_3 (Takahashi et al 1986b; Gowen and Mundy 1986; Gowen et al 1986; Takahashi et al 1987). Interferon-γ is also known to stimulate differentiation of the monocyte-macrophage lineage (Perussia et al 1983; Weinberg et al 1984). If the osteoclast and macrophage do share a common precursor, interferon-γ could therefore be involved in the switch from osteoclast formation to macrophage formation.
3. Parathyroid hormone – PTH increases the number of osteoclasts in bone, but the mechanism of this increase is uncertain (Bingham et al 1969; Feldman et al 1980; Baron and Vignery 1981; Hedland et al 1983; Takahashi et al 1988b; Yamashita et al 1990). It is thought that PTH stimulates osteoblasts to produce an osteoclastic

proliferation factor, or that it acts directly on preosteoclasts (Jilka 1986; Akatsu et al 1989; Hakeda et al 1989; Teti et al 1991).

4. $1,25(OH)_2 D_3$ – this hormone is thought to stimulate osteoclast progenitors to differentiate and fuse (Ibbotson et al 1984; Roodman et al 1985; Pharoah and Heersche 1985; MacDonald et al 1986; Takahashi et al 1988b; Thavarajah et al 1991; Takano-Yamamoto et al 1992; Braidman and Anderson 1993). It also increases the number of nuclei per osteoclast in bone organ cultures (Holtrop and Raisz 1979) and increases the activity of TRAP (Roodman et al 1985).
5. Calcitonin – CT decreases osteoclast numbers and the number of nuclei per osteoclast both *in vitro* and *in vivo* (Feldman et al 1980; Baron and Vignery 1981; Hedland et al 1983; Takahashi et al 1988b), although high concentrations are needed to show this effect. It also causes contraction of the cytoplasmic membrane which correlates with its ability to reduce bone resorption (Chambers and Magnus 1982). However, these effects are only transient and osteoclasts eventually become refractory to CT following continued exposure (Wener et al 1972).
6. Other factors – glucocorticoids inhibit osteoclast formation *in vitro* (Suda et al 1983). Prostaglandins of the E series have similar effects as PTH and $1,25(OH)_2 D_3$ (Rifkin et al 1980; Schelling et al 1980). Reactive oxygen species (ROS) such as hydrogen peroxide have been shown to promote the differentiation of osteoclast precursors (Suda 1991).

1.3.2.5 Osteoclast activation

Many factors are thought to play a role in activating osteoclasts, the most important of which are described here:

1. Mineral exposure – although there is some dispute as to whether osteoclasts can resorb osteoid (Reid 1986; Boyde and Jones 1987), it is generally accepted that, *in vivo*, osteoclasts will only resorb mineralised tissues (Chambers et al 1984a). Hence, the osteoid layer is thought to be a natural barrier to bone resorption *in vivo*. However, osteoid apparently only covers all bony surfaces in foetal and growing bones (Boyde

1972; Boyde and Jones 1987). In adult resting bones, the surface layer of collagen fibres appears to be mineralised, although, there is conflicting evidence to suggest that this is not the case (Chambers and Hall 1991). In situations where osteoid is present, it has been postulated, on the basis of results obtained in cell cultures, that neutral proteases are secreted by osteoblastic cells, to expose the mineralised surface to osteoclasts (Hamilton et al 1984; Heath et al 1984; Pfeilschifter et al 1990). However, this effect has not been demonstrated *in vivo*.

2. Cells of the osteoblastic lineage – osteocytes, osteoblasts and bone lining cells are connected via cytoplasmic processes, and it is thought that they can transmit positional and mechanical information to localise osteoclasts to regions of bone remodelling (Rodan et al 1975; Rubin and Lanyon 1984; Noda and Sato 1985).
3. Hormonal control via the osteoblast – there is accumulating evidence to suggest that the osteoblast plays a vital role in bone resorption. Osteoblasts have receptors for PTH (Silve et al 1982; Rouleau et al 1986) and for $1,25(OH)_2\ D_3$ (Manolagas et al 1980; Narabitz et al 1983), both of which are potent stimulators of bone resorption. This idea is supported by the fact that osteoclasts do not have receptors for PTH (Silve et al 1982; Rouleau et al 1986), and the lack of stimulation of bone resorption seen using the disaggregated osteoclast assay system in response to these agents (Chambers et al 1985a; Nicholson et al 1989; Zaidi et al 1993). Also, neither agent affects osteoclast motility or spreading (Braidman et al 1983, 1986; Chambers et al 1984a, 1984b).

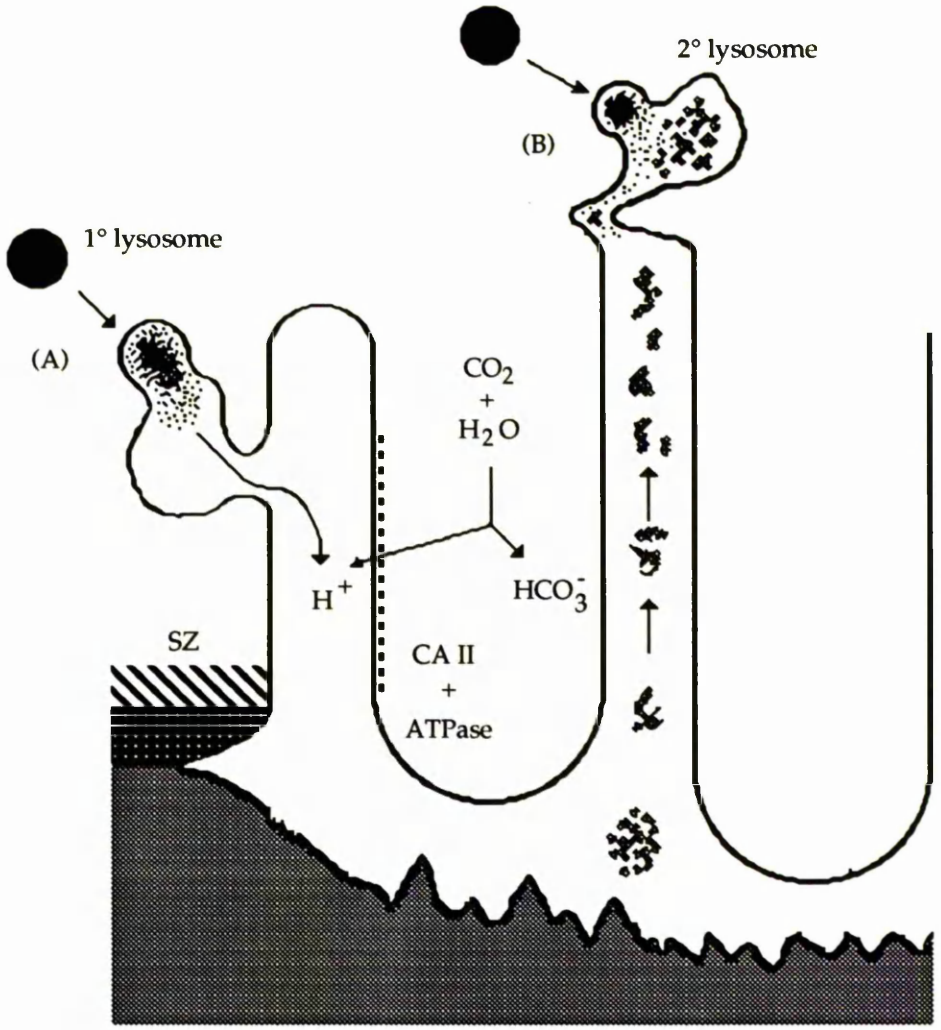
   The way in which the osteoblast influences the osteoclast in response to these hormones is still unknown. It is generally thought that it could either directly stimulate the osteoclast, or cause a decreased production of an osteoclast inhibitor. McSheehy and Chambers (1986a, 1986b) were the first to identify a soluble factor in osteoblast supernatants that stimulated bone resorption, and they termed this ORSA (osteoclast resorption-stimulating activity). However, the identity of this factor is still unknown, although it has recently been suggested that hydrogen peroxide is a possible candidate, as it has been shown to be a potent direct stimulator of osteoclasts

(Bax et al 1992). Hydrogen peroxide causes the production of ROS, which are known to stimulate osteoclasts both *in vitro* and *in vivo* (Garrett et al 1990).

1.3.2.6 Mechanisms of bone resorption

The essential features of the mechanisms of bone resorption are summarised in Figure 1.7.

Figure 1.7 The exocytic and endocytic mechanisms of bone resorption by osteoclasts



SZ – sealing zone, CAII – carbonic anhydrase II (cytoplasmic), ATPase – proton pump (intramembranous). High power view of the ruffled border (see Figure 1.3). Enzymes are (A) released from the primary lysosomes into the subosteoclastic space. Products of resorption are (B) taken into the osteoclast, digested further in secondary lysosomes, and released into adjacent blood vessels.
After Marks and Popoff (1988)

The resorption of bone involves exocytic and endocytic mechanisms (Lucht 1972; Lucht and Norgaard 1976; Marks and Popoff 1988). Acid hydrolases are produced in the golgi apparatus, packaged into primary lysosomes and transported to the ruffled border, where they are released into the subosteoclastic space (Lucht 1971, 1972). Products of cell respiration are broken down by carbonic anhydrase isozyme II (located adjacent to the ruffled border membrane) to produce hydrogen ions, which are released via proton pumps (Na^+, K^+ ATPase) (located in the membrane) to acidify the subosteoclastic space, providing the optimal environment for acid hydrolase activity (Gay et al 1983; Vaananen and Parvinen 1983; Baron et al 1986b, 1988). In the subosteoclastic space, the bone is degraded in the acid environment and the products are taken back into the osteoclast in secondary lysosomes, where they are further digested, before being released into local vascular spaces (see also, Figure 1.3).

The acid hydrolases include TRAP which is used as a histochemical marker of osteoclasts (see Section 1.3.2.3).

1.3.2.7 Modulation of resorptive activity

Many factors involved in the control of osteoclast formation and activation (both of which processes indirectly affect bone resorption) are also directly involved in the regulation of resorptive activity.

Thus, CT acts directly on osteoclasts to reduce bone resorption, by reducing cytoplasmic motility and spreading (Chambers and Magnus 1982; Chambers and Moore 1983; Chambers et al 1985a; Arnett and Dempster 1987). PTH does not act directly on osteoclasts, as they do not possess PTH receptors (see Section 1.3.2.5). Bone resorption by isolated osteoclasts is not increased by PTH (Chambers et al 1985a; Nicholson et al 1989), but can be restored by incubation of osteoclasts with osteoblast primary cultures or cell lines (McSheehy and Chambers 1986a; Nicholson et al 1989). $1,25(OH)_2$ D_3 also acts indirectly to increase bone resorption (Raisz et al 1972; Marie and Travers 1983; Herrmann-Erlee and Gaillard 1978). Various other agents which are secreted by osteoblasts in response to PTH and $1,25(OH)_2$ D_3,

including IL-1 (Hanazawa et al 1985, 1987; Lorenzo et al 1990), TGFß (Centrella and Canalis 1985; Robey et al 1987), CSF-M and CSF-GM (Felix et al 1988), cause an increase in bone resorption by osteoclasts.

1.4 Bone Growth and Development

1.4.1 Bone Growth

There are two types of bone histogenesis; intramembranous ossification (flat bones) and endochondral ossification (long bones) (Teitelbaum 1990; Baron 1993). The main difference between them is the presence or absence of a cartilaginous precursor.

1.4.1.1 Intramembranous ossification

In intramembranous ossification, there is no cartilaginous model for bone formation. The process starts with mesenchymal cells clustering together within highly vascularised areas of the embryonic connective tissue. These cells divide and differentiate directly into preosteoblasts, and then osteoblasts, which in turn produce bone matrix. Within this matrix, the collagen fibres are irregularly arranged, the osteocytes are large and numerous and mineralisation occurs irregularly, giving the characteristic appearance of woven bone (Baron 1993). Mesenchymal cells at the periphery keep differentiating and follow the same steps, hence intramembranous bone only develops by apposition. The bone marrow is formed by incorporation of blood vessels between the bony trabeculae. This woven bone is later remodelled (see below) and replaced by lamellar bone.

1.4.1.2 Endochondral ossification

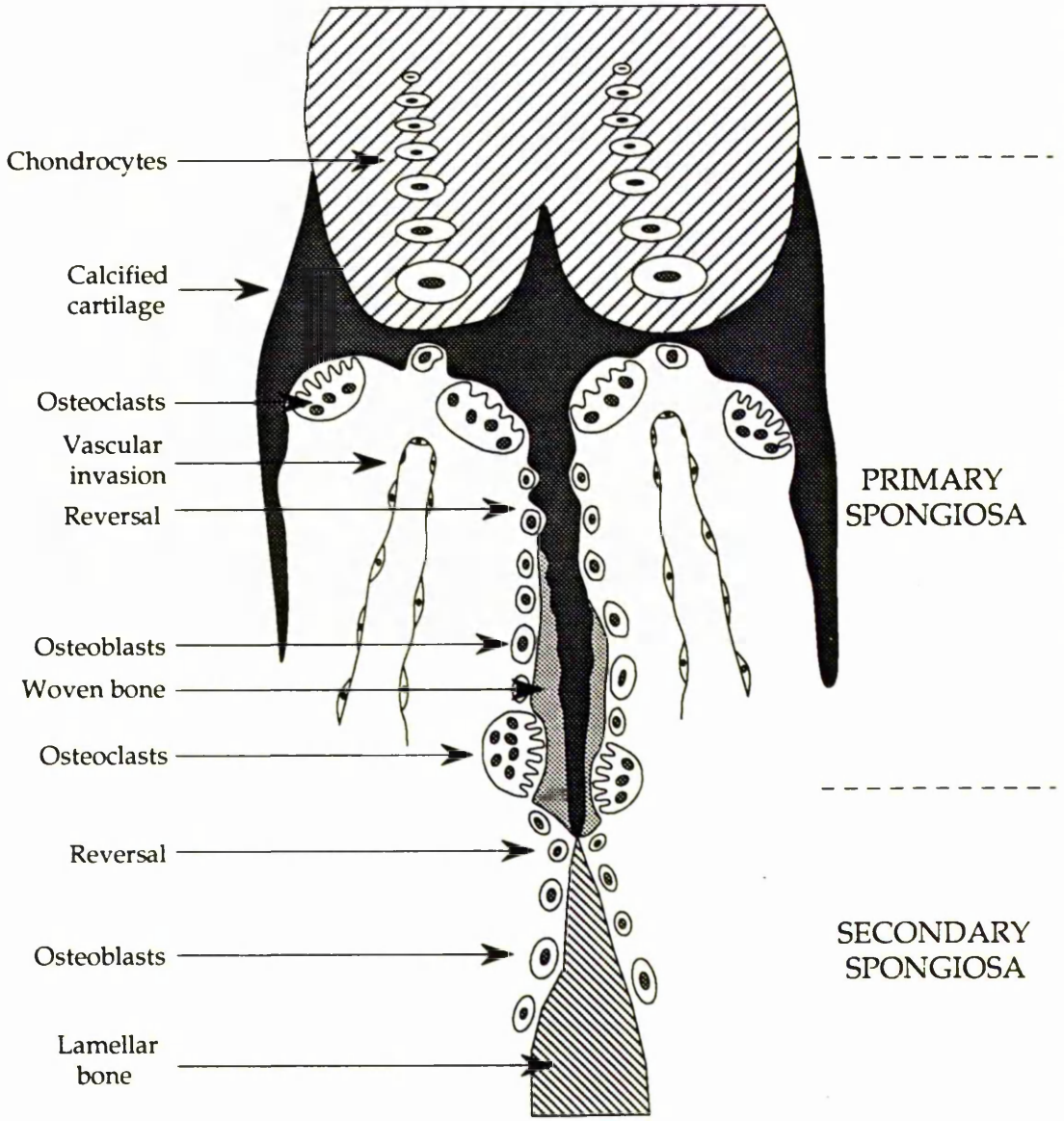
Endochondral ossification occurs in long bones, where the mesenchymal precursor cells differentiate into prechondroblasts and chondroblasts (Teitelbaum 1990; Baron 1993). Cartilage can thus grow by apposition, by proliferation of the mesenchymal cells, but cartilage can also grow interstitially, as the chondroblasts and chondrocytes can secrete their

own matrix (rich in type II collagen and mucopolysaccharides) to increase the cartilage mass. The transformation of cartilage to bone occurs within ossification centres within the cartilage model, and at the perichondrium. As longitudinal growth occurs, the growth plate cartilage (also known as the physis) forms (see Figure 1.1). This consists of regular columns of cells (called isogenous groups) which consist of four distinct anatomical zones (Siffert 1966; Brighton 1978):

1. Zone of resting cartilage – this is firmly adherent to the overlying epiphyseal bone, and consists of mesenchymal cells that have yet to undergo proliferation.
2. Zone of proliferating cartilage – this is characterised by vertical columns of actively replicating chondrocytes, which actively synthesise matrix and expand the physis interstitially until they enter the:
3. Zone of hypertrophic cartilage – in this zone, the chondrocytes enlarge and start to degenerate. The oxygen tension is lowest in this zone and cellular metabolism is anaerobic (Brighton and Heppenstall 1971). Alkaline phosphatase is produced by the hypertrophic cells closest to the metaphysis, and it is thought that this, along with the invasion of vasculature, promotes formation of the:
4. Zone of calcified cartilage – the matrix of the longitudinal cartilage septa calcifies, and this calcified matrix serves as a framework for the initial deposition of bone in the primary spongiosa (see Figure 1.8). The hypertrophic chondrocytes eventually become highly vacuolated and die. This mineralised cartilage is eventually resorbed by osteoclasts, which are accompanied by invading blood vessels. Osteoblasts differentiate and form a layer of woven bone covering the cartilaginous remnants. This woven bone is resorbed, along with the calcified cartilage, and replaced by lamellar bone, to form the metaphyseal trabeculae which make up the secondary spongiosa (see Figure 1.8).

As the physis expands by interstitial growth, it is replace by bone on the metaphyseal side, thus producing longitudinal bone growth. Growth is terminated by cessation of interstitial growth of the physis, which eventually becomes completely replaced by bone (Siffert 1966; Brighton 1978).

Figure 1.8 Bone remodelling and growth at the physis



Diagrammatic representation of the cellular events occurring at the growth plate in long bones.
Adapted from Baron 1993

Growth in the diameter of long bones occurs by the formation of new membranous bone beneath the periosteum. The metaphysis is wider than the midshaft, which is consequently continuously resorbed by osteoclasts beneath the endosteum as the bone grows. The

osteoclasts also progressively destroy the lower part of the metaphysis, transforming it into the diaphysis (Baron 1993).

1.4.2 Bone Modelling, Remodelling and Repair

Longitudinal growth and modelling of bone only occur in the growing skeleton, and are therefore not seen following closure of the growth plates (Teitelbaum 1990). As skeletal development requires bone formation at some surfaces and resorption at others, growth and modelling are characterised by dissociation of osteoblastic and osteoclastic activity. Remodelling and repair, however, occur throughout life (Teitelbaum 1990). Repair relates to fracture healing. Remodelling occurs as a turnover mechanism, replacing old bone with new, and also serves to control mineral homeostasis (Teitelbaum 1990; Baron 1993). In the normal adult skeleton, bone formation only occurs after, and is intimately linked to, bone resorption, a process known as "coupling" of the activities of osteoclasts and osteoblasts (Frost 1969). The sequence of events at the remodelling site is known as the ARF sequence (Activation-Resorption-Formation) (reviewed by Baron 1993). The sequence is initiated by recruitment of osteoclasts to a targeted site on the bone surface. The osteoclasts resorb bone, producing Howship's lacunae, and then depart, to be replaced by osteoblasts. In young individuals, the osteoblasts form an equal amount of bone as that which was resorbed. In elderly individuals, the amount of bone formed is slightly less than that removed, and this contributes to the bone loss of ageing. The period between resorption and formation is known as the reversal phase (Baron 1993).

1.5 Culture Systems for Examining Bone Cell Biology

Many systems have been developed for studying the biology of both osteoblasts and osteoclasts. Unfortunately, none of the systems completely mimic the *in vivo* situation, hence, often conflicting evidence is obtained. Another major problem with many of the *in vitro* systems is that, whilst attempts are made to obtain homogeneous cell lines or populations, contamination with other cell types is often, if not always, the outcome. Isolated cell systems

have been developed, but again, the problem with these is that they do not mimic the complete situation *in vivo*.

1.5.1 Osteoblasts

One of the first methods used to isolate osteoblasts was by sequential enzymatic digestion of flat bones (Wong and Cohn 1975). However, none of the cell populations studied is consistent, using morphological or histochemical data (Wong 1982; Burger et al 1986). Also, the effects of the enzymes used have not been adequately determined, particularly with reference to e.g. membrane receptors.

Osteoblast-like cell lines have been established from osteosarcomas (Majeska and Rodan 1982; Ibbotson et al 1986). However, the exact developmental stage of cells in many of these systems has not been satisfactorily established, although some cultures, particularly those in second or third passage, have a majority of cells expressing osteoblast phenotypes (Ecarot-Charrier et al 1983; Gerstenfeld et al 1987).

Osteoblast-like cells have also been cultured by outgrowth of cells from bone explants (Beresford et al 1984). Cells can be harvested at intervals and their biology examined. As with some of the osteosarcomatous culture systems, the main problems with cells grown from explants is their exact identification as osteoblastic, and their differentiation stage. The cells appear grossly as more fibroblast-like, although in later cultures (i.e. those derived from cells that grew after the first few harvests) the cells do express a more stable osteoblastic phenotype. As with all the systems, however, there is a problem with possible contamination.

1.5.2 Osteoclasts

Before osteoclasts could be reproducibly isolated, macrophages and foreign body giant cells were used, and related to the biology of osteoclasts (Mundy et al 1977; Kahn et al 1978; Teitelbaum et al 1979). However, numerous differences between these types of cell and osteoclasts have been demonstrated (Shapiro et al 1979; Hogg et al 1980; Chambers and

Magnus 1982; Horton et al 1984) and the validity of the results obtained from these experiments is now questionable. However, human leukaemic (HL-60) cells have recently been shown to be capable of differentiating into multinucleated osteoclast-like cells which can resorb bone (Yoneda et al 1991; 1993).

Primary cultures of osteoclasts are most readily obtained from avian sources (Zambonin-Zallone et al 1984; Nijweide et al 1985; Oursler et al 1985). However, the failure of isolated avian osteoclasts to respond to CT (Arnett and Dempster 1987; Nicholson et al 1987), has diminished their usefulness for studying mammalian osteoclasts. Mammalian osteoclasts can be isolated, and the ability of individual cells to resorb bone slices has been investigated (Chambers et al 1984a). Contaminating cells can be kept to a minimum to give reproducible responses to bone resorbing agents (Chambers et al 1985a, 1985b), although very few cells remain viable after isolation.

Bone marrow cultures from various species, including dogs, have been used extensively by some investigators to examine the regulation of osteoclast development (Testa et al 1981; Ibbotson et al 1984; Roodman et al 1985; MacDonald et al 1986, 1987; Takahashi et al 1986a, 1986b, 1987, 1988b; Seed et al 1988; Bird et al 1992). However, the difficulty of distinguishing osteoclasts from macrophage polykaryons in these systems has led to their usefulness being questioned (reviewed by Marks and Popoff 1988; Chambers and Hall 1991; Zaidi et al 1993). Contamination with other cells is also a major problem with bone marrow cultures.

1.5.3 Co-culture Systems and Living Organisms

A variation of the isolated cell systems has been to use co-cultures of osteoblasts and osteoclasts to examine their interactions (McSheehy and Chambers 1986a, 1986b; Thomson et al 1986; Nicholson et al 1989). Whilst these systems are better than using isolated cells, they still do not completely mimic the complex situation of the *in vivo* skeletal environment.

Organ cultures more closely resemble the *in vivo* situation, but it is sometimes difficult to pinpoint the cell type(s) involved in bringing about certain responses. There are three major

types of organ cultures that have been used; foetal long bones (Raisz and Niemann 1967), neonatal calvaria (Goldhaber 1958, 1960; Reynolds and Dingle 1970) and foetal metatarsals (Burger et al 1982, 1984). In the first two types, the net effect in culture is bone resorption, whereas *in vivo*, these areas would be undergoing net growth. The latter system has proved more useful, as the skeletal components of the foetal mouse hindpaw develop late, and osteoclast precursors arrive late at that site. Thus, if the periosteum is stripped from these bones at 17 days, osteoclasts do not develop. However, the stripped metatarsals can serve as inducers of osteoclast development and activation, if precursor cells are provided (Burger et al 1982, 1984; Scheven et al 1986; Thesingh 1986).

Living organisms have also been used to examine skeletal biology. However, as with organ cultures, it is difficult to ascribe effects to a definite cell type. Nevertheless, recent experiments with transgenic mice have proved useful in examining the role of the proto-oncogene c-*fos* in regulating bone cell function. Overexpression of c-*fos* in transgenic mice leads to increased bone resorption (Ruther et al 1987), and down-regulation of c-*fos* via gene knock-out leads to osteopetrosis (Johnson et al 1992; Wang et al 1992). A similar effect is seen if c-*src* is disrupted (Soriano et al 1991). Expression of a human T lymphotropic virus-1 long terminal repeat-tax (HTLV-1 LTR) transgene in mice also induces osteoclastic activity (Ruddle et al 1993). The various effects of these proto-oncogenes and tax are described and discussed in more detail in Section 3.3.

Thus, it seems that whole organisms might give useful information, if the exact effects, and the target cells, can be accurately determined.

Chapter 2

The Paramyxoviridae

2. The Paramyxoviridae

2.1 Introduction

Paramyxoviruses are enveloped, single stranded RNA viruses, with helical nucleocapsid symmetry (Kingsbury 1990). They are very pleomorphic, but usually spherical or globular, although filamentous forms also occur (Hosaka et al 1966, Galinski and Wechsler 1991). The helical nucleocapsid is enclosed in a lipid containing envelope which is studded with two types of glycoprotein projections, one with cell fusion activity and another with haemagglutination (HA) and neuraminidase (NA) activities in some viruses of the family (Galinski and Wechsler 1991). Another important biological property of these viruses is their ability to cause persistent infection upon passage in cell culture (Galinski and Wechsler 1991).

They are classified into three genera based on antigenic cross reactivities among the members of each genus and on the presence or absence of HA and NA activity (Matthews 1982):

1. Paramyxoviruses agglutinate mammalian and avian erythrocytes and have NA activity.
2. Morbilliviruses haemagglutinate but lack NA activity.
3. Pneumoviruses exhibit neither HA nor NA activities.

Paramyxoviruses are found in a wide variety of warm blooded vertebrate species, both wild and domesticated (see Table 2.1).

2.2 The Virion

The viral messenger RNAs are complementary to the virion RNA. Hence, the paramyxoviruses are called "negative-strand" viruses, as viral message is conventionally designated as positive (Kingsbury 1990). As is typical of negative-strand RNA viruses, the virion is made up of an internal ribonucleoprotein core (the nucleocapsid) and an outer

lipoprotein envelope (Kingsbury 1990) (see Figure 2.1). The envelope consists of a lipid bilayer, associated with a non-glycosylated protein and covered with glycoprotein spikes projecting from the outer surface. These outer spikes mediate virus attachment and penetration.

Table 2.1 Genera and some species of Paramyxoviridae

Genus	Species	Main Host
Paramyxovirus	Sendai virus (murine parainfluenza virus type 1)	Mouse
	Human parainfluenza viruses types 1-4	Human
	Mumps virus	Human
	Newcastle disease virus	Chicken
	Simian virus 5 (canine parainfluenza virus type 2)	Dog
Morbillivirus	Measles virus	Human
	Canine distemper virus	Dog
	Rinderpest virus	Cow
Pneumovirus	Respiratory syncytial virus	Human
	Bovine respiratory syncytial virus	Cow

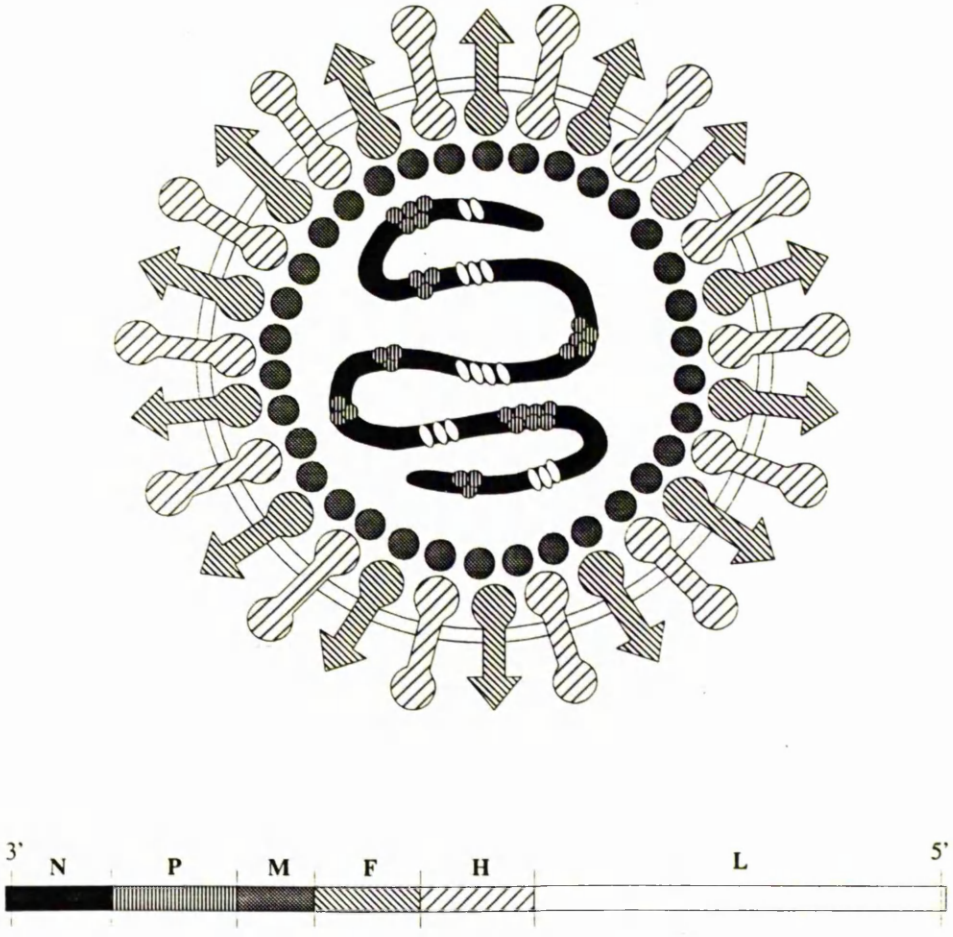
Adapted from Choppin and Compans (1975) and Kingsbury (1990)

Paramyxovirions are generally 150-300 nm in diameter (Kingsbury 1990, Galinski and Wechsler 1991), although filamentous forms upto several micrometres in length and larger virus particles are sometimes seen (Hosaka et al 1966). This reflects the relative lack of stringency in the budding stage of the virus assembly process (Kingsbury 1990), so that some particles contain the equivalent of two or more genomes (polyploidy) (Hosaka et al 1966, Kingsbury and Granoff 1970).

2.3 The Paramyxovirus Genome

The genome consists of a linear molecule of non-segmented, negative sense, single stranded (ss) RNA with molecular weight of about $5x10^6$ (approximately 15000 nucleotides) (Galinski and Wechsler 1991). Each genome occupies a single nucleocapsid and the RNA contains a set of six or more genes, linked covalently in tandem (see Figure 2.1).

Figure 2.1 Diagrammatic representation and genetic map of a typical Paramyxovirion



Genetic map of a Morbillivirus. N–nucleocapsid protein gene, P–phosphoprotein gene, M–matrix protein gene, F–fusion protein gene, H–haemagglutinin protein gene, L–large protein gene. Adapted from Kingsbury (1990)

2.3.1 The Nucleocapsid

The nucleocapsid comprises a single piece of RNA associated with many protein subunits in a rod shaped structure with helical symmetry (Kingsbury 1990). There are three nucleocapsid proteins:

1. The major nucleocapsid protein is the structural protein, referred to as N, NP or NC. It is phosphorylated and highly susceptible to proteolytic enzymes.
2. The very large L protein acts together with the smaller P (phosphoprotein) to synthesise RNA.
3. The P protein. Neither the L nor the P protein is capable individually of carrying out RNA synthesis.

2.3.2 The Envelope

The envelope is a modified piece of cell membrane and is composed of lipids and proteins (Kingsbury 1990). The lipids are essentially the same as those found in cell membranes of uninfected host cells, but the three proteins are derived from the virus (Galinski and Wechsler 1991):

1. The large attachment protein (H) causes haemagglutination in the Morbillivirus and Paramyxovirus genera. In the latter, it also has neuraminidase activity, hence it is termed HN (haemagglutinin-neuraminidase). In Pneumoviruses, the corresponding attachment protein lacks both haemagglutination and neuraminidase activity and has therefore been designated G (glycoprotein).
2. The fusion protein (F), as its name suggests, causes fusion of the virus envelope with the surface membrane of the host cell. It occurs in the form of either a continuous molecule (the inactive precursor known as F_0), or two products of proteolytic cleavage (referred to as F_1 and F_2) which are linked by disulphide bridges to produce the active form (Scheid and Choppin 1974, 1977).

3. The non-glycosylated membrane, or matrix (M), protein plays a role in nucleocapsid-envelope recognition during viral assembly and it may also participate in the formation of the envelope.

2.3.3 Non-structural Proteins

Several small proteins have been found encoded within the genome of paramyxoviruses e.g. the C and V proteins (encoded by the P gene) found in Paramyxoviruses and Morbilliviruses (Etkind et al 1980; Portner et al 1986; Norrby and Oxman 1990; Barrett et al 1991). They vary among the virus species and are of unknown function.

2.4 Replication

Viral replication is confined to the cytoplasm of the host cells (see Figure 2.2). The replication cycle can be sub-divided into several different stages (Choppin and Compans 1975; Kingsbury 1990):

2.4.1 Attachment and Penetration

The HN, H or G proteins mediate viral attachment and this is then followed by fusion of the virion envelope with the cell membrane, allowing delivery of the infective nucleocapsid into the cell (Choppin and Scheid 1980). The fusion process is dependent on the cleavage of F_0 to F_1 and F_2 (see Section 2.3.2).

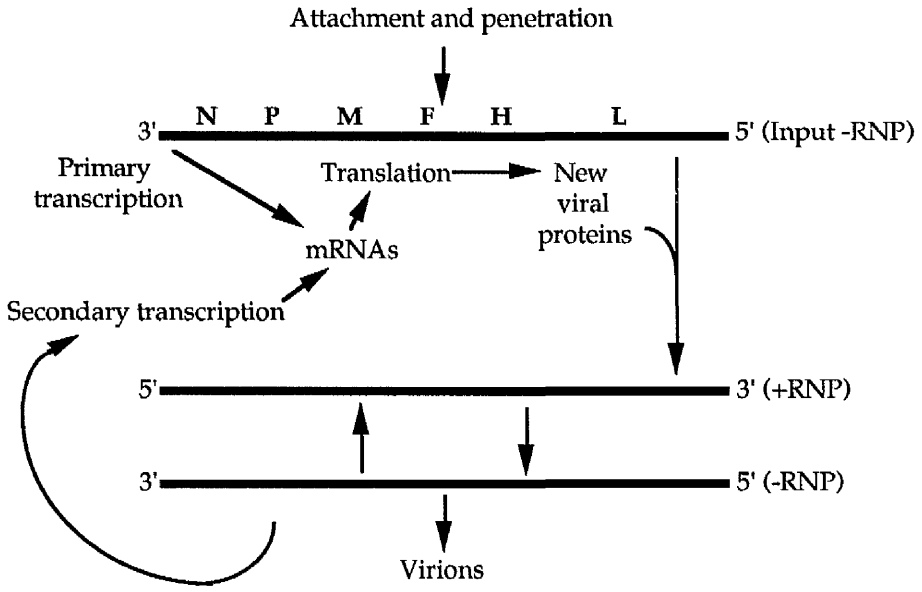
2.4.2 Transcription and Translation

As the base sequence of the genomic RNA is negative sense, it must first be transcribed before translation can occur (Kingsbury 1966; Bratt and Robinson 1967; Blair and Robinson 1968). The nucleocapsid acts as a template and, along with the proteins N, P and L, can transcribe the mRNA (Stone et al 1972; Marx et al 1974; Emerson and Yu 1975). Primary transcription takes place under the control of an RNA-dependent RNA polymerase whose activity is due to the L and P proteins.

During infection, each gene is coded for by a separate mRNA species. The mechanism for this is at the transcriptional level, by initiation and termination of RNA synthesis at specific consensus nucleotide sequences which are located at the gene boundaries (Gupta and Kingsbury 1985).

There is also transcriptional regulation of mRNA numbers governed by gene order, along with an attenuating effect of the transcriptional regulatory sequences (Kingsbury 1990; Norrby and Oxman 1990; Pringle 1991). Transcriptional efficiency declines with distance from the 3′ end of the genome. This "polarity" is a regulatory mechanism which determines the number of viral proteins, e.g. each nucleocapsid contains approximately 100-fold more N proteins than L proteins (Kingsbury 1990; Norrby and Oxman 1990); the relative numbers of the other virus proteins reflect their positions in the genome.

Figure 2.2 The major steps in paramyxovirus replication



RNP – Ribonucleoprotein; (-) – Negative-strand; (+) – Positive-strand.
Viral replication is confined to the cytoplasm of the host cell, no provirus is formed, and there is no integration into host cell DNA. Adapted from Kingsbury (1990)

Viral mRNAs bind to ribosomes and are translated into protein in the same way as cell mRNAs (Kingsbury 1990).

2.4.3 Replication of Viral RNA

During viral replication, the entire base sequence of the negative-strand RNA is conserved as a single template (Kingsbury 1990). Hence, the sequences that are not expressed during mRNA synthesis are incorporated into the positive-strand RNA (antigenome) which acts as a template for negative-strand synthesis. Therefore, the RNA synthesis machinery enters an antitermination mode, ignoring the signals at the gene boundaries (Kingsbury 1990; Galinski and Wechsler 1991). The exact mechanisms of this switch from mRNA synthesis to replication are not known, however it is thought that it involves the accumulation of a critical concentration of nucleocapsid structure units, which changes the way the RNA polymerase copies its template (Patton et al 1983; Carlsen et al 1985).

2.4.4 Assembly and Release

The initial step in nucleocapsid assembly involves combination of the N structure units with genomic (or, sometimes, antigenomic) RNA. This takes place in the cytoplasm and is followed by addition of the auxiliary nucleocapsid proteins (P and L) to this ribonucleoprotein template (Kingsbury et al 1978).

Assembly of the envelope occurs at the cell surface. Virus glycoproteins (HN/H and F) replace most of the normal cellular proteins in the plasma membrane (Kingsbury 1990; Wiley and Skehel 1990). This is followed by accumulation of M-protein molecules on the inner surface of the membrane. Nucleocapsids arrive and virions are formed by budding from the cell membrane (Kingsbury 1990; Wiley and Skehel 1990).

As budding takes place, the cell membrane surface becomes studded with viral glycoprotein spikes. Trypsin-like proteases, if present, will activate F_0 molecules present on the surface and this can cause fusion with associated cell membranes (Gallaher and Bratt 1974). Replicating virus genomes are thus able to spread from cell to cell, largely avoiding

circulating anti-viral antibodies. Fusion can take place between a large number of cells, leading to the formation of large multinucleated cells (syncytia) (Kingsbury 1990).

As a consequence of replication, intracytoplasmic inclusion bodies (consisting of N and possibly P, M and L proteins) form (Norrby and Oxman 1990). Morbilliviruses also cause the formation of intranuclear inclusion bodies (Norrby and Oxman 1990). The precise nature and function of the intranuclear inclusions is unclear, as there is no nuclear component to paramyxoviral replication (Kingsbury 1990). However, recent work with canine distemper virus has suggested that these inclusions consist of N protein associated with nucleolar derivatives known as nuclear bodies (Oglesbee 1992; Oglesbee and Krakowka 1993).

2.5 Paramyxovirus Persistence

Viral persistence can be defined as anything ranging from a simple low-grade infection, where infectious virus is continuously produced (such as lymphocytic choriomeningitis in mice and hepatitis B in humans) (Buchmeier et al 1980; Marion and Robinson 1983), to infections where the virus genome is present, but infectious virus is not produced, and the persistent virus genome may be defective in some way (Randall and Russell 1991). This latter type of persistence is different from latent infections, such as herpes virus infection, where reactivation of virus replication can occur (Roizman and Sears 1987).

The ability of paramyxoviruses to persist *in vitro* has been long established (Ahmed and Stevens 1990; Randall and Russell 1991), and it is now becoming clear that they can also persist *in vivo*. The mechanisms of paramyxovirus persistence are complex, and vary between different viruses of the family. However, several mechanisms are thought to play a general role in the establishment of persistent infections.

2.5.1 Mechanisms of Viral Persistence

There are two main mechanisms by which viruses can persist in tissues:

2.5.1.1 Regulation of cell lysis

For a virus to persist, there must be a critical number of infected cells (Oldstone 1989; Ahmed and Stevens 1990). Therefore, the virus must persist without rapidly damaging or destroying the host cells. For viruses that do not normally cause cell lysis, this is easily achieved. However, for those viruses which normally cause cell death, such as the paramyxoviruses, some regulation of this lytic potential must occur. To do this, these viruses can either regulate their gene expression, or generate variants (mutants) that are less lytic, or that interfere with the growth of the "normal" lytic virus. For example, there are temperature sensitive mutants of various paramyxoviruses, including Newcastle disease virus (Tsipis and Bratt 1976), Sendai virus (Portner et al 1974) and measles virus (Yamazi and Black 1972; Bergholz et al 1975). Plaque morphology mutants have also been described, including CDV (Cosby et al 1981, 1985). Defective interfering (DI) viruses spontaneously arise during infections, due to errors of the polymerase complex. These DI viruses are preferentially amplified and interfere in the replication of normal, nondefective, genomes, thereby favouring the establishment of persistent infections (Perrault 1981). DI paramyxoviruses have been commonly described, including MV (Morgan and Rapp 1977) and CDV (Tobler and Imagawa 1984). Subacute sclerosing panencephalitis (SSPE) is a chronic neurological disease resulting from MV persistence in the CNS, and is described in detail in Section 2.6.4. Gene-specific hypermutation is seen in MV isolates from some cases of SSPE (Cattaneo et al 1988a).

2.5.1.2 Evasion of the immune response

There are two main mechanisms by which the immune system can control viral infections (Whitton and Oldstone 1990): cytotoxic T lymphocytes, which destroy viral antigens associated with the major histocompatibility complex proteins (i.e. cell-associated virus),

and antibodies (often acting with complement), which can recognise both virally infected cells and free virus particles. There are many ways in which viruses can evade these two mechanisms.

One suggested mechanism is a reduction in the amount of virally-encoded antigens expressed on the cell surface (Oldstone and Fujinami 1982). This would decrease the risk of an immune response, and is thought to be due to natural selection of cells in which the virus only synthesises reduced amounts of viral proteins, possibly due to defects in the genome.

Another mechanism of viral persistence is that of antibody-induced antigenic modulation (Joseph and Oldstone 1974, 1975; Oldstone and Tishon 1978; Oldstone and Fujinami 1982; Fujinami and Oldstone 1984). The suggestion is that the actions of the immune system keep the levels of surface viral antigens below a critical point required for cell lysis by continuously removing the viral glycoproteins. That is, antibodies bind and remove the proteins from the cell surface, but there is insufficient antibody to cause cell lysis. The infected cells would still produce other viral proteins, and these could affect the physiological status of the cell (Oldstone and Fujinami 1982). Support for this theory has come from *in vitro* studies which have shown that the addition of measles-specific antibody, in the absence of complement, leads to a capping and rearrangement of viral proteins (Fujinami and Oldstone 1979, 1980). These proteins are then shed from the cell surface as antigen-antibody complexes. In addition, the levels of other intracellular viral proteins are also reduced (Fujinami and Oldstone 1979, 1980), and this may increase the possibility of a persistent infection (see Section 2.6.4.6). The possibility that this mechanism may also be involved *in vivo* has been demonstrated. In new-born hamsters, the presence of maternal neutralising antibody to measles virus (MV) protects against acute encephalitis following intra-cerebral inoculation of virus, but allows the development of a persistent MV infection in the CNS (Wear and Rapp 1971). Also, in monkeys, it has been shown that a persistent infection is only established in those monkeys with pre-existing anti-MV antibodies, and not in non-immune animals (Albrecht et al 1977). It has also been suggested that this effect may result from the presence of cross-reacting antibodies against

other viruses, resulting in a persistent infection without any specific antibody response to that virus (Russell and Goswami 1984).

Cell-mediated immunity is also important in the pathogenesis of persistent paramyxovirus infections. It has been shown, for example, that $CD8^+$ cytotoxic T cells are required to eliminate persistent MV infections in rats (Maehlen et al 1989). It has also been shown that MV can alter the functions of T cells, and possibly lead to persistence (McChesney and Oldstone 1987). Patients with SSPE have a history of recovering from an initial infection with MV, and have normal immune responses to other infectious agents (Blaese and Hofstrand 1975; Sell and Ahmed 1975). It appears, therefore, that these patients develop an impaired T-cell response to MV (Dhib-Jalbut et al 1989). The mechanisms of this are unknown, but it has been suggested that an inappropriate T suppresser cell activity might induce tolerance to MV (Oldstone 1989), or that persistent cells are naturally selected by the immune response, as they contain defective genomes in which the mutations are such that the immune response no longer recognises the target antigens (Randall and Russell 1991).

Paramyxoviruses can establish persistent infections in sites other than those initially involved in the infection. To do this, they must be transported to these sites of persistence, and this is usually achieved by infection of circulating lymphoid cells. Both measles and canine distemper virus can infect and replicate in lymphocytes and macrophages (Joseph et al 1975; Sullivan et al 1975; Appel 1987). Measles virus has also been isolated from lymphocytes of patients with SSPE (Horta-Barbosa et al 1971). Simian virus 5 (SV5) has also been isolated from the peripheral lymphocytes of a patient with SSPE (Robbins et al 1981), and this virus has also been found in the bone marrow cells of patients with multiple sclerosis (Goswami et al 1984). Interestingly, the latter study also found SV5 in 25% of control patients, and found that parainfluenza virus types 1 and 3 were present in the bone marrow cells of 50% of controls and 25% of the multiple sclerosis patients. These results have led to the suggestion that several of the paramyxoviruses may persist in human

tissues, and that they might cause widespread, though clinically inapparent, infections (Robbins et al 1981; Randall and Russell 1991).

2.6 Measles Virus

2.6.1 Introduction

Measles is a highly contagious, acute viral infection which is widespread throughout the world (Robbins 1962). The use of vaccines has substantially reduced the prevalence of the disease, but, in third world countries, there are still many deaths due to measles (Assaad 1983; Norrby and Oxman 1990). It is thought that the virus originated some time around the second century, possibly by adaptation of closely related animal viruses such as rinderpest (McNeill 1976; Norrby et al 1985a; Norrby and Oxman 1990).

Measles virus (MV) belongs to the genus Morbillivirus, in the family Paramyxoviridae (Kingsbury 1990). This genus includes three other important viruses, canine distemper (CDV), peste des petits ruminants (PPRV), and rinderpest (RV). All four are closely related immunologically, and it is thought that rinderpest is the oldest member of the genus (Imagawa 1968; Stephenson and ter Meulen 1979; Hall et al 1980; Norrby et al 1985a; Rozenblatt et al 1985; Sheshberadaran et al 1986). Each virus has only one serotype, although minor epitopic variations have been demonstrated between different strains of MV and CDV (Orvell and Norrby 1980; Sheshberadaran et al 1983; Orvell et al 1985; Appel 1987; Giraudon et al 1988; Taylor et al 1991). Morbilliviruses do not exhibit neuraminidase activity (see Section 2.1) and, as part of their cytopathology, they cause the formation of intranuclear inclusion bodies (composed of nucleocapsid protein associated with nuclear bodies) as well as intracytoplasmic inclusions (Appel 1987; Kingsbury 1990; Norrby and Oxman 1990; Oglesbee 1992; Oglesbee and Krakowka 1993) (see Section 2.4.4.).

Humans are the only natural host for measles, although the virus has been studied experimentally in monkeys, dogs, calves, ferrets and rodents (Frazer and Martin 1978; Albrecht et al 1980; Appel et al 1981). The rodents, in which acute encephalitis is readily

established, have been most extensively used. Establishment of persistent infections requires special conditions, which include, in monkeys and ferrets, the use of cell-associated SSPE virus injected intracerebrally into animals with preinoculation passive immunity (Albrecht et al 1977; Thormar et al 1977). In rodents, the establishment of persistent infections depends on the properties of the virus inoculated, the strain of animals used and the age of animals at the time of injection (Janda et al 1971; Neighbour et al 1978; Carrigan 1986a, 1986b; Norrby and Oxman 1990).

2.6.2 Epidemiology and Pathogenesis

After infection with wild type measles, antibody titres can be measured throughout life, even without re-exposure to the virus (Brown et al 1969; Black 1989; Norrby and Oxman 1990). Maternal antibodies protect against disease for approximately 6 months (sometimes longer), and this can interfere with vaccination programmes (Black 1989; Norrby and Oxman 1990).

In developed countries, if vaccines are not widely used, most measles cases occur between 5 and 9 years of age, with over 95% of cases occurring before 15 years of age (Norrby and Oxman 1990). In developing countries, measles occurs much earlier; in some areas of Africa, more than 50% of children are infected at less than 2 years of age, and 100% by the age of 5 (Assaad 1983; Lamb 1988; Black 1989; Norrby and Oxman 1990). This appears to be due to a more rapid loss of maternal antibody, social practices, family size, and prolonged shedding of virus by malnourished children (Hull 1981; Assaad 1983; Aaby 1988; Aaby et al 1988; Pison and Bonneuil 1988; Norrby and Oxman 1990).

If measles is introduced into a population with no previous exposure, all age groups are affected, and the severity of disease is increased (Beck 1962; Neel et al 1970; Black 1989).

Measles virus has no animal reservoirs, and is usually spread by face to face contact via droplets, although airborne transmission and transmission by fomites can also occur (Black 1989; Chen et al 1989; Norrby and Oxman 1990). The virus is shed in respiratory secretions and the urine, and shedding is prolonged in immunosuppressed and malnourished

individuals (Aaby 1988; Black 1989; Norrby and Oxman 1990). Affected individuals are infectious from the onset of symptoms to the fourth day of rash (see Section 2.6.3), with the most infectious period occurring just prior to the appearance of the rash (Black 1989; Norrby and Oxman 1990).

Although both humoral and cell-mediated immunity can prevent infection in normal individuals, cell-mediated immunity is needed to clear an established measles virus infection (Norrby and Oxman 1990). Cytotoxic T cells have been found in peripheral blood samples from measles patients and following *in vitro* culturing of peripheral blood lymphocytes (Kreth et al 1979; Wright and Levy 1979). The cytotoxic T cells show specificity for both surface and internal viral antigens (Jacobson et al 1987). Long term immunity requires antibodies to both the fusion and haemagglutinin proteins (Norrby 1975, Norrby et al 1975).

The primary mode of transmission of measles virus is via aerosols, which are produced by talking, coughing and sneezing (Black 1989; Norrby and Oxman 1990). This leads to infection of the epithelial cells of the conjunctivae, oropharynx or respiratory tract (Robbins 1962; Hall et al 1971). Following 2 to 4 days of replication in the respiratory epithelia, the virus then spreads, probably in pulmonary macrophages, to drainage lymph nodes, where further replication occurs (Robbins 1962; Hall et al 1971). Virus then enters the blood stream, where it is carried in leucocytes to reticuloendothelial sites throughout the body, producing a primary viraemia (Joseph et al 1975; Frazer and Martin 1978). Viral replication at these sites is clinically inapparent, although it does produce lymphoid hyperplasia and multinucleated giant cell formation, and a secondary viraemia which spreads the virus, via lymphocytes and monocytes, to tissues throughout the body (Hathaway 1935; Corbett 1945). This viral replication, coupled with the host immune response, produces clinical signs approximately 8 to 12 days after infection. When clinical signs appear, the virus is widely spread throughout the lymphoid and epithelial cells of the body, and one of the most striking features of this infection (also seen *in vitro*) is the appearance of multinucleated giant cells, which are formed by fusion of infected cells with

uninfected cells and other infected cells (Archibald et al 1971; Hall et al 1971; Norrby and Oxman 1990).

2.6.3 Clinical Signs

The initial infection and incubation period are not usually apparent clinically, although mild respiratory symptoms have been reported following infection in some patients (Robbins 1962; Norrby and Oxman 1990). In association with the secondary viraemia, there is often a leucopenia (affecting mainly the lymphocytes) (Benjamin and Ward 1932). Inflammation of the lymphoid tissues produces a generalised lymphadenopathy and mild splenomegaly (Babbott and Gordon 1954).

Following the incubation period of about 8-12 days, symptoms of pyrexia, malaise, coryza, coughing and conjunctivitis are seen. The cough and coryza result from an intense inflammatory reaction that involves most of the respiratory tract. These symptoms are followed a few days later by the appearance of a rash, which reaches a peak on about the fifth day of symptoms (Norrby and Oxman 1990). Pulmonary infiltrates are commonly found in association with the appearance of the rash (Babbott and Gordon 1954). The first rash lesions appear as 3-4 mm diameter macropapules around the ears and on the forehead and upper parts of the neck (Christie 1974). The rash then spreads to the rest of the body and reaches the feet about three days later. The rash usually starts to fade approximately 3 days after appearing, hence the lesions on the face fade as those on the feet appear (Christie 1974).

Two or three days prior to the appearance of the rash, Koplik's spots appear on the buccal mucosa (Koplik 1896). These are small, bright red spots with a bluish-white centre. They increase in number to cover the whole of the buccal and labial mucosa by the first day of the rash. The red areas then coalesce and slough to leave a normal appearance to the mucosa on about the third day of the rash (Koplik 1896).

The pyrexia increases until about the fourth day of the rash, and then the temperature usually returns to normal (Christie 1974).

Complications of measles virus infection include secondary bacterial infections, progression and extension of viral replication and abnormal immune responses (Norrby and Oxman 1990). These can lead to otitis media (affecting 5-9% of cases) (Preblud and Katz 1988), pneumonia (affecting up to 7% of cases) (Gremillion and Crawford 1981; Preblud and Katz 1988) and an immunopathological response (known as atypical measles syndrome), caused by exposure to wild type measles 2-4 years following vaccination with inactivated measles vaccine (Fulginiti et al 1967; Nader et al 1968; Hall and Hall 1979; Frey and Krugman 1981).

In humans, there are three pathologically distinct forms of encephalitis associated with measles infection:

1. Acute postinfectious encephalitis – the most common neurological complication, usually developing during the first week following the onset of the rash (Miller et al 1956). The neurological signs are variable and 10-20% of affected individuals die. It is thought that the disease results from autoimmune demyelination, rather than direct infection of the brain with measles (Norrby and Oxman 1990).
2. Acute progressive infectious encephalitis – also known as measles inclusion body encephalitis (MIBE), this occurs in immunosuppressed patients, producing an acute or subacute fatal encephalitis (Breitfield et al 1973; Aicardi et al 1977; Wolinsky et al 1977).
3. SSPE – a rare, late complication of measles infection, is described in more detail in Section 2.6.4.

2.6.4 Subacute Sclerosing Panencephalitis

2.6.4.1 Introduction

SSPE is a slowly progressing, fatal disease of the CNS, affecting children and young adults (reviewed by Swoveland and Johnson 1989). The first thorough description of the disease, which was then termed lethargic encephalitis, reported the presence of intracytoplasmic and intranuclear eosinophilic inclusion bodies, primarily in the grey matter (Dawson 1933). A similar condition affecting primarily the white matter, called subacute sclerosing leukoencephalopathy, was also described (Van Bogaert 1945), and it was five years later that the two conditions were recognised as one disease, which was termed SSPE (Greenfield 1950). The inclusions have since been studied electron microscopically and found to resemble paramyxovirus inclusion bodies (Bouteille et al 1965; Tellez-Nagel and Harter 1966; Dayan et al 1967; Herndon and Rubinstein 1967). Further evidence to support a paramyxoviral aetiology came when elevated levels of anti-measles virus antibodies were detected in the serum and cerebrospinal fluid (CSF) of SSPE patients (Connolly et al 1967), and immunofluorescence studies demonstrated viral antigens in diseased brains (Connolly et al 1967; Freeman et al 1967). These studies were followed by isolation of measles virus from affected brain tissue, although, in all cases, co-cultivation of brain tissue with measles virus-susceptible cell lines was required for isolation (Chen et al 1969; Horta-Barbosa et al 1969a, 1969b; Payne et al 1969). Following these initial reports, further isolates have also been described (Degre et al 1972; ter Meulen et al 1972; Ueda et al 1975; Wechsler and Fields 1978; Hall et al 1979a; Wechsler and Meissner 1982; Carter et al 1983; ter Meulen and Carter 1984; Sheppard et al 1986). More recently, full length cDNA clones have been isolated and sequenced from SSPE cases (Cattaneo et al 1986, 1988a; reviewed by Billeter and Cattaneo 1991).

2.6.4.2 Epidemiology

SSPE affects approximately one in one million individuals under the age of 20 worldwide (Swoveland and Johnson 1989). Patients with SSPE have a history of having suffered from

measles at an early age, usually before the age of 2 (Halsey et al 1980; Aaby et al 1984; Cianchetti et al 1986). The average incubation period is 6-7 years, although cases have been reported as young as 9 months and as old as 30 years (Dayan et al 1967; Cape et al 1973). Males are more commonly affected, with male:female ratios ranging from 1.4:1 to 3.5:1 (Aaby et al 1984). Geographic and racial variations have also been reported. In South Africa, the Cape Province has an above average prevalence (Moodie et al 1980), and in Italy, SSPE is more common in Sardinia than the rest of the country (Cianchetti et al 1986). In the USA, the prevalence is highest in the southeast and midwest states, and whites are more commonly affected than blacks (Modlin et al 1979).

2.6.4.3 Clinical signs and diagnosis

The clinical signs of SSPE can be variable, but tend to follow a pattern of severity (Jabbour et al 1969). The initial signs, of intellectual and psychological dysfunctions, are subtle, and the disease is rarely diagnosed in these stages, which can last several months or longer. The signs then progress, indicating more severe neurological involvement, with myoclonic jerks, motor disability and seizures becoming more common. After several weeks or months, patients progress towards coma. Some patients may then appear to improve slightly, but death, due to vasomotor collapse or infectious complications, usually occurs (Jabbour et al 1969). Remissions lasting for up to several years can occur (Risk et al 1978), although relapses usually result in death.

Several criteria are used to confirm the clinical signs of SSPE, including electroencephalography and magnetic resonance imaging. However, the most useful and consistent diagnostic tests are measurements of CSF IgG and anti-measles virus antibodies. IgG levels within the CSF are almost always elevated (Cutler et al 1968; Link et al 1973; Mehta et al 1977), and approximately 50-80% of this IgG is measles virus specific (Link et al 1973; Vandvik et al 1976; Trotter and Brooks 1980).

2.6.4.4 Pathology

SSPE is characterised pathologically by a mild to moderate inflammatory infiltrate of both white and grey matter (Ohya et al 1974; ter Meulen et al 1983). This infiltrate may be focal or widespread, and, in cases where there is white matter involvement, focal demyelination is seen, accompanied by large numbers of reactive astrocytes (Ohya et al 1974; ter Meulen et al 1983). Inclusions are found primarily in the nuclei and cytoplasm of neurons and oligodendroglial cells (Esiri et al 1981). Most areas of the brain can be involved, although involvement of the spinal cord is inconsistent, and the cerebellum is usually spared (Ohya et al 1974; Esiri et al 1981). In the inclusion bodies, two types of nucleocapsids have been demonstrated electron microscopically. Intranuclear nucleocapsids are always "smooth", with sharply delineated tubules, whereas cytoplasmic nucleocapsids are usually "fuzzy", with a diffuse electron dense material coating the tubules, although smooth nucleocapsids and intermediates are also seen (Jenis et al 1973; Dubois-Dalcq et al 1974a). The significance of this finding is unknown, although it has been postulated that it may relate to viral persistence in some way (Dubois-Dalcq et al 1974b). Budding virions have not been identified by electron microscopy (Swoveland and Johnson 1989).

2.6.4.5 Biology of MV isolates from SSPE brains

Measles virus isolates from "normal" (lytic) and SSPE cases have been studied extensively to try and identify differences that might explain the persistence of virus seen in SSPE. However, no single abnormality has been shown to be responsible in all cases.

Using monoclonal antibodies, N and P proteins have been shown to be present in all SSPE brains, whereas M protein has been reported in 4 of 4 brains in one study (Norrby et al 1985b), but only 1 of 4 in another (Baczko et al 1986). Other studies have failed to detect M protein using immunoprecipitation (Hall and Choppin 1981) and immunoperoxidase labelling (Haase et al 1985). Also, F and H proteins are sometimes undetectable, suggesting that more than one protein can be affected (Norrby et al 1985b; Baczko et al 1986). It has

also been shown that there is a lack of antibodies against M protein in patients with SSPE (Hall et al 1979a).

In situ hybridisation studies have shown the presence of mRNA for the M protein (Haase et al 1981; Swoveland and Johnson 1989). However, bicistronic P-M transcripts have also been demonstrated (Cattaneo et al 1986, 1987), hence the mRNA might not always be functional. Northern analysis has shown that message for all viral proteins is present in SSPE brains, but F and H mRNAs are reduced in comparison with N, P and M (Baczko et al 1986). However, *in vitro* translation studies revealed that M and H proteins are not always identifiable, suggesting that some mRNAs cannot be efficiently translated (Baczko et al 1986).

Copy numbers per cell of genomic and messenger RNA have been shown to be greatly reduced in SSPE brains compared with lytically infected tissue culture cells (Haase et al 1985), suggesting that restricted viral replication may play a role in persistence.

Isolation of viruses by co-culture with susceptible cell lines (see Section 2.6.4.1), and sequencing of cDNA clones from MV RNAs of SSPE brains, has allowed the characterisation of several different MV isolates from SSPE tissue (reviewed by Billeter and Cattaneo 1991). Virus isolates from different SSPE cases vary from each other in their gene expression. Mutations due to errors of the viral RNA polymerase have been found in all genes of MV, although not all genes are necessarily affected in any one case. From these studies, it is apparent that up to 2% of nucleotides are mutated during persistence (Cattaneo et al 1988a; Billeter and Cattaneo 1991). Of these mutations, approximately 35% result in amino acid changes. Most of the mutations occur in and around the M gene (Cattaneo et al 1986, 1988a; Billeter and Cattaneo 1991), and it has been suggested that, as M protein is involved in viral assembly (see Section 2.4.4), loss of M protein expression could account for lack of viral budding, and thus favour persistence. In some cases of SSPE, and in one case of MIBE, up to 50% of U residues have been shown to be mutated to C (Cattaneo et al 1986, 1988a, 1988b; Ayata et al 1989; Enami et al 1989; Wong et al 1989). It has been suggested that these changes result from hyper-mutation events, rather than accumulation of several

mutations of single nucleotides. The exact mechanism of hyper-mutation is not known, although it has been postulated that it results from aberrant formation of a double-stranded RNA structure involving the MV genome and M mRNA (Bass et al 1989; reviewed by Billeter and Cattaneo 1991). After unwinding/modification of this structure, biased mutations would occur by introduction of C residues. Whilst these mutations might play a role in persistence in some cases of SSPE, it has been suggested that these events also occur during "normal" MV infections, resulting in defective virions which fail to survive (Billeter and Cattaneo 1991).

Although DI RNA species have been found in some cell lines persistently infected with MV, they have never been clearly identified in SSPE material (Billeter and Cattaneo 1991).

The mutations seen in SSPE MV isolates are complex and differ between cases, suggesting that several factors may play a role in MV persistence. However, it does seem that alterations in the M protein might be important in establishing a persistent infection, although not all cases of SSPE examined have been shown to have mutations in, or lack of function of, the M protein (reviewed by Billeter and Cattaneo 1991).

2.6.4.6 Anti-MV antibodies and SSPE

It has previously been shown, in weanling hamsters, that levels of anti-MV antibodies correlate with levels of recoverable extracellular virus (Johnson and Byington 1977), and that treatment with anti-lymphocyte serum or thymectomy maintains acute infection, without conversion to persistence, in hamsters (Johnson et al 1975). If measles-infected cell cultures are incubated with anti-MV antiserum, persistently infected cell lines are produced (Rustigian 1966). The presence of anti-MV antibodies also results in persistence following viral inoculation in hamsters (Wear and Rapp 1971), in ferrets (Thormar et al 1977) and in monkeys (Albrecht et al 1977) (see Section 2.5.1.2). It has also been shown that HeLa cells inoculated in the presence of monoclonal anti-H antibodies have decreased levels of all viral proteins, especially F, M and P (Fujinami and Oldstone 1979, 1980). Similar

experiments using rat glioma cells have shown a complete loss of expression of all viral proteins in the presence of anti-H antibodies or SSPE serum (Barrett et al 1985), and that it took up to 9 months for protein expression to be resumed following removal of the serum. A subacute encephalitis can be induced in weanling mice following MV inoculation, using monoclonal anti-H antibodies (Rammohan et al 1981a, 1981b). The mice developed an acute encephalitis if antibodies were absent.

These experiments suggest that anti-MV antibodies may play a direct role in the development of persistent MV infections.

2.6.4.7 The host cell and SSPE

The possible role of the host cell in the development of persistent neurological infections has also been studied. Persistently measles-infected HeLa cells and hamster cells can induce an acute lytic infection when co-cultured with vero cells (Wild and Dugre 1978; Wechsler et al 1979). MV strains which produce lytic infections in vero cells produce persistent infections in mouse neuron cultures (Rentier et al 1981). Agents such as papaverine, which increase intracellular cAMP levels, induce differentiation in human and mouse neuroblastoma cells infected with MV (Miller and Carrigan 1982). This is accompanied by an increase in N, and a decrease in M protein levels, and by a reduction in the levels of extracellular virus production. Different strains of MV can induce acute encephalitis in newborn mice (Rammohan et al 1980) and newborn hamsters (Swoveland and Johnson 1986), whereas persistent infections are seen, using the same strains in adult mice and weanling hamsters.

It therefore seems that age at MV infection is important with regard to the establishment of persistence, possibly as a result of the developmental state of the CNS and/or the immune response. Differences between strains of MV may also be important.

2.7 Canine Distemper Virus

2.7.1 Introduction

Canine distemper is a highly contagious, acute to subacute disease, affecting dogs and other carnivores worldwide (reviewed by Appel 1987). The disease was first thoroughly described in the early 19th century (Jenner 1809), but it was almost 100 years later before the aetiologic agent was shown to be a filterable virus (Carré 1905). The introduction of live, attenuated vaccines has largely controlled the disease, although outbreaks still occur in susceptible populations (Cabasso et al 1951; Haig 1956; Rockborn 1960).

Dogs and other carnivores are the natural hosts for CDV, although disease signs have been seen in non-human primates (Yoshikawa et al 1989) and seals (Visser et al 1990; Rima BK, personal communication). Also, a human has been injected subcutaneously with blood obtained from a distemper-infected dog (Nicolle 1931). Although no clinical signs were seen in the human, when his blood was injected intraperitoneally into two previously uninfected dogs, they developed clinical signs of distemper, suggesting that the virus was capable of surviving and replicating in human tissues.

Only one serotype of CDV is currently recognised, although a variety of biotypes are found which vary in their pathogenicity and tissue tropism within the CNS (McCullough et al 1974a, 1974b, 1974c; Shapshak et al 1982; Summers et al 1984a; Appel 1987). Using monoclonal antibodies, minor epitopic variations have been found between three CDV vaccine strains (Orvell et al 1985), and vaccine strains have been differentiated from virulent strains (Appel 1987). CDV shows antigenic cross-reaction with MV, PPRV and RV. Polyclonal antibodies have revealed a close serological relationship between all antigens of MV and CDV, except the H protein (Orvell and Norrby 1974; Gibbs et al 1979; Stephenson and ter Meulen 1979; Hall et al 1980; Orvell and Norrby 1980; Appel 1987), whereas monoclonal antibodies only revealed cross reactions between F and M proteins (Appel 1987). Studies with RNA have revealed areas of varying homology between the

morbilliviruses, with some areas of e.g. the N gene showing over 90% homology between MV and CDV (Rozenblatt et al 1985).

2.7.2 Epidemiology and Pathogenesis

Distemper is enzootic in most parts of the world, except in dry, hot areas (reviewed by Appel 1987). Outbreaks can occur in areas where the disease has been absent for a few years if a susceptible population (of either domestic or wild animals) emerges (Gorham 1960). The use of attenuated live vaccines since the 1950's has greatly reduced the incidence of distemper, although eradication in dogs is not possible due to wildlife reservoir hosts (Appel 1987).

As well as animals in the Canidae, Mustelidae and Procyonidae families, members of the Felidae family are also susceptible to infection, although they do not normally develop clinical signs (Appel et al 1974). However, clinical signs of chronic encephalitis have been associated with CDV in a Bengal tiger (Blythe et al 1983) and a Siberian tiger (Gould and Fenner 1983). Natural and experimental disease has also been reported in non-human primates (Yoshikawa et al 1989) and members of the Viverridae such as binturongs (Goss 1948) and a masked palm civet (Machida et al 1992). Distemper-like viruses, which are thought to have originated from CDV, have also been described in several marine mammal species (Osterhaus et al 1988; Kennedy et al 1988; Domingo et al 1990). Based on viral antigenic and genetic properties, a new morbillivirus, phocid distemper virus (PDV), which is closely related to CDV, has been shown to be the cause of the disease in seals (Cosby et al 1988; Mahy et al 1988; Orvell et al 1990; Visser et al 1990; Haas et al 1991; Harder et al 1991; Kövamees et al 1991; Curran et al 1992; Harder et al 1993). However, some cases of seal distemper are thought to result from direct infection with CDV (Visser et al 1990; Rima BK, personal communication), and distemper-like symptoms in a mink farm were caused by a morbillivirus that was antigenically indistinguishable from PDV (Blixenkrone-Møller et al 1990).

Approximately 7 days following infection, acutely affected dogs shed virus in all body secretions and excretions, whether they show clinical signs or not (Appel 1987). Virus can not be isolated from dogs with subacute encephalomyelitis or persistent infection, though these animals can still transmit the virus (Appel 1987). Following recovery, dogs no longer transmit CDV. Transmission occurs primarily by aerosol, directly from dog to dog. The risk of infection is therefore increased in densely populated areas (Appel 1987). There is also an apparent increased prevalence during cold seasons (Gorham 1960, 1966).

Immune responses of infected dogs depend on the strain of CDV and the host (reviewed by Appel 1987). Affected dogs are always initially immunosuppressed and lymphopenic, apparently due to a direct effect of CDV on lymphocytes (Krakowka et al 1980). Dogs that recover early exhibit both humoral and cellular immune responses (Appel 1969; Krakowka et al 1975; Krakowka and Wallace 1979; Appel et al 1982, 1984). Neutralising antibody appears between 10 and 20 days post infection (Appel 1969; Krakowka et al 1975). Virus-specific IgM and IgG appear early in infection. IgG appears to be more important for immunity (Appel 1987). Circulating virus-specific cytotoxic T cells appear between 10 and 14 days post infection, and reach maximal levels within a further 7 days (Krakowka and Wallace 1979; Appel et al 1982). The humoral response persists in recovered dogs for several years, perhaps for life, whereas the cell mediated responses are of shorter duration (Appel et al 1982). In dogs that succumb to disease, there are little or no levels of neutralising antibodies in their serum, and cell mediated responses are either delayed or absent (Appel et al 1982). Dogs that succumb to subacute or chronic infection, or become persistently infected, show great variability in their immune responses (Appel et al 1982; Appel 1987).

Prior to the widespread use of vaccines, the highest incidence of distemper occurred between 2 months and 2 years of age (Gorham 1960, 1966). Younger animals were protected by maternal antibodies and older dogs had survived the disease. In vaccinated populations, dogs are protected by maternal antibodies until about 6 to 12 weeks, and are then susceptible to infection until they are protected by vaccination.

Distemper is usually spread by inhalation of airborne virus (Laidlaw and Dunkin 1926). As with MV (see Section 2.6.3), the virus first replicates in the respiratory tract lymphatic tissues, and then spreads via circulating cells to all other lymphatic tissues (Laidlaw and Dunkin 1926). Approximately 7 days post infection, the virus can be isolated from all lymphatic tissues and from circulating lymphocytes (Appel 1987). Pyrexia is usually detected 3 to 4 days post infection. Depending on virus strain, approximately 7 to 14 days post infection, dogs either successfully mount a humoral and cellular immune response and recover, or they die from acute or subacute disease, or become persistently infected (Appel 1987). In dogs that fail to recover, the virus spreads, via circulating lymphocytes and macrophages, to various epithelia, including those of the respiratory, alimentary and urogenital tracts. CDV first appears in the CNS approximately 8 to 10 days following infection, initially in perivascular lymphocytes and then in meningeal macrophages, and later in neurons and glial cells (Appel 1987). Susceptible dogs usually die between 2 and 4 weeks, depending on the virus strain. Some dogs mount a late immune response, and either succumb to a subacute disease (usually encephalitis), or become persistently infected for 2 to 3 months (Appel 1987). Many of these dogs eventually recover.

The CNS signs depend on the distribution of CDV. Both grey and white matter may be involved, although some strains of CDV show a particular affinity for grey or white matter (Summers et al 1984a, 1984b). In most cases, CDV can be found in, and can be isolated from, affected areas of the CNS (Wisniewski et al 1972; Raine 1976; Vandevelde and Kristensen 1977; Summers et al 1978; Hall et al 1979b; Imagawa et al 1980). Most commonly affected sites include the cerebellar peduncles, medulla, midbrain, basal ganglia, cerebral cortex, optic tracts and meninges (reviewed by Appel 1987). Acute encephalitis and death is usually associated with grey matter infection, whereas in subacute and chronic cases, demyelination is also seen (Appel 1987). Demyelination has been postulated to be due to "bystander effects" rather than direct CDV infection (reviewed by Appel 1987). It is thought that infected macrophages or astrocytes, which are often found in close proximity to demyelinating axons, release cytotoxic enzymes to cause demyelination. It has also been

postulated that ROS may be involved, as CDV stimulates the production of ROS in cultures of canine brain cells *in vivo* (Bürge et al 1989).

2.7.3 Clinical Signs

Variations in the duration and severity of clinical signs have been reported in both experimental and natural distemper (Laidlaw and Dunkin 1926; Cornwell et al 1965a; Appel 1969; McCullough et al 1974a, 1974b, 1974c). The variations appear to depend on virus strain, age of the animal and individual host resistance. In natural infections, secondary bacterial and viral infections also increase the severity of clinical signs. The mortality rate in very young susceptible dogs is higher than in weaned or adult dogs (Krakowka and Koestner 1976).

The incubation period of CDV may range from approximately 7 days to 4 weeks or more (Laidlaw and Dunkin 1926; Lauder et al 1954; Cornwell et al 1965a, 1965b). The initial pyrexia, at about 3 to 6 days post infection, is usually inapparent clinically. However, the second pyrexia, associated with viraemia, is usually accompanied by coryza, conjunctivitis and anorexia. Affected dogs are also often lymphopenic. Gastrointestinal and respiratory disease usually follow, often complicated by secondary infection (reviewed by Appel 1987).

As with MV infection (see Section 2.6.3), syncytia formation is found affecting various cells, including lymphocytes, haematopoietic precursor cells and alveolar macrophages (Krakowka et al 1980; Gossett et al 1982; Summers and Appel 1985).

Some dogs develop neurological signs, usually accompanied by, or following, systemic disease. Seizures and incoordination are most commonly seen. Other CNS signs include myoclonus, tremors, chorea, paresis, nystagmus and hyperaesthesia (Innes and Saunders 1962; Boenig et al 1974; Appel 1987). Retinal lesions and optic neuritis are also seen (Fischer 1971; Fischer and Jones 1972; Blanchard et al 1973), and skin pustules have been reported in natural cases (Cornwell et al 1965a). Dogs that develop CNS signs usually die or are euthanased, but some do recover, often with residual signs of disease, such as

persistent tremors (reviewed by Appel 1987). As well as acute encephalitis in young dogs, CDV can also cause a chronic encephalitis in more mature animals. This disease is not preceded, or accompanied, by systemic signs (Vandevelde et al 1980). The areas of the CNS primarily affected in chronic distemper encephalitis are the cerebellar and cerebral peduncles, the central cerebellar white matter, the optic tracts and the spinal cord white matter (Vandevelde et al 1980; Braund et al 1987). Also, old dog encephalitis, a rare, chronic neurological disease has been postulated to be caused by CDV (Lincoln et al 1971, 1973; Adams et al 1975; Imagawa et al 1980) (see Section 2.7.4). Hyperkeratosis of the footpads ("hard pad") is often seen with CDV infection (MacIntyre et al 1948) and tooth enamel hypoplasia is a common finding in growing dogs (Bodingbauer 1960; Dubielzig et al 1981).

2.7.4 Old Dog Encephalitis

2.7.4.1 Introduction

Old dog encephalitis (ODE) is a subacute or chronic, progressive panencephalitis that occurs in mature dogs (Cordy 1942; Adams et al 1975; Vandevelde et al 1980; Appel 1987; reviewed by Braund et al 1987). ODE can sometimes be confused with chronic distemper encephalitis (see Section 2.7.3), but is usually differentiated on clinical and pathological differences (Vandevelde et al 1980; Braund et al 1987). In ODE, lesions occur primarily in the upper brain stem and cerebral cortex, and the cerebellum is usually not involved, whereas, in chronic distemper encephalitis, the lower brain stem and spinal cord are most commonly affected. Diffuse, sclerotic lesions are seen in ODE, compared with multifocal necrosis seen in chronic distemper encephalitis. Interest in ODE comes mainly from the clinical, pathologic and immunologic similarities between ODE and SSPE (Adams et al 1975).

Serological, immunocytochemical, electron microscopic and viral isolation techniques have all implicated CDV as the aetiologic agent in ODE (Lincoln et al 1971, 1973; Imagawa et al 1980).

2.7.4.2 Epidemiology

Affected dogs are usually over 6 years of age, although younger dogs with ODE have been reported (Cordy 1942; Lincoln et al 1971, 1973; Adams et al 1975; Imagawa et al 1980; Vandevelde et al 1980). There appears to be no breed or sex predisposition, and there are no concurrent systemic signs associated with the disease. ODE can affect both vaccinated and unvaccinated dogs.

2.7.4.3 Clinical signs and diagnosis

The most common initial finding in dogs with ODE is visual impairment, due to optic neuritis (Adams et al 1975; Vandevelde et al 1980; Braund et al 1987). Dogs then show progressive signs of mental depression, hyperkinesia, compulsive circling and head pressing. Affected dogs show bilateral menace defects and may exhibit personality changes.

In living animals, diagnosis is based on these clinical signs, as haematologic, biochemical and electroencephalographic data are nonspecific (Adams et al 1975; Vandevelde et al 1980; Braund et al 1987). The diagnosis can be confirmed using autopsy material.

2.7.4.4 Pathology

Pathologic changes in ODE are restricted to the CNS, and are characterised by diffuse involvement of the cerebral cortex and often the basal nuclei, thalamus, hypothalamus and midbrain (Adams et al 1975; Vandevelde et al 1980; Imagawa et al 1980; Braund et al 1987). The lesions consist of perivascular mononuclear cell infiltrates (lymphocytes and plasma cells), microglial proliferation, astrocytosis, neuronal degeneration, demyelination and areas of focal necrosis. Inclusion bodies are found in the nuclei and cytoplasm of affected neurons and glial cells (Lincoln et al 1973; Adams et al 1975; Vandevelde et al 1980; Braund et al 1987).

2.7.4.5 Evidence for CDV involvement in ODE

When Cordy first described ODE (1942), he also reported attempts to transmit the disease to other dogs. Whilst he suspected that the disease had an infectious origin, he was unable to confirm this by transmission experiments. To this day, nobody has been able to successfully transmit encephalitis to other animals using tissues derived from dogs with ODE. However, further evidence has emerged to implicate CDV in the aetiopathogenesis of ODE. CDV antigens have been shown in the brains of dogs with ODE (Lincoln et al 1971), and anti-CDV antibody levels were also shown to be raised in the serum of these dogs. However, attempts to recover virus and to transmit the disease to other animals were unsuccessful, suggesting that the virus was in some way defective (Lincoln et al 1971). The same group have also provided electron microscopic evidence to support their original findings. Paramyxovirus-like inclusion bodies were found in the nuclei of nerve cells (Lincoln et al 1973). More recently, CDV has been isolated from the brain cells of 2 of 6 cases of ODE, however, it was not possible to transmit the disease to ferrets (Imagawa et al 1980). Interestingly, CDV was also isolated from the bladder of one of the ODE cases. The authors suggested that this might reflect the ability of CDV to persist in other canine tissues, or that accidental infection from leucocytes might have occurred during *in vitro* cultivation. Another possibility is that the dog was suffering from chronic distemper encephalitis, and not ODE.

2.7.4.6 Comparison of ODE and SSPE

As can be seen, similarities exist between ODE and SSPE, and between the biology of CDV and MV regarding persistence. However, there are several differences that have been demonstrated between the two conditions. Isolation of CDV from the brains of dogs with ODE was carried out without the need for cocultivation (Imagawa et al 1980). Also, using immunoprecipitation techniques, all proteins of CDV have been shown to be present in ODE brains (Hall et al 1979b). This differs markedly from SSPE, where cocultivation techniques

are required for isolation, and M protein is absent, or defective (see Section 2.6.4.5), and suggests that different mechanisms exist to cause persistence.

2.7.5 CDV and Canine Rheumatoid Arthritis

Canine rheumatoid-like arthritis (CRA) is a naturally occurring symmetrical, erosive, inflammatory polyarthropathy (Halliwell et al 1972, Newton et al 1976, Pedersen et al 1976, Bennett 1987a, 1987b). On the basis of clinical, pathological and radiological similarities, CRA has been proposed as a model of human rheumatoid arthritis (RA) (Bell et al 1991, May et al 1993). Indeed, human criteria for the diagnosis of RA have been adapted for the diagnosis of CRA and this adaptation remains in common use in the veterinary field (Bennett 1987a).

Dogs with CRA are usually middle aged and present with multiple joint pain, swelling and stiffness. The disease is characterised pathologically by an inflammatory polysynovitis consisting predominantly of a mononuclear cell infiltrate. As the disease progresses, pannus formation can occur, leading to destruction of articular cartilage and erosion of subchondral bone (Bennett 1987b). Sera and synovial fluids from affected dogs contain high levels of immune complexes and autoantibodies, with serum IgM rheumatoid factor detectable in about 75% of cases (Bari et al 1989, Carter et al 1989). IgA rheumatoid factors are raised in severe disease (Bell et al 1993). Immunocytochemical studies have shown distinct T and B lymphocyte areas within the affected synovium (May et al 1992).

The first evidence implicating paramyxoviruses in CRA came from the detection, by electron microscopy, of inclusion bodies, resembling paramyxovirus nucleocapsid proteins, in the synovial membranes of dogs with CRA (Schumacher et al 1980). CDV has been more specifically implicated by the detection, using an enzyme linked immunosorbent assay, of increased levels of anti-CDV antibodies in the synovial fluids of dogs with CRA compared with those of dogs with osteoarthritis (OA) (Bell et al 1991). When the immune complexes were examined by Western blot analysis, those from dogs with CRA were found to react with anti-CDV sera, whilst those from dogs with OA did not react (Bell et al 1991). More

recently, further evidence has supported these initial findings (May et al 1993). Levels of anti-CDV antibodies in the sera and synovial fluids of dogs with CRA were found to correlate with the respective total levels of immune complexes. Also, both the synovial fluid anti-CDV antibody levels and the synovial fluid immune-complex anti-CDV antibody levels correlated with the synovial polymorphonuclear cell count, a useful indicator of synovial inflammation. By immunocytochemistry of CRA synovial tissues, distemper viral antigens have been demonstrated within synovial macrophages with morphological characteristics of antigen presenting cells (May 1992). The onset of clinical polyarthritis has also been associated with recent vaccination in young dogs (Bennett D and May C, personal communication).

These results suggest an association between CDV and ongoing synovial inflammation in dogs with CRA.

CDV has also been implicated in human RA. Similar inclusion bodies to those found in canine synovial cells are found in the circulating lymphocytes of human RA patients (Dryll et al 1977), and epidemiologic studies have revealed an association between RA and dog ownership (Gottlieb et al 1974). Also, anti-CDV antibodies and immune complexes containing CDV antigens have been found within the synovial fluids of humans with RA (Bennett D, May C and Carter SD, personal communication).

2.8 Paramyxovirus Persistence and Other Diseases

As well as SSPE and ODE, there are several other diseases in which the persistence of paramyxoviruses is thought to play a role. Paget's disease of bone, a chronic, focal disorder, in which several paramyxoviruses have been implicated, is described in detail in Section 3.3. The other diseases in which paramyxoviruses have been implicated include:

2.8.1 Autoimmune chronic active hepatitis

As well as in SSPE, measles virus is also thought to be involved in autoimmune chronic active hepatitis (AICAH). The liver is a target organ for MV and, although jaundice is not

a clinical feature of acute MV infection, subclinical hepatitis is thought to be present in up to 80% of affected adults (Gavish et al 1983). Anti-measles antibody levels are higher in patients with AICAH than those with natural measles, and can be as high as those seen with SSPE (Triger et al 1972; Christie and Haukenes 1983; Robertson et al 1987). Furthermore, studies have shown that, since the introduction of measles vaccination programmes, the disease is seen much less commonly in younger age groups (i.e. those that have been vaccinated) (Randall and Russell 1991). *In situ* hybridisation studies have revealed the presence of MV within leucocytes of approximately 70% of patients with AICAH (Robertson et al 1987). However, several of the control patients also had MV detectable, and this was also the case when the polymerase chain reaction (PCR) was used (Kalland et al 1989). This again suggests that persistent paramyxovirus infections may be more common than previously thought, although these infections are usually not detectable in normal individuals.

2.8.2 Multiple sclerosis

After it was found that MV was the cause of SSPE, a great deal of attention focused on the possible role of paramyxoviruses in other neurological conditions, particularly multiple sclerosis (MS) (Norrby 1978; ter Meulen and Stephenson 1981; Russell 1983; Goodman and McFarlin 1987). The first signs of MS are usually seen around 30 years of age, and the resulting loss in CNS function is variable, with relapses being a common feature (Randall and Russell 1991). The lesions in the CNS are small plaques which cause breakdown of myelin and can be widespread, although they are usually confined to the white matter (McFarlin and McFarland 1982; Waksman and Reynolds 1984). There appears also to be some immune component to the disease. Many white cells (T and B lymphocytes, and macrophages) are found in the lesions (Traugott et al 1983; Hauser et al 1986), and immunoglobulin levels, particularly IgG, are raised in the cerebrospinal fluid, suggesting intrathecal synthesis of immunoglobulins (Whitaker and Snyder 1984).

Based on epidemiological and serological studies, various paramyxoviruses have been implicated in MS. These include MV, CDV and SV5 (Adams and Imagawa 1962; Haire et al 1973; Norrby et al 1974; Cook et al 1978; Hughes et al 1980; Goswami et al 1987). However, the evidence for CDV and SV5 involvement has been questioned, and attention has focused on MV involvement. More recently, molecular techniques have been used to examine MS tissues. The first study, using solution hybridisation, found no evidence of MV (Stevens et al 1980), and this was later confirmed by dot blot analysis (Dowling et al 1986). However, some evidence has been found for the presence of MV sequences. Haase et al (1981) found MV in 1 out of 4 MS brains using *in situ* hybridisation, and in a later study found MV in 13 out of 25 MS brains (Haase et al 1984). However, they also found MV in 4 out of 7 control brains. In a similar study, Cosby et al (1989) found MV in 2 out of 8 MS patients and in 1 out of 56 control patients. They found no evidence of rubella virus, CDV, or SV5, and concluded that widespread sampling of diseased brains was needed before drawing firm conclusions as to the presence or absence of MV.

The finding of MV in control tissues, particularly in normal brains, might merely reflect the ability of MV to persist without causing any symptoms. It seems likely that, as well as in cases of SSPE, MV can persist in neurological tissues and that this persistence may be responsible for disease only in some cases.

2.8.3 Crohn's disease

Crohn's disease is an inflammatory bowel disorder that is characterised by granulomatous lesions in the intestinal submucosa (Wakefield et al 1989, 1990). Recently, it has been suggested that Crohn's disease is the result of a persistent MV infection of the mesenteric microvascular endothelium (Wakefield et al 1990, 1993). Supportive evidence for this hypothesis has come from electron microscopy, *in situ* hybridisation and immunohistochemistry (Wakefield et al 1993). However, control samples of intestine were also found to be positive for MV. The authors concluded that MV is capable of persistently

infecting the intestine, and that Crohn's disease may be the result of a granulomatous vasculitis in response to the virus.

As can be seen from these studies, MV (and other paramyxoviruses) can persist in human tissues. What has still to be proved, however, is that this persistence actually causes the diseases being studied. The possibility still remains that persistence does not cause pathology, and that the virus is only found when diseased tissue (hence active, leading to activation of the virus) is examined.

Chapter 3

Bone Disorders

3. Bone Disorders

3.1 Introduction

There are many bone disorders of both humans and animals, some of which have proven aetiologies, and others whose aetiology is yet to be determined. As the work detailed in this thesis concerns the possible role of a virus, namely CDV, in bone disorders, this chapter will concentrate on osteomyelitis in humans and animals, Paget's disease of bone in humans and several idiopathic canine bone disorders.

3.2 Osteomyelitis

Osteomyelitis is an infectious inflammation of bone marrow and adjacent bone. Clinical signs associated with the condition include pyrexia, swelling, abscessation, pain and lameness (Waldvogel et al 1970). Most cases in both humans and animals result from bacterial infection, although bone lesions are sometimes seen with viral and fungal infections.

3.2.1 Bacterial Osteomyelitis in Humans and Dogs

There are three ways in which bacteria reach bone (Kahn and Pritzker 1973) – via haematogenous routes from a distant site of infection, by spread from a source external to bone (i.e. post-operative or post-traumatic infections), or by direct spread from an adjacent soft tissue infection. In humans, localisation and characteristics of haematogenous osteomyelitis vary with age of the patient (Trueta 1959). In infants, the metaphyses are involved, multiple foci are frequently seen and common organisms are Streptococci or Staphylococci. In children aged 1 to 16 years, the metaphyses are again involved, however, multiple foci are rare and Staphylococci are mainly isolated. In adults (>16 years), subchondral bone is usually involved, the organisms vary (though they are usually Staphylococci) and the infection usually involves only one site. The metaphyseal localisation in children has been attributed to the hair-pin loops of the metaphyseal

arteries draining into larger metaphyseal veins (see Section 1.2.3.1) (Trueta 1959). The larger size of these veins results in a slowing down of the blood flow at this level, allowing bacterial emboli (if present) to attach to the vascular endothelium and so start infection. In the adult, this vascular arrangement changes following resorption of the growth plate, hence the lack of metaphyseal osteomyelitis in this age group (Trueta 1959; Kahn and Pritzker 1973). In humans, the apparent susceptibility of bones such as the femur and tibia has been explained by the fact that the growth plates in these bones are subject to greater mechanical stress than other bones (Starr 1922; Robertson 1927). This would predispose them to repeated minor trauma, which could result in mild metaphyseal haemorrhage and necrosis without clinical signs. These sites could provide a focus for any circulating bacteria. The blood supply to adult subchondral bone has been shown to have some similarities to the metaphyseal supply in children (Trueta 1959), thus explaining the localisation of adult osteomyelitis.

Although bacterial osteomyelitis is common in humans, the condition is rarely described in dogs (reviewed by Caywood et al 1978). Most cases of osteomyelitis in dogs result either directly from trauma, or following surgical repair of fractures, hence organisms isolated consist mainly of Staphylococii and Streptococci, and multiple species (including gram negative organisms) are commonly isolated. The majority of affected dogs are aged between 1 and 4 years. In the study of 67 cases by Caywood et al (1978), only 6% of infections were haematogenous in origin, and none of these 4 affected dogs were <2 years of age. However, osteomyelitis has been reported in young dogs (Denholm 1966) and metaphyseal localisation of infection has also been reported (Dunn et al 1992). In the latter report, the radiographic features were similar to those of metaphyseal osteopathy (MO) (see Section 3.4.3.2). The metaphyseal blood supply in young dogs is similar to that in children (see Section 1.2.3.1), hence, bacteria (and viruses) could settle in these areas and initiate infection. The fact that metaphyseal osteomyelitis appears to be rarer in dogs than in humans might be explained by the fact that dogs are quadripeds and each of their long bones are thus subjected to less stress and trauma than the femurs and tibiae of humans.

3.2.2 Human Viral Osteomyelitis

Viral osteomyelitis is rare in humans, although several viruses have been shown to produce bone lesions. Metaphyseal radiolucencies are seen with congenital rubella virus (Rudolph et al 1965a, 1965b; Singer et al 1967; Reed 1969; Sekeles and Ornoy 1975) and human cytomegalovirus (HCMV) (Sacrez et al 1960; Graham et al 1970; Merton and Gooding 1970; McCandless et al 1975) infections. Most reports of rubella infection suggest that these lesions are a result of metabolic or nutritional insufficiency during gestation, although inflammatory infiltrates have been reported within the affected areas (Sekeles and Ornoy 1975). Metaphyseal lesions are also seen with variola infection (Brown and Brown 1923; Sheldon 1923; Huenekens and Rigler 1926; Cockshott and MacGregor 1959; Davidson et al 1963; Eeckels et al 1964) and following vaccination with vaccinia (Sewall 1949; Cochran et al 1963). The lesions caused by variola are usually symmetrical and inflammation is commonly seen. Relapses frequently occur. Periosteal new bone often forms around the metaphyses and this usually extends to involve the diaphyses.

3.2.3 Viral Osteomyelitis in Animals

Whilst bacterial osteomyelitis has been reported in the dog, there are no previous clinical reports of viral osteomyelitis, although Boyce et al (1983) (and Krakowka S, personal communication) reported metaphyseal lesions associated with CDV infection.

Experimental herpesvirus infection in cats has also been shown to produce necrosis of metaphyseal bone (Hoover and Griesemer 1971). The authors reported "an irregular, transverse grey zone, 1 to 2 mm wide" in the affected metaphyses. Inclusion bodies were found in osteoclasts, osteoblasts and periosteal lining cells, but not in osteocytes.

Avian osteopetrosis, a disease characterised by symmetrical thickening of the shafts of long bones, has been shown experimentally to be the result of infection by a filterable agent obtained from infected birds (Simpson and Sanger 1968). The lesions appear to be the result of hyperplasia and hypertrophy of osteoblasts lining the periosteum. Osteoblasts lining

the medullary cavities may also be affected. The new woven bone formed as a result of this increase in osteoblastic activity is poorly mineralised and, hence, prone to deformity.

3.3 Paget's Disease of Bone

3.3.1 Introduction

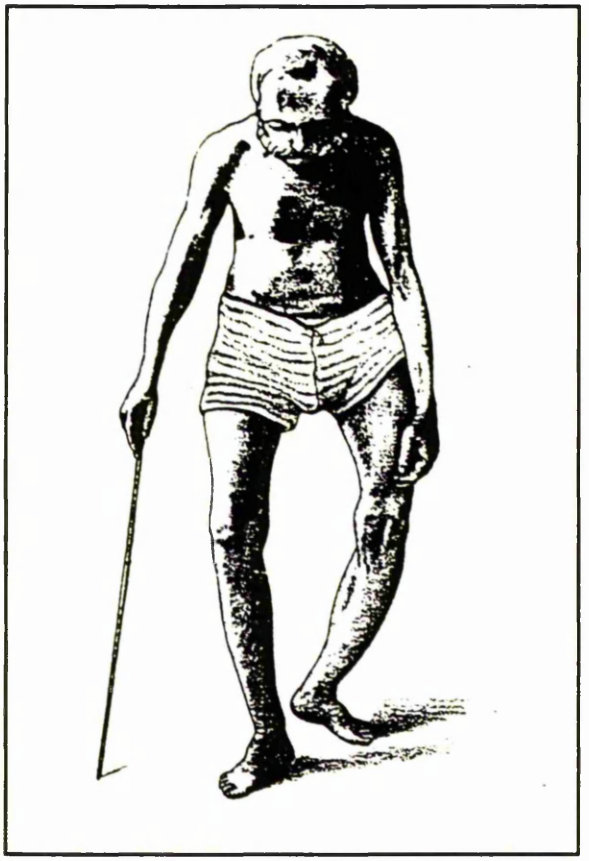
Paget's disease is a chronic, focal skeletal disorder, which is particularly prevalent among the elderly populations of the United Kingdom, America and Australasia (reviewed by Kanis 1991; Anderson 1993). The condition was first reported by Wilks (1869), but it was a few years later before the disease was classically described by Sir James Paget (1877). Based on macroscopic and microscopic examinations, he used the term "osteitis deformans" to describe in detail the pathological changes in a patient from the North of England, whom he had studied for 22 years. He also reported the disease in 4 other patients, one of whom was the patient initially described by Wilks. Following these initial reports, he later presented details of a further 7 patients (Paget 1882) and the disease has since become widely known as Paget's disease of bone (see Figure 3.1).

The term "osteitis deformans" is something of a misnomer, as there are rarely any inflammatory changes in the disease (Kanis 1991; Anderson 1993), but Paget's initial description of the condition still holds true to this day: "It begins in middle age or later, is very slow in progress, may continue for many years without influence on the general health, and may give no other trouble than those which are due to the changes of shape, size and direction of the diseased bones. The bones enlarge and soften, and those bearing weight yield and become unnaturally curved and misshapen".

Although Paget was the first to fully document the condition, it is now evident that the disease existed many years before the nineteenth century. Pagetic changes have been described in bones from Ancient Egypt (Hutchinson 1889), the Gallo-Roman era (Astre 1957), Anglo-Saxon burial grounds (Wells and Woodhouse 1975; Price 1975), and, more recently, in bones from medieval burial grounds (Aaron et al 1992). It has also been suggested that

Beethoven's deafness may have been a result of Paget's disease affecting his skull (Naiken 1971).

Figure 3.1 First reported case of Paget's disease



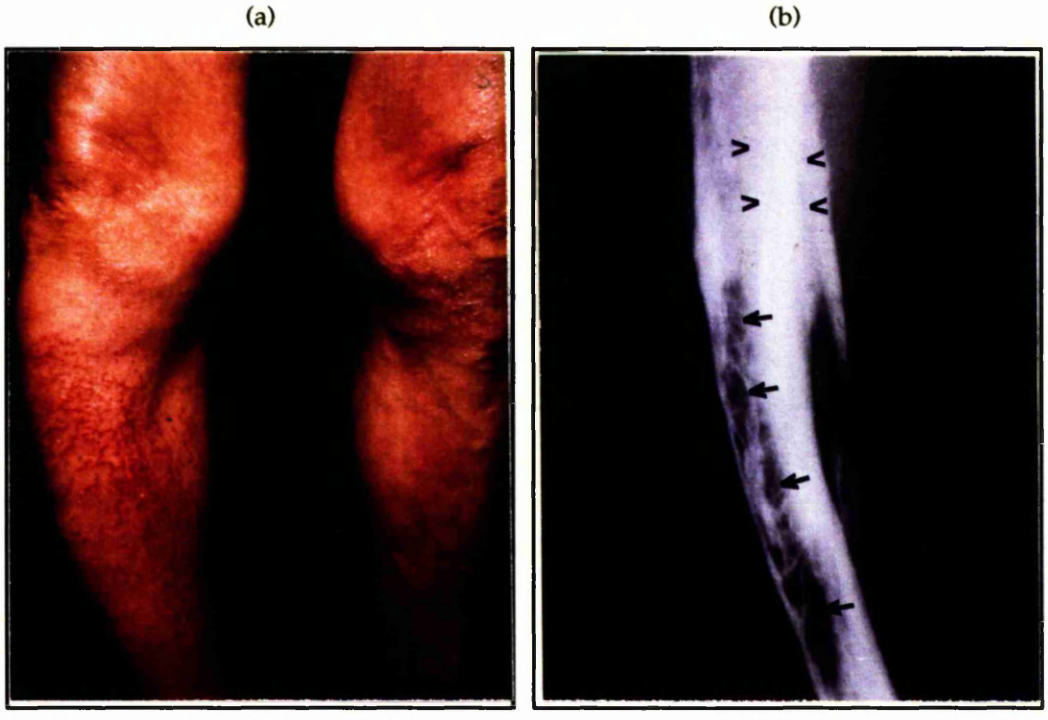
Elderly patient with extensive Paget's disease, first described by Sir James Paget. Note enlargement of the skull, and bowing of the spine and tibiae. From Paget (1877).

3.3.2 Pathology, Clinical Signs and Treatment

The pathology of Paget's disease is related to disturbances in the rate of bone turnover and remodelling (reviewed by Kanis 1991; Anderson 1993). The initial lesion is one of excessive resorption by an increased number of abnormally large osteoclasts. The activity of individual osteoclasts in Paget's disease is, however, impaired, and the increase in bone resorption is thought to result purely from the increase in number and size of osteoclasts

(reviewed by Kanis 1991). The increase in resorption is followed by a secondary increase in osteoblastic activity. Affected bones become enlarged due to the increased osseous activity (see Figure 3.2).

Figure 3.2 Paget's disease – gross and radiographic features



(a) gross and (b) radiographic features of Paget's disease affecting the right tibia. Note swelling and deformity of the affected limb grossly (a). Note areas of bone resorption (arrows) and new bone formation (arrow heads).

The net effect of this abnormal turnover of bone is increased vascularity and intraosseous fibrosis, and a disturbed bony architecture consisting of variable amounts of immature (woven) bone and irregular, mature lamellar bone (due to irregular orientation of collagen). The diseased bone is hence weak and, therefore, prone to deformity and fractures (see Figure 3.2). Other common problems associated with the disease are bone pain, cardiac problems (due to the increased cardiac output to the diseased bones), and a higher risk of bone neoplasia (both osteosarcomas and giant cell tumours) (Kanis 1991; Anderson 1993).

Until fairly recently, no specific drugs were used in the treatment of Paget's disease. Purely symptomatic treatment was given in the form of analgesics and anti-inflammatory drugs (Kanis 1991; Anderson 1993). However, effective treatments are now available in the form of calcitonin, and the more useful bisphosphonates, such as etidronate and pamidronate (Cantrill and Anderson 1990; Kanis 1991; Anderson 1993).

3.3.3 Epidemiology

Various epidemiologic studies have been carried out, and it is evident from these that Paget's disease has extremely variable geographic and ethnic distributions.

3.3.3.1 Geographic distribution

The disorder appears to be most prevalent in the United Kingdom, affecting approximately 5% of the population aged over 55 years, with a particularly high prevalence (up to 8.3%) in some towns of the North West of the country (Barker et al 1977, 1980). The disease is common in Western Europe, although the prevalence is less than in the United Kingdom (Detheridge et al 1982). It is also fairly common in Australia (Barker 1984), New Zealand (Reasbeck et al 1983) and America (Guyer and Chamberlain 1980; Polednak 1987). Countries where the disease is rare include Scandinavia, China, Japan and the middle East (Kanis 1991). In Australia, the disease is rare among aboriginals (Kanis 1991) and the prevalence among British immigrants is higher than that in the native white population (Gardner et al 1978).

3.3.3.2 Genetic studies

Whilst a high familial component to Paget's disease has frequently been recorded (Kanis 1991; Siris et al 1991), studies on the genetic basis of the disease have proved relatively unrewarding. Several investigations have proposed an association between Paget's disease and HLA type, most notably DR2 and DQw1 (Fotino et al 1977; Tilyard et al 1982; Singer et al 1985). However, one study in the UK failed to confirm these findings, but instead

suggested that HLA A8 and B15 frequencies were increased (Kanis 1991), and another suggested that DPB4 might be involved (Gordon et al 1994). Overall, the reports are conflicting and the numbers of patients studied are probably not sufficient to draw proper conclusions. Nevertheless, it remains an attractive possibility that there is a genetic predisposition to Paget's disease.

3.3.4 Aetiology

Despite the many studies which have been carried out, the cause of Paget's disease is still unknown. Several theories have been put forward, including inflammatory, autoimmune, endocrinological and neoplastic aetiologies (reviewed by Kanis 1991). However, for quite some time, the consensus of opinion has been that one or more of the paramyxoviruses might be responsible. It is generally thought that, if paramyxoviruses are responsible for the disease, infection occurs in early childhood, and the virus persists in the bone cells to cause disease later in life (Kanis 1991; Anderson 1993).

3.3.4.1 Evidence for a viral aetiology

The first evidence for a possible viral aetiology came from Rebel et al (1974) using electron microscopic studies which demonstrated viral nucleocapsid-like inclusion bodies in pagetic osteoclasts. These findings have since been independently confirmed by several groups (Mills and Singer 1976; Gheradi et al 1980; Howatson and Fornasier 1982). Although the presence of inclusions was once regarded as specific for Paget's disease, viral-like inclusion bodies have since been found in other skeletal disorders, including giant cell tumour (Schajowicz et al 1985), pycnodysostosis (Beneton et al 1987), osteopetrosis (Mills et al 1988), familial expansile osteolysis (Dickson et al 1991) and primary oxalosis (Bianco et al 1992). Viral-like inclusion bodies are therefore associated with several bone disorders, and there appears to be little correlation between the presence of inclusions and the degree of resorption. However, the functional activity of individual osteoclasts is impaired in Paget's disease, pycnodysostosis and probably osteopetrosis, suggesting that the inclusions may be in some way related to osteoclast activity (reviewed by Kanis 1991).

Despite a recent study using reverse transcriptase-polymerase chain reaction (RT-PCR) with degenerate primers, and a further study using primers designed to detect either MV or CDV, both of which failed to detect any paramyxovirus sequences (Ralston et al 1991; Birch et al 1994), further evidence for the presence of paramyxoviruses has been demonstrated, mainly from immunohistochemical studies, where the paramyxoviruses implicated include MV (Rebel et al 1980; Singer and Mills 1983; Basle et al 1985), respiratory syncytial virus (RSV) (Mills et al 1981; Singer and Mills 1983; Pringle et al 1985), parainfluenza virus type 3 and SV5 (Basle et al 1985). There has also been a study showing positive *in situ* hybridisation with a cDNA probe to MV (Basle et al 1986).

RT-PCR has been used to demonstrate MV transcripts in marrow mononuclear osteoclast precursor cells (Roodman 1994). These cells have been shown to be hyper-responsive to $1,25(OH)_2D_3$ in long term cultures (Kukita et al 1990), and, more recently, CFU-GM cells from Paget's patients have been shown to be similarly hyper-responsive to $1,25(OH)_2D_3$ (Demulder et al 1993).

These viral findings are, however, difficult to reconcile with the startling differences in geographic distribution of Paget's disease (see Section 3.3.3.1). Human paramyxoviruses are fairly ubiquitous in all of the countries mentioned, suggesting that some other agent might be involved. Recent work has therefore moved towards investigating a possible animal connection. Paramyxoviruses can infect a wide range of species, including birds and dogs (see Section 2.1), and these species do show a more varied geographic distribution, for example, domestic dogs were, until recently, uncommon among the Chinese in Hong Kong (Anderson DC, personal communication).

3.3.4.2 Canine distemper virus and Paget's disease

Recent evidence has suggested that CDV might be involved in Paget's disease. Several studies have demonstrated an increased incidence of dog ownership amongst Paget's patients compared with controls (O'Driscoll and Anderson 1985; Holdaway et al 1990;

O'Driscoll et al 1990). However, these findings have been disputed by others (Barker and Detheridge 1985; Siris et al 1990).

A further epidemiological study found an association between Paget's disease and both pet ownership and eating lamb or goat meat not subject to official meat inspection (Piga et al 1988). After variance analysis, only the eating of lamb or goat was found to still be significant, and the authors suggested that this could be a possible risk factor for Paget's disease. However, the average age of the control group was less than that of the Paget's patients , and so on average, the control patients could not have eaten contaminated meat, as they were born after the introduction of legislation to control meat hygiene.

More convincing evidence implicating CDV in the aetiology of Paget's disease has come from molecular studies demonstrating the presence of CDV in pagetic bone cells (Gordon et al 1991, 1992; Cartwright et al 1993). Positive *in situ* hybridisation to CDV mRNA has been shown in both osteoblasts and osteoclasts from up to 65% of Paget's patients, using probes to three separate CDV genes (N, F and P genes) (Gordon et al 1991; Cartwright et al 1993). No hybridisation was seen with any of the probes to genomic RNA of CDV. This finding could be explained by the fact that viral transcription without replication is a known method of viral persistence, or that the virus is replication deficient (see Section 2.5.1). No patients studied had MV or SV5 viral sequences and only one patient had RSV detectable. More definitive proof implicating CDV has come from cDNA sequencing following RT-PCR (Gordon et al 1992). Using specific primers to the CDV-N gene, RT-PCR and subsequent Southern blotting and probing showed 8 of 13 Paget's patients had CDV nucleic acids sequestered within their bone cells. Interestingly, RT-PCR also demonstrated that one patient had both CDV and MV present in the bone cells. The presence of more than one paramyxovirus within the same patient has been documented previously for MV and RSV (Mills et al 1984). It was possible to clone and sequence CDV products from two patients and these clones revealed approximately 2% base pair changes (5 of 206bp) in the nucleic acid sequences relative to the Genbank Onderstepoort strain of the CDV-N gene. Included in these was a single base pair change (0.5%) compared with the sequence of the

cDNA clone present in the laboratory (Mee and Sharpe 1993, 1994). In SSPE, there is a similar 2% mutation rate (see Section 2.6.4.5). The mutations seen in CDV in Paget's disease could favour persistence at the expense of viral replication, and might explain the lack of genomic RNA found in the *in situ* hybridisation studies.

3.3.4.3 Anti-virus antibody measurements

There are usually increased levels of specific antibodies in viral infections, however measurements of circulating antibodies to several paramyxoviruses have failed to show any differences between Paget's patients and controls (Morgan-Capner et al 1981; Winfield and Sutherland 1981; Basle et al 1982; Hamill et al 1986). A recent study investigating 83 Paget's patients also failed to find any significant difference in anti-CDV antibody levels between patients and controls (Gordon et al 1993). There was also no apparent change in antibody levels following treatment with bisphosphonates. However, several patients and some of the controls did have markedly elevated levels of anti-CDV antibodies. Also, it was possible with some patients to correlate anti-CDV antibody levels with results from *in situ* hybridisation studies. This revealed that patients positive for CDV by *in situ* had significantly lower levels of anti-CDV antibodies when compared with those that were negative using the same technique. It was concluded that anti-CDV antibodies probably play little or no direct role in the pathogenesis of Paget's disease (which might be expected due to the lack of inflammatory changes seen in Paget's disease (Kanis 1991; Anderson 1993)), and that failure to clear CDV during an initial infection might favour the sequestration of virus within bone cells and lead eventually to Paget's disease.

3.3.5 Biochemistry of the Pagetic Osteoclast

The role of paramyxoviruses in Paget's disease will remain unresolved until it can be shown that viral infection or gene expression in human bone cells produces a pagetic phenotype. However, what has never been in any doubt is the importance of osteoclasts in their role in initiating and maintaining the disease (Kanis 1991; Anderson 1993). However, it is only

recently that attention has focused on the biochemistry and cell biology of osteoclasts in Paget's disease.

3.3.5.1 Interleukin-6 and c-Fos

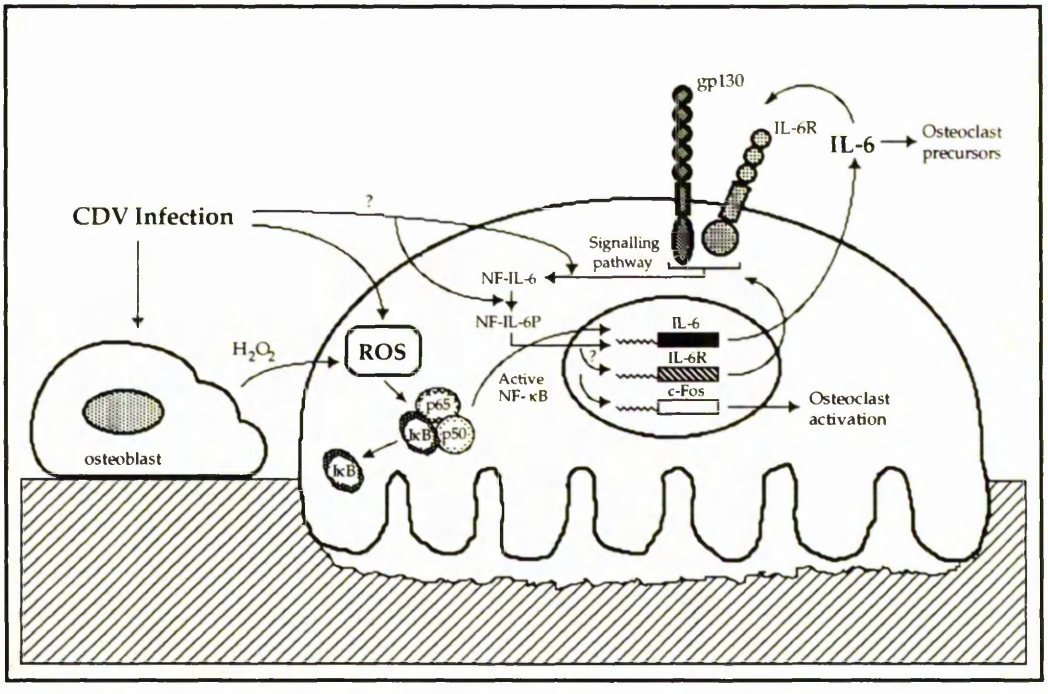
Interleukin 6 (IL-6) has recently been proposed as an important regulator of bone cells (Roodman 1992). IL-6 has been shown to be produced by osteoblasts and osteoblast-like cells *in vitro* (Feyen et al 1989; Lowik et al 1989; Ishimi et al 1990; Fang and Hahn 1991; Linkhart et al 1991; Littlewood et al 1991), and has been shown to be involved in the regulation of osteoclast formation and function. IL-6 has been shown to promote osteoclastogenesis and bone resorption in both murine (Lowik et al 1989; Ishimi et al 1990), and human (Kurihara et al 1990b; Ohsaki et al 1991; Roodman et al 1992) culture systems. Conditioned medium from long-term pagetic marrow cultures, which was shown to contain high levels of IL-6, has been shown to stimulate osteoclast-like cell formation in normal marrow cultures (Roodman et al 1992). The Paget's patients also had elevated IL-6 levels in their serum. These observations led to a proposal that IL-6 acts as an autocrine/paracrine factor, promoting the recruitment of osteoclast precursors from the marrow to the bone surface.

Further evidence for the importance of IL-6 has come from *in situ* hybridisation studies of expression of components of the IL-6 regulatory pathway, namely IL-6, IL-6 receptor (IL-6R) and IL-6 nuclear factor (NF-IL6) (Hoyland et al 1994). These have shown that *IL-6* gene expression is greatly elevated in pagetic osteoclasts compared with active non-pagetic osteoclasts, where it can hardly be detected. *IL-6R* gene expression is also elevated in pagetic osteoclasts and *NF-IL6* is equally expressed in pagetic and non-pagetic osteoclasts. Pagetic osteoclasts thus contain molecules necessary for IL-6 to act in an autocrine or paracrine manner and the increased expression of IL-6 itself can explain the elevated serum levels.

These results led to a proposed model for the pagetic osteoclast phenotype based on altered IL-6 biochemistry (Figure 3.3) (Hoyland et al 1994). The important feature of this model is

that it predicts a dual role for IL-6 in both osteoclast recruitment and activation. Infection of osteoclasts, or their precursors, by CDV or some other paramyxovirus results in transcription of viral genes whose products interfere with the activity of NF-IL6 post-translationally, possibly by protein phosphorylation which is known to activate NF-IL6 (Akira et al 1992; Trautwein et al 1993). The active NF-IL6 is then able to regulate expression of its target genes which include *IL-6* (leading to the observed increase in IL-6) and possibly *IL-6R*, although this link has not been established.

Figure 3.3 Proposed model of Paget's disease



Viral infection of osteoclasts and/or osteoblasts stimulates the production of ROS which in turn activate NF-κB by removal of the inhibitory IκB. NF-κB then up-regulates *IL-6* gene expression. Alternatively, the virus might act directly on the signalling pathway to NF-IL-6, or in activation of NF-IL-6, possibly by phosphorylation. Increased activity of NF-IL-6 then up-regulates *IL-6* and *IL-6R* gene expression. Increased IL-6 up-regulates c-*Fos* gene expression. Increased IL-6R levels result in an increased response to IL-6, increased levels of c-Fos stimulate osteoclast activity, and IL-6 in the serum promotes osteoclast precursor recruitment and/or fusion. (Adapted from Hoyland et al 1994).

Infection by RNA viruses such as human immunodeficiency virus and DNA viruses such as HCMV, for example, are known to induce IL-6 synthesis (Akira and Kishimoto 1992; Almeida et al 1994). Recently, MV has also been shown to induce IL-6 production in brain

cell cultures (Schneider-Schaulies et al 1993), with particularly high levels induced in persistently infected cells.

The activation of osteoclasts was proposed to be mediated by the action of IL-6 on expression of c-*Fos* (Hoyland et al 1994). This proto-oncogene is the cellular homologue of v-*fos*, which was originally isolated from murine osteosarcomas (Finkel et al 1966). IL-6 can up-regulate c-*Fos* expression in B cell differentiation (Korholz et al 1992) and in pheochromocytoma cells (Metz and Ziff 1991), and HCMV has also been shown to induce c-*Fos* (Boldogh et al 1990). Elevated levels of c-*Fos* gene expression have also been detected in pagetic osteoclasts (Hoyland and Sharpe 1994). These observations predict that increased c-Fos in pagetic osteoclasts would result in increased bone resorption. Although not shown directly, manipulation of c-*fos* expression in transgenic mice supports this prediction (see also Section 1.5.3). Overexpression of c-*fos* results in transgenic mice with osteosarcomas and increased bone resorption, particularly in the area of the growth plates (Rüther et al 1987, 1989), that has similarities to the histology of both Paget's disease (Kanis 1991; Anderson 1993) and MO (see Section 3.4.2). A similar effect is seen if c-*src* is disrupted (Soriano et al 1991). Down-regulation of c-fos via gene knock-out in transgenic mice results in osteopetrosis (Johnson et al 1992; Wang et al 1992). Thus, c-Fos appears to be essential for normal bone homeostasis, and alteration of the level of c-Fos shifts the balance between deposition and resorption.

3.3.5.2 Reactive oxygen species and NF-κB

Recent evidence has shown that hydrogen peroxide is a potent stimulator of bone resorption via a direct effect on osteoclasts (Bax et al 1992). The mechanism of this stimulation was not shown, although it was postulated that it might be due to activation of the transcription factor, NF-κB. NF-κB is present in many cells, including those of the immune system, and consists of three subunits: two are stimulatory, p50 and p65, the third is inhibitory, IκB. NF-κB p65 has been shown to potentiate the effects of c-Fos in HeLa cells (Stein et al 1993). Many agents, including viruses and hydrogen peroxide, are known to

activate NF-κB by releasing IκB from the active subunits (Schreck et al 1991; Roulston et al 1993). It has been postulated that these agents might all act via ROS (Schreck et al 1991). ROS have been shown to stimulate the formation of osteoclasts and bone resorption (Garrett et al 1990; Suda 1991) (see Sections 1.3.2.4 and 1.3.2.5) and have also been demonstrated in cultured canine brain cells infected with CDV (Bürge et al 1989) (see Section 2.7.2). ROS have also been shown to induce c-*fos* expression in mouse epidermal cells (Crawford et al 1988). These findings, together with the fact that NF-κB is known to induce IL-6 production (Libermann and Baltimore 1990; Shimizu et al 1990; Brach et al 1992; Gruss et al 1992; Brach et al 1993), can be incorporated into the IL-6-based model of Paget's disease (Figure 3.3), providing an alternative pathway for viral action, leading to up-regulation of IL-6. Support for this virus–NF-κB–IL-6 link has come from transgenic mice experiments, where expression of an HTLV-1 LTR tax transgene results in a phenotype with characteristics of Paget's disease (Ruddle et al 1993). Tax is known to stimulate NF-κB activity and production of a number of cytokines.

3.3.6 Paget's Disease – The Paramyxovirus Enigma

Persistent infection of cells *in vitro* is a long established property of paramyxoviruses (see Section 2.5). The most dramatic example of persistence *in vivo* is of MV in neuronal cells causing SSPE (see Section 2.6.4). Evidence for similar persistence of paramyxoviruses in other human diseases such as MS, Crohn's disease and AICAH and the canine disease, ODE has also been reported (see Sections 2.7.4.5 and 2.8). However, unlike in SSPE, ODE and possibly Crohn's disease, viral persistence has not yet been shown definitively to be the causative factor. Indeed it is possible that isolation of virus from infected tissues merely reflects the ability of paramyxoviruses to establish persistent infections in human tissues and is unrelated to the disease itself. Thus, with the exception of SSPE and possibly Crohn's disease, persistent infection of human tissues may be entirely passive and have no disease consequences. Indeed, the infection may arise as a consequence of the onset of disease.

Despite the now numerous reports of the connection between Paget's disease and paramyxovirus infection, the jury will remain out until a definite link can be shown. The challenge now is to establish any causative link, either by directly inducing viral gene expression in bone cells, or by the use of animal models or *in vivo* and *in vitro* studies which might help in the understanding of the biochemical changes in the pagetic osteoclasts that lead to their altered characteristics.

3.4 Idiopathic Canine Bone Disorders

There is no canine equivalent of human Paget's disease; this might be related to a number of factors, especially the shorter lifespan of dogs. However, several idiopathic canine bone diseases do exist which are characterised by excessive bone remodelling, for example, the conditions of panosteitis, craniomandibular osteopathy (CMO) and MO. Whilst it would have been of great interest to examine bone samples from all of these disorders, only samples from cases of MO became available, hence this condition will be described in more detail.

3.4.1 Panosteitis

Panosteitis (also known as enostosis and eosinophilic panosteitis) is a disease of unknown aetiology that usually affects dogs of the large breeds between 5 and 12 months of age (Bohning et al 1970; Watson 1990; Palmer 1993). Approximately 75% of reported cases involve German Shepherds, and over 60% of affected dogs are male.

The disease is characterised by episodes of mild to severe pain and lameness, which tends to be intermittent and shifting. Remissions and exacerbations are common, although the disease is usually self-limiting after a few months (Watson 1990; Palmer 1993). Anorexia and unwillingness to stand may be seen in severely affected dogs. The lameness is associated with abnormalities in the diaphyses of affected bones.

Radiographically, the disease is characterised initially by blurring and accentuation of the trabecular pattern, increased density of the medullary cavity and endosteum, and reduced contrast between the medullary cavity and the cortex (Bohning et al 1970). This is followed by the appearance of patchy or mottled radiodensities in the medullary cavity which are often associated with the nutrient artery. Periosteal new bone is seen in approximately one third of cases (Watson 1990). These changes gradually recede over several months to leave a normal appearance.

Histology reveals areas of increased osteoblastic and fibroblastic activity involving the endosteum, periosteum and medullary cavity. The fibrous tissue is replaced by woven bone which is then remodelled to form lamellar bone, before being removed during the following months (Bohning et al 1970; Palmer 1993). Most cases show no evidence of inflammatory cell infiltrates.

Diagnosis is based on clinical and radiographic features. Eosinophilia may be found in some cases, although this is not a reliable diagnostic feature. There is no specific treatment. Analgesics are used during periods of lameness, but, regardless of treatment, the disease usually continues for several months.

3.4.2 Craniomandibular Osteopathy

Craniomandibular osteopathy is a proliferative disease affecting primarily the mandibles, tympanic bullae and occasionally other bones of the head (Riser et al 1967; Alexander 1978; Watson 1990; Palmer 1993). Rarely, limb bones may also be affected (Riser et al 1967). Scottish and West Highland white terriers are most commonly affected, although the disease has been reported in Cairn and Boston terriers, and in larger breeds of dog such as the Doberman pinscher (Watson et al 1975). Affected dogs usually present between 4 and 10 months of age, with a history of discomfort in chewing, drooling of saliva and sometimes obvious mandibular swelling (Watson et al 1975; Alexander 1978; Watson 1990; Palmer 1993). The disease is usually self-limiting, although exacerbations can occur at intervals during the course of the disease, but these usually cease after about 1 year of

age. Pyrexia often accompanies these exacerbations, although haematological and biochemical data are normal (Palmer 1993). The bone changes usually then gradually regress, although persistent abnormalities can occur in some cases (Riser et al 1967; Alexander 1978; Watson 1990).

Radiographs are characterisitic and show varying degrees of bony thickening and proliferation around the mandibles, and often involving the tympanic and petrous temporal bones (Riser et al 1967; Alexander 1978; Watson 1990; Palmer 1993). If the limbs are affected, the lesions radiographically resemble those of the chronic stages of MO (Riser et al 1967; Watson 1990).

Histologic changes involve the endosteum, periosteum and trabecular bone, which show complex patterns of concurrent bone formation and resorption (Palmer 1993). This can give rise to an appearance similar to that of Paget's disease. Lymphocytes, plasma cells and neutrophils are sometimes seen in these areas. The areas of woven bone are eventually replaced by lamellar bone, which is then gradually resorbed.

The increased prevalence seen in related breeds is consistent with some genetic predisposition, and an autosomal recessive trait has been suggested in West Highland white terriers (Padgett and Mostosky 1986). Despite the presence of an inflammatory infiltrate, no infectious agent has ever been demonstrated.

Corticosteroids have been used in the treatment of CMO to relieve clinical signs and to reduce the bony lesions (Watson 1990). Analgesics also help to relieve the pain associated with the condition.

3.4.3 Metaphyseal Osteopathy

3.4.3.1 Introduction

Metaphyseal osteopathy is a skeletal disease of unknown aetiology, seen in young, fast-growing dogs, usually of the large and giant breeds. It has also been referred to as

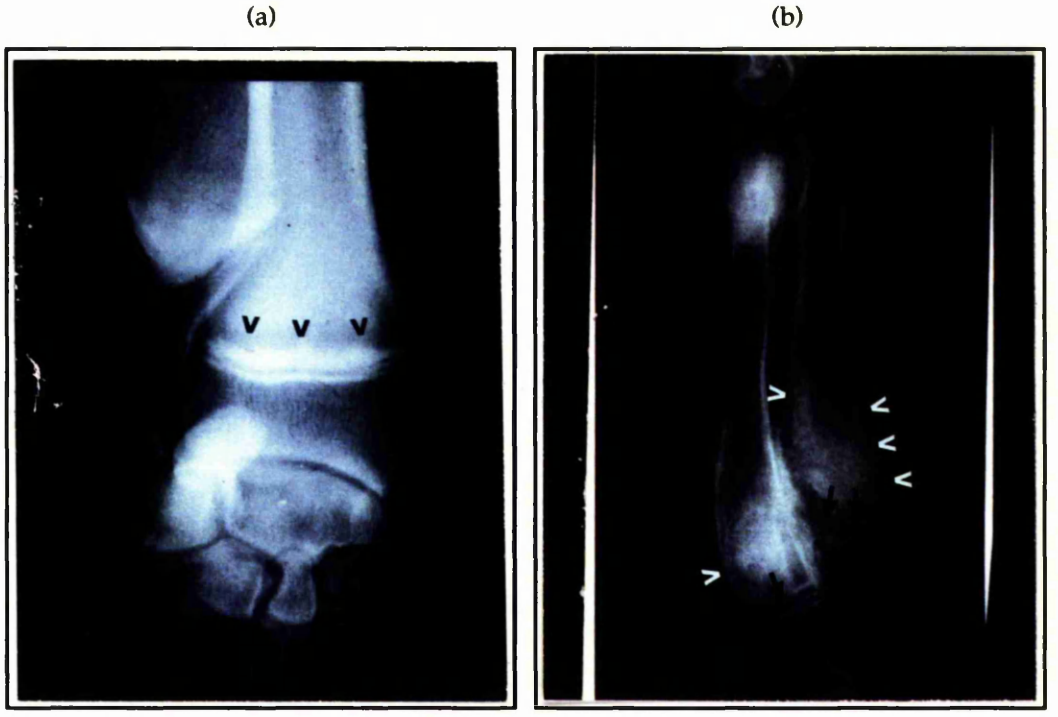
hypertrophic osteodystrophy (HOD) (Riser 1964), Möller-Barlow's disease, Barlow's disease (Merillat 1936), skeletal scurvy (Holmes 1962), hypovitaminosis C, osteodystrophy I and osteodystrophy II (Riser 1964).

3.4.3.2 Clinical signs

Clinical signs are usually seen between 3 and 6 months of age, though they can occur any time from 2 months upto 2 years. There is often a history of some other disease, such as diarrhoea, occurring several days before the onset of MO. In the acute stages, these signs consist of fever, anorexia and painful, swollen metaphyses (Meier et al 1957; Grøndalen 1976; Woodard 1982). The pain may be so great that affected animals cannot stand. Usually, the faster growing bones, such as the distal radius and ulna, are most severely affected, though many other bones, including the ribs and mandibles, may be involved. Radiographs taken in the acute stages show irregular, alternating radiodense and radiolucent lines parallel to the growth plate (Meier et al 1957; Grøndalen 1976; Woodard 1982) (see Figure 3.4). Soft tissue swelling is also usually seen. Most dogs recover clinically within a few days, but some go on to develop areas of periosteal and extraperiosteal ossification, resulting in hard, swollen metaphyses (hence the term HOD). In severe cases, this bone formation may progress to involve almost the whole length of the affected bone (Meier et al 1957; Grøndalen 1976; Woodard 1982) (see Figure 3.5). These changes can cause permanent deformity, though, if dogs do survive, the excessive bone is usually remodelled and gradually removed. Remissions and relapses sometimes occur and affected dogs can die, though they are more commonly euthanased on humane grounds. If relapses do occur, radiographs may reveal several radiolucent lines, corresponding to the repeated incidents of disease. Dogs that suffer from relapses also tend to show signs of new bone deposition.

The variations in clinical signs seen at different stages of the disease suggest that MO is probably better described as a disease syndrome, with several recognised types or subtypes.

Figure 3.4 Metaphyseal osteopathy – radiographic features

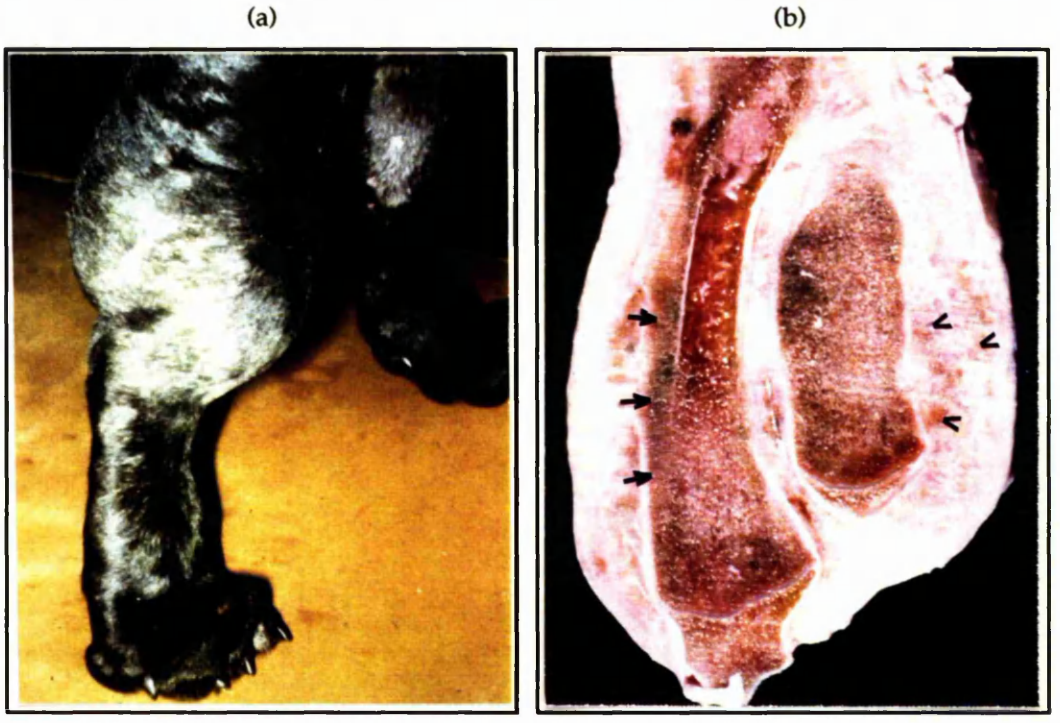


(a) acute and (b) chronic radiographic features of MO. Note in (a) radiolucent lines parallel to growth plates (arrow heads). Note in (b) areas of bone resorption (arrows) and new bone formation (arrow heads).

3.4.3.3 Pathology and treatment

Histologically, changes are seen most prominently in the primary spongiosa, where there is elongation and failure of ossification of the cartilaginous lattice (Grøndalen 1976; Woodard 1982). There is also a marked inflammatory infiltrate (neutrophils and lymphocytes) adjacent to the primary spongiosa and extending between the trabeculae, and this is accompanied by trabecular necrosis and microfractures which may cause infraction across the metaphysis. Defective bone formation occurs on the diaphyseal side of the infraction, due to osseous tissue being deposited on fractured and necrotic trabeculae. Subperiosteal inflammation and haemorrhages occur, leading to the new bone formation seen in the later stages of the disease. The characteristic histological features of MO are shown in Chapter 5 (Results).

Figure 3.5 Metaphyseal osteopathy (chronic) – gross features



(a) gross and (b) post-mortem features of MO. Note swelling and deformity of the affected limb grossly (a). Note new bone formation (arrows) and soft tissue calcification (arrow heads) (b).

Neutrophilia and lymphopenia may be found during the active stages of the disease, but are not consistent and are thought to be due to stress and the inflammatory changes.

Ascorbic acid has been advocated as a treatment for MO, but is now generally considered to be of no use (see Section 3.4.3.4). As the cause of the disease is still unknown, treatment is symptomatic, and consists of the use of anti-inflammatory and analgesic drugs (Bennett 1976; Grøndalen 1976; Woodard 1982).

3.4.3.4 Aetiology

Many suggestions have been put forward as to the cause of MO, but none have been proved.

The disease was originally thought to be caused by a lack of vitamin C, due to the radiographic similarities to infantile scurvy in humans, and to findings of low serum levels

of the vitamin (Merillat 1936; Meier et al 1957; Holmes 1962; Watson et al 1973; Grøndalen 1976). Several authors have claimed successful treatment with ascorbic acid (Rendano et al 1977; Vaananen and Wikman 1979), however, others have found that treatment with ascorbic acid had no significant effect on recovery from the disease, as treated and untreated dogs had similar rates of recovery (Bennett 1976; Grøndalen 1976; Woodard 1982). In Woodard's study of the disease in a litter of Weimaraners (1982), the levels of liver ascorbic acid (which are a more reliable marker of vitamin C status) were measured and were found to be within normal limits. One group even found that treatment with vitamin C exacerbated the bony lesions (Teare et al 1979), and suggested that vitamin C is contra-indicated in the treatment. In humans with scurvy, treatment with vitamin C leads to a rapid, complete recovery, which is not consistently seen with treatment in dogs. Also, in humans, fragility of capillaries leads to bleeding, often seen in the mouth, which also does not occur in dogs. Since MO usually spontaneously regresses, and vitamin C levels vary with stress and malnutrition (both of which occur in metaphyseal osteopathy), supposed successful treatments with vitamin C are difficult to assess, and it is now generally accepted that there is some other cause.

Another suggestion by some authors, is that overnutrition plays a role in the disease (Hedhammar et al 1974; Hazewinkel et al 1985; Goedegebuure and Hazewinkel 1986). However, the lesions produced experimentally differed both radographically and histologically from the natural disease and pyrexia was not seen. Bennett (1976) suggested that excess vitamin and mineral intake may play a role. However, not all dogs affected with the disease have a history of vitamin and mineral supplementation, and many dogs that do receive supplementation do not develop clinical signs of MO. The fast growth that occurs due to oversupplementation may play some part in the disease process, but there must be some other factor involved.

Copper deficiency in rats, caused by feeding them thiomolybdate, produces radiographic changes similar to those of MO (Spence et al 1980). Similar experiments in dogs produced thickening of the growth plates and sometimes metaphyseal radiolucencies and bone pain

(Read 1984). However, the histological changes produced were not the same as those seen in MO.

The history and clinical signs associated with MO are suggestive of an infectious cause. The disease is often seen after a previous incident such as diarrhoea or upper respiratory tract infection. The occurence of pain and swelling, pyrexia, neutrophilia and the histological evidence of suppurative inflammation all support the theory that some infectious agent is partly or wholly responsible for the disease. However, attempts to isolate an organism and to transmit the disease to other dogs have proved unsuccessful (Grøndalen 1979; Woodard 1982). Despite this, some authors suggest that the possibility of an infectious cause deserves more investigation (Watson 1990; Palmer 1993). Palmer (1993) suggested that infectious canine hepatitis virus (ICHV) might be involved in MO, as the virus is known to cause metaphyseal haemorrhages, however, no scientific evidence exists to support this hypothesis.

3.5 Aims of Thesis

The aims of this thesis were threefold:

1. To establish whether CDV can infect and actively replicate in canine bone cells of dogs naturally infected with the virus and, if so, what pathological effects this might have.
2. To determine whether CDV is involved in any naturally occurring canine bone disorders, and whether any of these might serve as a model of Paget's disease.
3. To attempt to establish an *in vitro* canine model of Paget's disease.

It was felt that *in situ* hybridisation and RT-PCR techniques could be used to examine for the presence of CDV in bones from dogs with natural distemper to satisfy the first aim. Histological examination of the same samples would show any pathological changes.

To satisfy the second aim, several idiopathic canine bone disorders with a possible infectious aetiology were identified (panosteitis, CMO and MO), and the same molecular

techniques were to be used to examine for the presence of CDV in bone samples from dogs with these disorders. However, no samples from dogs with panosteitis or CMO could be obtained, and so only samples from dogs with MO were examined.

It was decided that any prospective model of Paget's disease would have to involve the osteoclast. Of the current culture systems available for examining osteoclast behaviour, it was felt that marrow culture systems would prove the most useful, as they would hopefully allow the examination of the effects of CDV on both osteoclast formation and activity. This system would allow the comparison of cultures of marrow cells from cases of natural distemper and from normal dogs, and would also allow the examination of the effects of *in vitro* addition of CDV to cultures from both sets of dogs. The presence or absence of virus would be confirmed using *in situ* hybridisation and RT-PCR techniques. It was hoped to be able to also culture marrow cells from dogs with MO, but no samples became available for this.

To further establish whether this culture system could serve as a model for Paget's disease, it was decided to examine the effects of CDV on both *IL-6* and c-*Fos* induction in the marrow cells. Again, *in situ* hybridisation techniques would be used to identify these factors in the cells.

Chapter 4

Materials and Methods

4. Materials and Methods

4.1 Abbreviations

BSA	Bovine serum albumin
$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
FCS	Foetal calf serum
IPTG	Isopropyl-ß-D-thio-galactopyranoside
MEM	Minimum essential medium
OD	Optical density
PBS	Phosphate buffered saline
RT-PCR	Reverse transcriptase-polymerase chain reaction
RNase	Ribonuclease
RNasin	RNase inhibitor
SDS	Sodium dodecyl sulphate
TRAP	Tartrate resistant acid phosphatase
Tris	Tris (hydroxymethylamino) methane
X-gal	5-bromo-4-chloro-3-indolyl-ß-galactoside

4.2 Solutions and Media

Denaturing solution	4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% N-laurylsarcosine, 0.1M ß-mercaptoethanol
Denhardts	1% ficoll, 1% polyvinylpyrrolidone, 1% BSA

Hydrolysis buffer	0.4M sodium hydrogen carbonate, 0.6M sodium carbonate pH 10.0
LB medium	1% bactotryptone (Oxoid), 0.5% yeast extract (Oxoid), 0.25M sodium chloride, pH 7.5
LB agar	as LB medium, with 1.5% bacto agar (Oxoid)
Neutralising solution	0.5M Tris-HCl pH 7.6, 1M sodium chloride
NTE buffer	0.5M sodium chloride, 10mM Tris-HCl pH 8.0, 1mM EDTA
Solution I	50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA
Solution II	1% SDS, 0.2M sodium hydroxide
Solution III	3M potassium acetate, 11.5% glacial acetic acid
1X SSC	150mM sodium chloride, 15mM sodium citrate, pH 7.0
1X TBE	0.089M Tris, 0.089M boric acid, 0.002M EDTA
TE	10mM Tris-HCl, 1mM EDTA pH 8.0
Transfer solution	0.4M sodium hydroxide, 0.6M sodium chloride
Wash buffer	50% de-ionised formamide, 0,3M sodium chloride, 20mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 10mM DTT, 1X Denhardts

4.3 *Escherichia coli* Strains

All *E. coli* were grown at 37°C either in a orbital shaking incubator (New Brunswick Scientific) at 280rpm (media), or in an oven (plates). They were grown in autoclaved flasks, sterile tubes (Northern Media) or on culture plates (Northern Media or Falcon).

4.3.1 Transformation of *E. coli*

Cells used were of two types:

1. JM101 – Genotype: Δ*lacpro*, *thi*, *sup*E, F', *tra*D36, *pro*AB, *lac*IqZΔM15 (Amersham)
2. DH5 – Genotype: *sup*E44, *hsd*R17, *rec*A1, *gyr*A96, *thi*-1, *rel*A1 (BCL)

Cells were stored streaked on LB plates at 4°C. A single colony of cells was inoculated into 5ml LB and grown overnight. Twenty ml LB was then inoculated with 1ml of overnight

culture, and incubated for 90 minutes. The cells were then centrifuged at 2000g for 10 minutes and resuspended in 5ml 5mM sodium chloride. They were then centrifuged again for 5 minutes and resuspended in 2ml 100mM calcium chloride and incubated on ice for 20 minutes. After a further centrifugation for 5 minutes, the cells were resuspended in 1ml calcium chloride and, for each transformation, 100µl of resuspended cells was incubated with 0.5-5µl DNA on ice for 60 minutes. The cells were then heat shocked for 5 minutes at 42°C and kept on ice for a further 2 minutes, prior to the addition of 800µl LB, and incubated at 37°C for 60 minutes. The cells were then smeared on LB plates containing 20mg/ml ampicillin (Sigma) and incubated overnight.

If blue/white colour selection was required (JM101s), the LB plates were also treated with 50mg/ml X-gal in DMSO and 23.3mg/ml IPTG prior to incubation.

4.4 Plasmid DNA Preparation

4.4.1 Mini-preparation

Individual E. coli colonies were inoculated into 5ml LB containing 50µg/ml ampicillin, and grown overnight. Aliquots of 150µl were removed and stored at 4°C for subsequent large-preparations, whilst the remainder of the cells were centrifuged at 2000g for 10 minutes. The pellet was then resuspended in 100µl solution 1, containing 2mg/ml lysozyme (Sigma), and incubated for 5 minutes at room temperature. Then, 200µl solution 2 was added and incubated for a further 5 minutes. Following this, 150µl ice cold solution 3 was added and the mixture was left on ice for 5 minutes, prior to centrifugation at 10000g for 5 minutes. The supernatant was then phenol/chloroform extracted and the DNA was precipitated in ethanol for 20 minutes at -20°C. The DNA was then washed in 70% ethanol, dried under vacuum and resuspended in 20µl TE. Plasmid DNA was analysed by restriction enzyme digest, visualised on 1% agarose gels.

4.4.2 Maxi-preparation

The 150µl aliquot of cells (see above) containing the desired insert was then inoculated into 500ml LB containing 50µg/ml ampicillin and incubated overnight. The cells were centrifuged at 5000rpm in a Beckman JA10 rotor at 4°C for 20 minutes, resuspended in 20ml solution 1, containing 5mg/ml lysozyme, and incubated for 10 minutes at room temperature. Forty ml solution 2 was then added and the mixture incubated for 5 minutes on ice, prior to the addition of 20ml solution 3 and a further incubation on ice for 10 minutes. After centrifugation for 15 minutes at 5000rpm at 4°C, the supernatant was mixed with 0.6vol propan-2-ol and left at room temperature for 60 minutes. The mixture was then centrifuged at 5000rpm for 25 minutes at 4°C, and the air-dried pellet was resuspended in 4ml TE, 4.25g caesium chloride and 330µl ethidium bromide (10mg/ml). The mixture was sealed into Beckman quickseal tubes and centrifuged for a minimum of 8hr at 50000rpm in a Beckman L8-70M ultracentrifuge. The supercoiled plasmid band was removed from the tube with a needle and syringe diluted with water to 5ml, and the ethidium bromide removed by repeated extraction with water-saturated butan-2-ol. The DNA was then precipitated overnight at -20°C in 2vol ethanol, and then centrifuged at 2000g for 10 minutes, washed in 70% ethanol, dried under vacuum and resuspended in 100µl TE.

Alternatively, mini- and maxi- preparations were performed using Promega Magic™ Prep kits, according to the manufacturer's instructions.

Purity and concentration of DNA was determined by spectrophotometry (Maniatis et al 1982). The OD of the DNA was recorded at 260nm and 280nm and the purity determined by the OD_{260}:OD_{280} ratio. Pure DNA gives a ratio of 1.8. The OD_{260} also gives a value for the concentration; an OD_{260} of 1 is equivalent to 50µg/ml DNA.

4.5 *In Situ* Hybridisation

All buffers were DEPC treated (0.1%) for 3 hours before autoclaving, and all glassware was rinsed in 0.1% DEPC prior to baking at 200°C for at least 4 hours.

4.5.1 Tissue Samples and Preparation

The samples taken from each case are summarised in Table 4.1.

Table 4.1 Samples obtained for *in situ* hybridisation

Case Number	Age	Sex	Breed	Diagnosis	Samples
1	4 weeks	Male	X	CDV+	L proximal femur Bladder Spleen
2	9 weeks	Female	X	CDV+	L proximaltibia Bladder
3	20 weeks	Male	X	CDV+	R proximal tibia Bladder
4	20 weeks	Female	X	CDV+	L distal radius Bladder
5	8 weeks	Male	GSD	CDV-	R distal radius
6	20 weeks	Male	Shar Pei	CDV-	L distal femur Bladder
7	20 weeks	Female	X	CDV-	R proximal tibia
8	16 weeks	Male	Japanese Akita	MO	L proximal tibia R proximal tibia L distal tibia R distal radius R distal ulna
9	13 weeks	Female	Boxer	MO	R distal radius R distal ulna Bladder Spleen
10	20 weeks	Male	Border Collie	MO	L distal radius
11	24 weeks	Male	GSHP	MO	R distal radius
12	24 weeks	Male	Great Dane	MO	R distal radius
13	16 weeks	Female	GSD	MO	L distal radius
14	9 weeks	Female	Gordon Setter	CDV-	L distal radius Bladder Marrow
15	24 weeks	Male	Beagle*	CDV-	R distal radius Bladder Marrow
16	24 weeks	Female	Beagle*	CDV-	L distal radius Bladder Marrow
17	24 weeks	Female	GSD	CDV-	L distal radius Bladder Marrow
18	12 weeks	Female	X	CDV+	L distal radius Bladder Marrow
19	19 weeks	Male	Greyhound	CDV-	R distal radius Bladder Marrow

Breed: X – Crossbreed, GSD – German Shepherd (Alsatian), GSHP – German Short haired Pointer, * Beagles obtained from Intervet, Diagnosis – Based on clinical signs: CDV+ distemper infection, CDV- no signs of distemper infection, MO Metaphyseal osteopathy,
Samples: L – left, R – right

Bone tissue was obtained from the long bones, under sterile conditions, either at autopsy (within 30 minutes of euthanasia), or at surgery. Samples were taken at surgery using a

bone trephine which produced cores of approximately 7mm diameter. Bladder and spleen samples were taken only from those dogs that were autopsied.

Samples were fixed in 10% buffered formalin for 24 hours, and the bone samples were then decalcified in 10% EDTA at 4°C until there was radiographic evidence of complete decalcification. The samples were then processed for routine paraffin-wax embedded histology, and sections were cut to a thickness of 7μm.

4.5.2 Slide and Coverslip Preparation

4.5.2.1 Organosilanation of slides

Slides were prepared according to the method of Rentrop et al (1986). Slides were washed overnight in Decon and then rinsed in RNase-free water, prior to baking at 200°C for 3hr. They were then dipped in 3% γ-aminopropyl-triethoxysilane (Sigma) in dry acetone, rinsed in 2 changes of acetone, then RNase-free water and baked at 37°C overnight. Slides were used up to 2 months after treatment.

4.5.2.2 Siliconisation of coverslips

Coverslips were washed in 1M HCl for 15 minutes, rinsed in RNase-free water and baked at 80°C until dry. They were then dipped in 5% dimethylsilane (Sigma) in xylene, dried at 80°C, rinsed in RNase-free water and then baked at 200°C overnight.

4.5.3 Preparation of Riboprobe

The required DNA templates were linearised with the appropriate restriction enzyme, extracted with phenol/chloroform and then precipitated with sodium acetate and ethanol. The DNA pellet was then washed with 70% ethanol, dried under vacuum and resuspended in 10μl TE. A small aliquot was then run on a 1% agarose gel in 1X TBE to check that the template was completely linearised.

Sense and antisense riboprobes were generated using the appropriate SP6, T3 or T7 RNA polymerase and ^{35}S-UTP (>37Bq/mM>1000Ci/mM) (Amersham), using a riboprobe kit according to the manufacturers instructions (Boehringer Mannheim). Briefly, probes were transcribed at 37°C for 90 minutes in a reaction containing 1X transcription buffer, 10mM DTT, 1u/µl RNasin, 25mM each of rATP, rCTP and rGTP, 12µM rUTP, 0.2-1µg linearised template, 50µCi ^{35}S-UTP (>400 Ci/mM) and 1µl RNA polymerase in a final volume of 20µl. The transcription reaction was terminated by adding 1u/µl RNase-free DNase, 1u/µl RNasin, 50µg tRNA (10mg/ml) and RNase-free water to 100µl and incubating for 10 minutes at 37°C. The RNA was then precipitated overnight at -20°C with 50µl 7.5M ammonium acetate and 375µl ethanol. The RNA pellet was then washed in 70% ethanol and either resuspended in 20µl 100mM DTT, 1u/µl RNasin (if the probe was less than 250bp long), or hydrolysed (if the probe was greater than 250bp). Probes were reduced to approximately 250 base pairs by limited alkaline hydrolysis (Angerer et al 1987) prior to hybridisation. For this, the RNA was dissolved in 160µl RNase-free water and 40µl hydrolysis buffer, and incubated at 60°C for a time defined by the equation:

$$t = \frac{L_o - L_f}{k L_o L_f}$$

Where t = time in minutes, L_o = initial probe length in kb, L_f = final probe length in kb and k = rate constant of hydrolysis = 0.11. This reaction was terminated by adding 3µl 3M sodium acetate and 1.3µl glacial acetic acid. The RNA was then precipitated with 50µg tRNA and 500µl ethanol, overnight at -20°C. The probes were then resuspended in 20µl 100mM DTT, 1 u/µl RNasin, as above, and 1µl was taken from each sample for radioactivity counting.

Probes were generated to the CDV-N gene (consisting of the final 500 base pairs of the 3' end of mRNA), and to the MV-N gene (consisting of a 183bp fragment, from base pairs 1379-1559) (both obtained from Dr SL Cosby, Queen's University, Belfast). A probe to the human ß-actin gene (a 1kb fragment) served as a positive control, and a probe transcribed from a random SP6 vector sequence (Promega) served as a further negative control.

4.5.4 *In Situ* Hybridisation

Sections were floated in RNase-free water at 50°C, onto organosilanated slides, and the slides were placed on a slide warmer at 50°C for 2 hours. The sections were then adhered to the slides in a 60°C oven overnight.

The hybridisation techniques used were those described by Angerer et al (1987) and modified by Gordon et al (1991,1992). Briefly, the sections were dewaxed in 2 changes of xylene for 10 minutes each and then rehydrated through a series of ethanol (99%, 90%, 70%, 50%) for 2 minutes each, and finally into water. The sections were then permeabilised with 0.2M HCl for 20 minutes at room temperature, followed by 10µg/ml proteinase K in 100mM Tris, 50mM EDTA, pH 8.0 for 30 minutes at 37°C. Non-specific background was reduced by treatment with 0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0, for 10 minutes at room temperature. Control slides were rendered free of RNA by treatment with 100µg/ml RNase A for 30 minutes at 37°C.

Hybridisation was performed at 50°C in 50% de-ionised formamide, 0.3M sodium chloride, 20mM Tris, pH 8.0, 5mM EDTA, pH 8.0, 10mM DTT, 10% (w/v) dextran sulphate, 1X Denhardts, 1mg/ml tRNA, overnight in a sealed, humidified container. Approximately $1x10^5$ counts of riboprobe was added to each section. Sections were covered with siliconised coverslips to prevent evaporation and ensure complete coverage of the section.

Post-hybridisation washes were in 2X SSC, 10mM DTT at room temperature for 60 minutes, wash buffer for 4 hours at 50°C, NTE containing 20µg/ml RNase A at 37°C for 30 minutes, NTE at 37°C for 30 minutes and then wash buffer at 50°C overnight.
The sections were then washed at room temperature in 2X SSC for 30 minutes, followed by a further 30 minutes in 0.1X SSC. They were then dehydrated in 70% ethanol, 300mM ammonium acetate for 2 minutes, 90% ethanol, 300mM ammonium acetate for 2 minutes and finally, 100% ethanol for 2 minutes.

After air drying, the sections were dipped in Ilford K5 emulsion that had been melted at 45°C and diluted 1:1 with distilled water. The slides were dried in a light-tight box for 1

hour at room temperature and then placed in a light-tight container and left at 4°C for 1 week.

The slides were then developed for 5 minutes in Ilford Phenisol (diluted 1+4 with water), rinsed in Ilford stop bath for 30 seconds and then fixed for 5 minutes in Ilford fixer. They were then washed for 15 minutes in distilled water prior to staining.

The sections were counterstained with filtered Harris' haematoxylin for 3 minutes, rinsed in running tap water for 5 minutes, then stained with eosin/yellow (2%) for 15 seconds and rinsed in tap water. Sections were then dehydrated through a series of ethanol, cleared in xylene and mounted using Practamount, prior to visualisation by light- and dark- field microscopy.

4.6 Polymerase Chain Reaction and Southern Blotting

All molecular biology reagents and enzymes were purchased from Promega UK, or Boehringer Mannheim. To prevent contamination, all reagents were aliquoted and discarded following single usage (Innis et al 1990).

4.6.1 Tissue Samples and Preparation

The samples taken from each case are summarised in Table 4.2.

Bone tissue was obtained from the metaphyseal regions of the long bones, under sterile conditions, either at autopsy (within 30 minutes of euthanasia), or at surgery. Samples were taken at surgery using a bone trephine which produced cores of approximately 7mm diameter. At autopsy, the whole of the metaphysis was removed from the bone using a hacksaw. The samples were placed into liquid nitrogen and stored at -70°C until subsequent RNA extraction.

Table 4.2 Samples obtained for PCR

Case Number	Age	Sex	Breed	Diagnosis	Samples
8	16 weeks	Male	Japanese Akita	MO	L proximal tibia R proximal tibia
14	9 weeks	Female	Gordon Setter	CDV-	R proximal tibia
15	24 weeks	Male	Beagle*	CDV-	R proximal tibia Marrow
16	24 weeks	Female	Beagle*	CDV-	L proximal tibia Marrow
17	24 weeks	Female	GSD	CDV-	L distal radius Marrow
18	12 weeks	Female	X	CDV+	L distal radius Marrow
19	19 weeks	Male	Greyhound	CDV-	R distal radius Marrow

Breed: *Beagles obtained from Intervet, GSD – German Shepherd (Alsatian), X – Crossbreed, Diagnosis – based on clinical signs: CDV+ distemper infection, CDV- no signs of distemper infection, MO Metaphyseal osteopathy, Samples: L – left, R – right

4.6.2 Total RNA Extraction

After fragmentation in a liquid nitrogen cooled 'bomb', total RNA was extracted according to a modification of the method of Chomczynski and Sacchi (1987). Briefly, the bone particles were homogenised in denaturing solution (approximately 10ml/g tissue), for 15 seconds in a polytron. Then 0.1vol 2M sodium acetate pH 4.0 was added, followed by 1vol water-equilibrated phenol and 0.2vol chloroform:methylbutan-1-ol (49:1). The tube was shaken and the mixture was then centrifuged for 10 minutes at 2000g. The aqueous layer was then mixed with 1vol propan-2-ol and centrifuged at 2000g for 10 minutes. The pellet was dried under vacuum and resuspended in 500µl denaturing solution. An equal volume of propan-2-ol was added and the mixture was incubated for 60 minutes at -20°C, prior to centrifugation at 2000g for 10 minutes. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 500µl RNase-free water, and incubated with 10u RNase-free DNase for 30 minutes at 37°C. The mixture was then phenol/chloroform extracted and precipitated in ethanol at -20°C overnight. After centrifugation at 10000g for 10 minutes, the pellet was washed with 70% ethanol, dried under vacuum and resuspended in RNase-free water.

The purity and concentration of RNA was determined by spectrophotometry as before (Section 4.4.2), except that the OD_{260}:OD_{280} for pure RNA is 2, and an OD_{260} of 1 is equivalent to 40µg/ml RNA.

4.6.3 Reverse Transcription

Single-stranded DNA was synthesised by incubating approximately 20µg total RNA with 10u avian reverse transcriptase enzyme, 1µg random hexamers, 1X reverse transcriptase buffer, 10mM DTT, 1u/µl RNasin, 2mM sodium pyrophosphate and 10mM each of dATP, dCTP, dGTP and dTTP, in a final volume of 20µl, at 42°C for 60 minutes. A further 10u of enzyme was then added and the incubation continued for a further 60 minutes. The mixture was then heated to 95°C and cooled on ice, to terminate the reaction.

4.6.4 PCR Primers

The details of the primers used are summarised in Table 4.3.

Table 4.3 Details of the primers used for PCR

Primer	Sequence 5′–3′	Location		Product Size
1	GCTGACAATTCAATATATT	CDV-N	450-470	
2	GCATAACTCCAGAGCAGTGG	CDV-N	961-980	556bp*
3	GTGCACTCGGAAAGATCCGA	CDV-N	1231-1250	
4	CCTCATCTTGGCGATTGCTT	CDV-N	1444-1464	249bp*
5	CAACAAAGAAGGGTAGGTTGG TGAATTTAGA	MV-N	601-630	
6	TGGAGGGTAGGCGGATGTTGTT GTTCTGGCCCT	MV-N	1596-1620	1019bp
7	CGTTGCTATCCAGGCTGTGC	ß-actin	2130-2150	
8	GTAGTTTCGTGGATGCCACA	ß-actin	2641-2661	434bp

CDV-N – Canine distemper virus nucleocapsid gene, MV-N – Measles virus nucleocapsid gene.
* Includes cloning sites.

Specific primers were manufactured (Cruachem Ltd, Glasgow, UK) to encompass the region of the CDV-N gene from position 450 to 980, and from position 1231 to 1464. Cloning sites were included, hence the expected fragment sizes were 556bp and 249bp, respectively. Primers were also made for the MV-N gene (601 to 1620) and (as a positive control) for exons 4 and 5 of the ß-actin gene. These gave fragments of 1019bp and 434bp, respectively.

4.6.5 PCR Reaction

Aliquots (5µl) from the reverse transcription reaction were amplified in a solution of 20mM Tris-HCl pH 8.0, 1.5mM $MgCl_2$, 100mM each of dATP, dCTP, dGTP and dTTP, 10pmol each of the specific primers and 2.5u of Taq DNA polymerase. The reaction volume was made up to 100µl with RNase-free water and 2 drops of mineral oil were added to each tube, to prevent evaporation during the amplification reactions. The denaturation (94°C for 50 seconds), annealing (55°C(CDV and ß-actin) or 65°C(MV) for 1 minute) and extension (72°C for 1 minute) reactions were repeated 40 times in a Techne cycler. To ensure complete strand synthesis, a final extension step at 72°C for 10 minutes was carried out. As a negative control, reverse transcribed tRNA was also used with each set of primers.

After the final extension reaction, 20µl aliquots from each reaction were run in a 2.5% agarose gel in 1X TBE.

4.6.6 Southern Blotting

To confirm the identity of the bands of expected size, the DNA was transferred from the gel onto Gene Screen Plus membrane (NEN® Research Products) and Southern blotted according to standard procedures (Ausubel et al 1987). Briefly, after the desired band separation had occurred, the gel was soaked in transfer solution for 30 minutes, and blotted onto the membrane overnight. The membrane was then soaked in neutralising solution for 30 minutes and dried.

The cDNA probe to CDV was labelled with ^{32}P-dCTP using a Prime-a-Gene kit (Promega). The probe was denatured, and hybridisation was carried out overnight at 65°C in 1M

sodium chloride, 1% SDS, 10% dextran sulphate, 50mM Tris-HCl pH 7.5 and 25µl of denatured, sonicated salmon sperm (10mg/ml) per 10ml hybridisation mixture. Membranes were washed in 2X SSC for 15 minutes at room temperature, 2X SSC/1% SDS for 15 minutes at 65°C twice, and 0.1X SSC for 15 minutes at room temperature. Autoradiography was then carried out at -70°C.

4.7 Bone Marrow Cultures

4.7.1 Tissue Samples and Preparation

The case details are summarised in Table 4.3.

Table 4.4 Samples obtained for marrow cultures

Case Number	Age	Sex	Breed	Diagnosis	Samples
14	9 weeks	Female	Gordon Setter	CDV-	Marrow
15	24 weeks	Male	Beagle*	CDV-	Marrow
16	24 weeks	Female	Beagle*	CDV-	Marrow
17	24 weeks	Female	GSD	CDV-	Marrow
18	12 weeks	Female	X	CDV+	Marrow
19	19 weeks	Male	Greyhound	CDV-	Marrow

Breed: * Beagles obtained from Intervet, GSD – German Shepherd (Alsatian), X – Crossbreed, Diagnosis – based on clinical signs: CDV+ distemper infection, CDV- no signs of distemper infection

Following euthanasia, the humeri, femora and tibiae were excised and the epiphyses were removed. Bone marrow cells were flushed from the diaphyses with a 1.1% EDTA/0.9% sodium chloride solution and then an equal volume of culture medium was added (MEM supplemented with 20% heat inactivated FCS, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin (GIBCO)). The cell suspension was then layered on a Ficoll-Hypaque density gradient (Histopaque-1077, Sigma) and centrifuged at 500g for 10 minutes at room temperature. The mononuclear cells were carefully removed and resuspended to 10^7 cells/ml in fresh culture medium. The cells were incubated in the presence or absence of varying concentrations (see Chapter 5) of 1,25-$(OH)_2$ D_3 (obtained from Dr M Hayes, Department of Medicine, University of Manchester, UK) and the Onderstepoort strain of

CDV (obtained from Dr S Chalmers, Intervet, UK) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were grown in 24 well tissue culture plates (Falcon Multiwell, Becton Dickinson Labware, USA) (1 ml/well), 8 well tissue culture slides (Labtek chamber/slides, Miles Scientific, USA) or 25 cm^2 culture flasks (Costar Corporation, USA). Cultures were replenished the following day, and then every three days, by replacing half the medium with fresh medium and 1,25-$(OH)_2$ D_3.

4.7.2 Demonstration of TRAP Activity

After 8 days incubation, the cells were washed twice with Dulbecco's Ca^{2+}- and Mg^{2+}-free PBS (GIBCO BRL) and then fixed with acetone-formaldehyde (Sigma). The cells were then stained for the presence of TRAP activity using the Acid Phosphatase, Leukocyte kit (Sigma) according to the manufacturer's instructions.

Cells were counted in five high power fields, in duplicate samples from each dog, and the final results were expressed as an average percentage. Cells were classified as TRAP negative (-) mononuclear cells, TRAP positive (+) mononuclear cells, small TRAP+ multinucleated cells (3-5 nuclei) or large TRAP+ multinucleated cells (>5 nuclei). There were a few fibroblasts and TRAP- multinucleated cells, but they were randomly distributed and were not present in all fields examined, so they were not counted.

4.7.3 Estimation of the Size of Multinucleated Cells

The size of the large multinucleated cells was estimated in two ways. The nuclei in fifty cells in a random field were counted and the mean calculated for each sample. Also, the total surface area covered by the multinucleated cells was compared by counting the number of squares on a grid that were occupied by multinucleated cells in five high power fields, and expressing this as an average percentage.

4.7.4 Measurement of Bone Resorption

Slices of devitalised bovine cortical bone (5 x 5 x 0.5 mm) were prepared using a low speed Isomet saw (Buehler Ltd, USA) (provided by Dr R Cooper, Department of Pathological Sciences, University of Manchester, UK). Slices were added to the wells prior to the addition of cells. Following incubation for two weeks, the cells were removed by gently rubbing the surface. The slices were then briefly counterstained with toluidine blue and examined by conventional light microscopy for the presence of resorption pits.

4.7.5 *In Situ* Hybridisation and PCR on Marrow Cells

These experiments were essentially the same as those described previously, except that:

1. *In situ* hybridisation – cells were fixed using acetone-formaldehyde, and the proteinase K step was omitted during permeabilisation (as it was not possible to organosilanate the slides).
2. PCR – cells were washed twice in PBS, treated with 1X trypsin/EDTA (GIBCO) for 5 minutes at 37°C and then centrifuged at 1000g for 3 minutes. The pellet of cells was then homogenised in denaturing solution, as described in Section 4.6.2.

Also, *in situ* hybridisations were carried out on the marrow cells using radioactively labelled cDNA probes to IL-6 and c-Fos. These experiments were carried out by Dr JA Hoyland (Department of Rheumatology, University of Manchester, UK) using established procedures (Hoyland et al 1994; Hoyland and Sharpe 1994).

Chapter 5

Results

5. Results

5.1 *In Situ* Hybridisation

The figures shown are representative of all of the samples examined.

5.1.1 Distemper-infected and Uninfected Dogs

Photographs showing positive hybridisation to the CDV-N gene are from experiments with the CDV-N sense probe (i.e. to genomic RNA). In all positive cases, hybridisation of a similar intensity was also seen using the antisense probe.

5.1.1.1 Histology

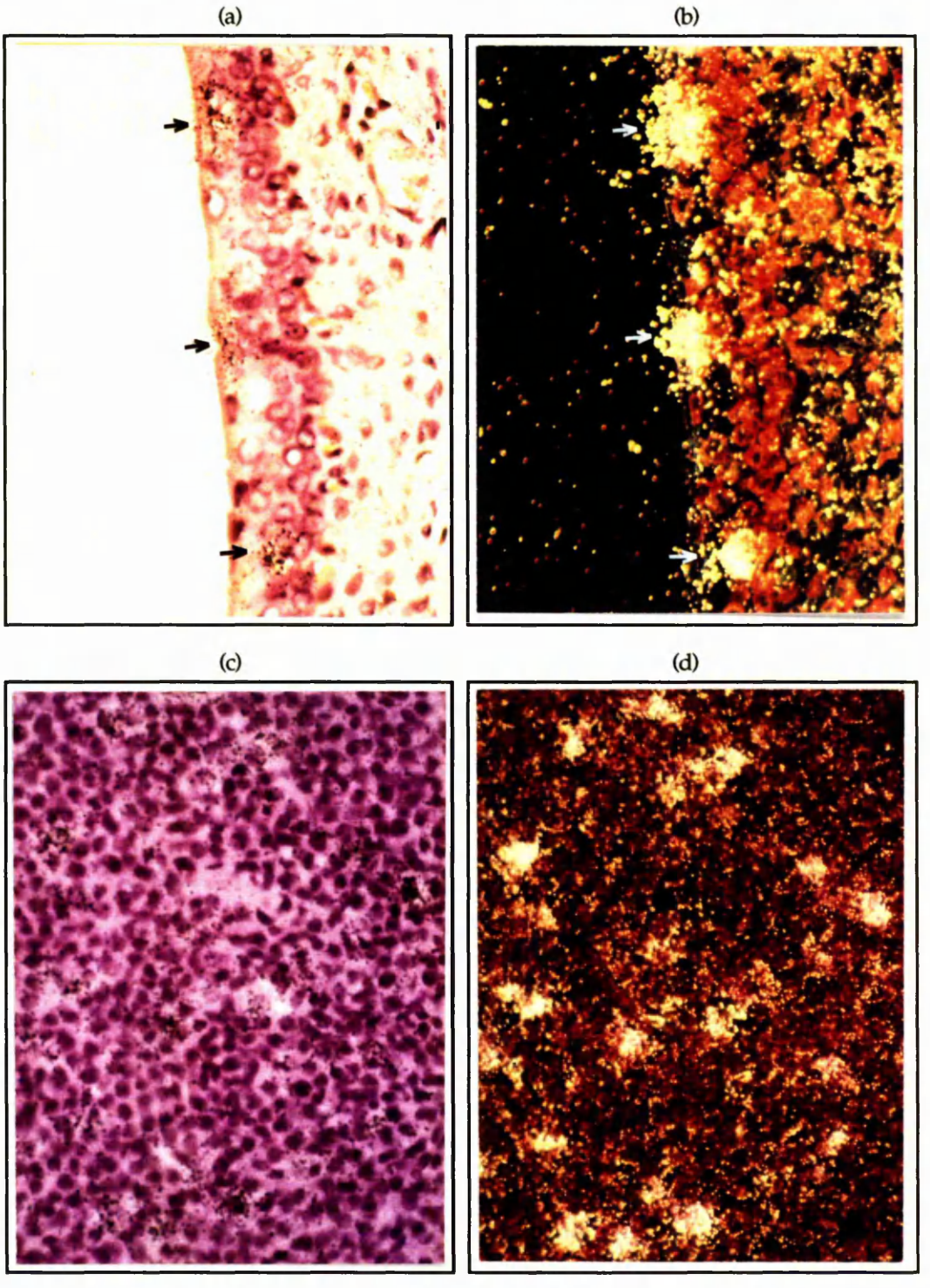
Bone samples were examined histologically for any evidence of distemper infection. Three of the samples from distemper-infected dogs (Cases 1, 3 and 18) showed areas of inflammatory infiltrate (predominantly neutrophils and a small number of lymphocytes) and bony necrosis, within the metaphyseal regions of the long bones. Necrotic osteoblasts, osteoclasts and osteocytes were also seen. These changes were also seen in two of the uninfected dogs (Cases 5 and 6).

5.1.1.2 *In situ* hybridisation experiments

In all distemper cases, bladder, and/or spleen samples served as control tissues, as both of these are known to be target organs for CDV (Appel 1987). Bladder and spleen samples from the infected dogs were examined and were found to be strongly positive with the CDV-N, but not MV-N probe (Figure 5.1), confirming the diagnosis of distemper, and the specificity of the reaction.

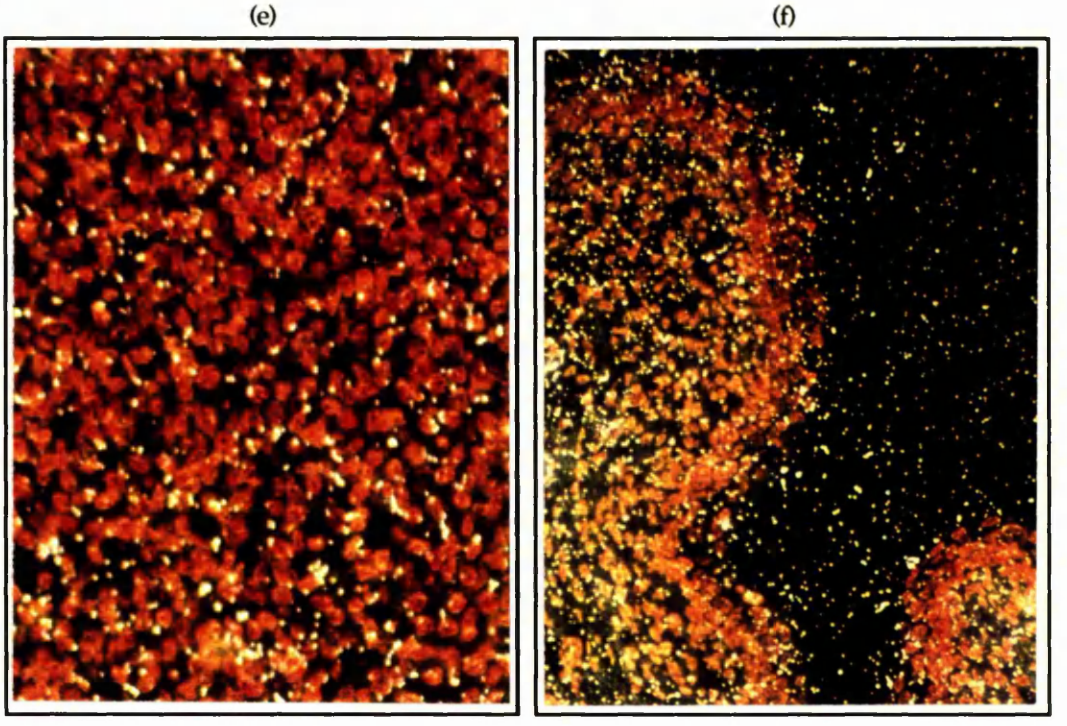
A particularly distinct pattern of hybridisation was observed in the bladder, in which discrete epithelial cells were strongly labelled (Figure 5.1 (a) and (b)).

Figure 5.1 *In situ* hybridisation results – distemper-infected dog bladder and spleen



Positive hybridisation with the CDV-N sense probe viewed by (a) and (c) light-field, and (b) and (d) dark field microscopy. Note distinct epithelial areas of hybridisation in the bladder (arrows) (Mx125).

Figure 5.1 *In situ* hybridisation results – distemper-infected dog bladder and spleen



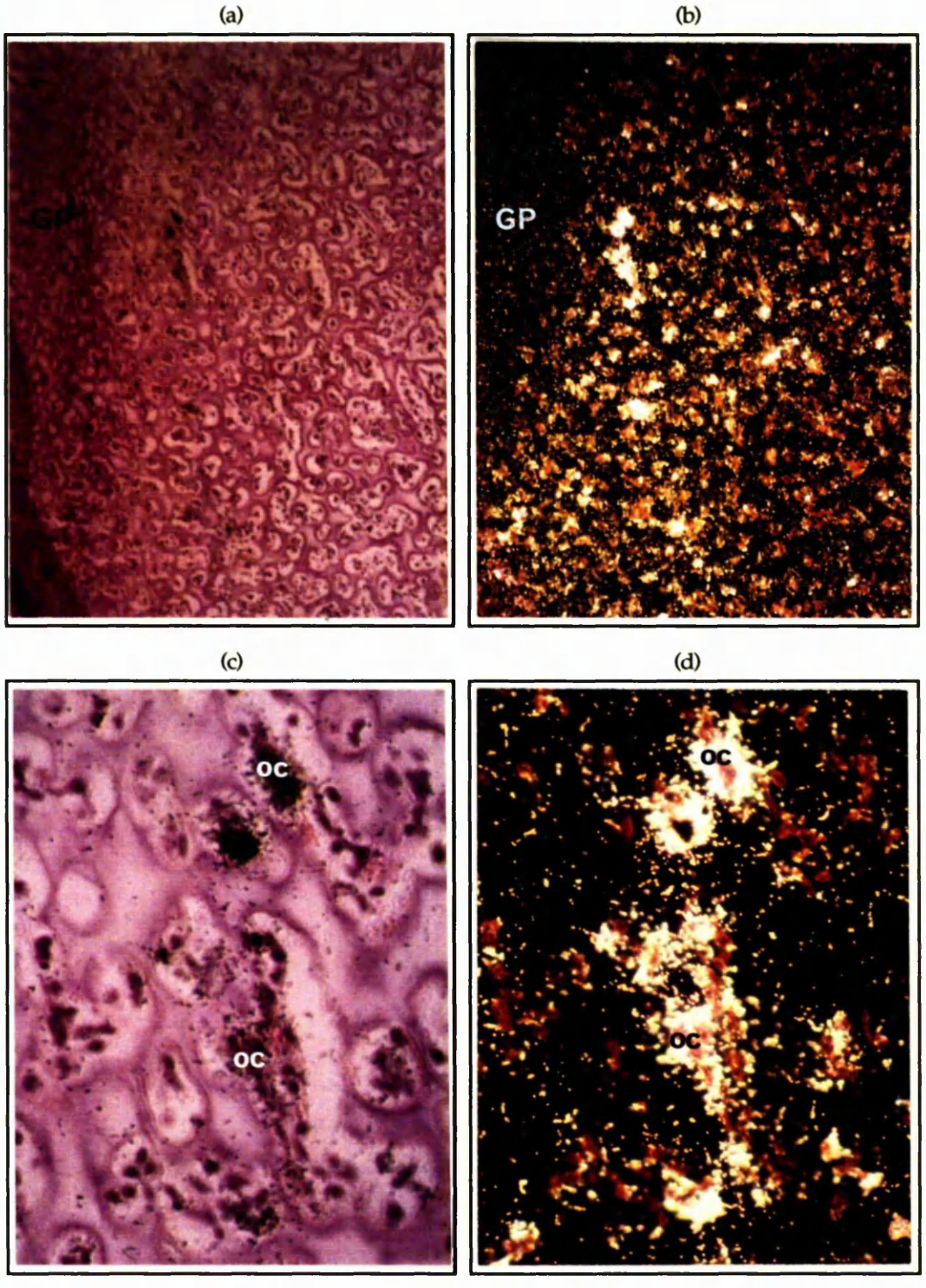
No hybridisation with the MV-N sense probe (background level of silver grains) (e) spleen and (f) bladder, viewed by dark-field microscopy (Mx125).

The addition of hybridisation solution alone, or the pre-treatment of sections with RNase, resulted in only background levels of silver grains in the sections (not shown).

Of the distemper-infected dogs, three (Cases 1, 2 and 18) showed positive hybridisation with the probes to the CDV-N gene (Figure 5.2), but not with the MV-N probes. Where present, hybridisation was found in all bone cell types, including osteoblasts and osteoclasts, but not in adjacent chondrocytes.

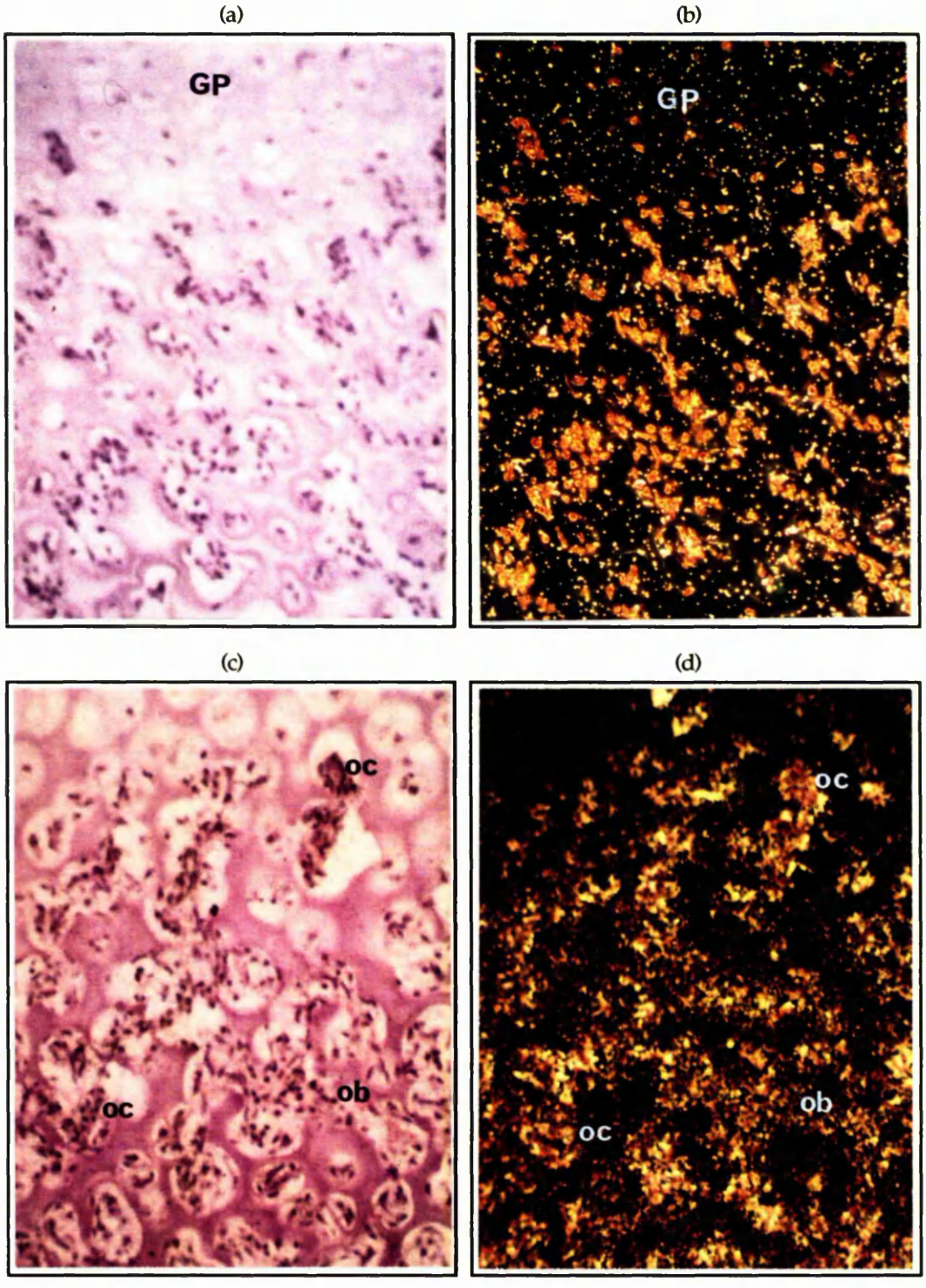
The signal was concentrated in the growth plate region of the long bones (Figure 5.2 (a) and (b)), and appeared particularly prominent in the multinucleated osteoclasts (Figure 5.2 (c) and (d)). However, not all of the osteoclasts in any one region were labelled to the same extent, if at all.

Figure 5.2 *In situ* hybridisation results – distemper-infected dog bone



Positive hybridisation with the CDV-N sense probe. Growth plate (GP) region viewed by (a) light-field and (b) dark-field microscopy (Mx50). Strong positive hybridisation, particularly in osteoclasts (oc) viewed by (c) light-field and (d) dark-field microscopy (Mx160).

Figure 5.3 *In situ* hybridisation results – uninfected dog bone



Growth plate (GP) region. No hybridisation with the CDV-N sense probe viewed by (a) light-field and (b) dark-field microscopy. Positive hybridisation with the ß-actin probe viewed by (c) light-field and (d) dark-field microscopy. Osteoblasts (ob) and osteoclasts (oc) (Mx160).

Tissue samples from uninfected dogs and from the two dogs with osteosarcoma showed no hybridisation (only background level of silver grains) with any of the viral probes (Figure 5.3 (a) and (b)), although accessibility to hybridisation was confirmed in all tissues by a positive reaction with a probe to ß-actin (Figure 5.3 (c) and (d)).

No hybridisation was seen following the addition of hybridisation solution alone, the SP6 probe, or pre-treatment of sections with RNase.

5.1.2 Dogs with Metaphyseal Osteopathy

Photographs showing positive hybridisation to the CDV-N gene are from experiments with the CDV-N antisense probe (i.e. to mRNA). In all positive cases, hybridisation was not seen using the sense probe.

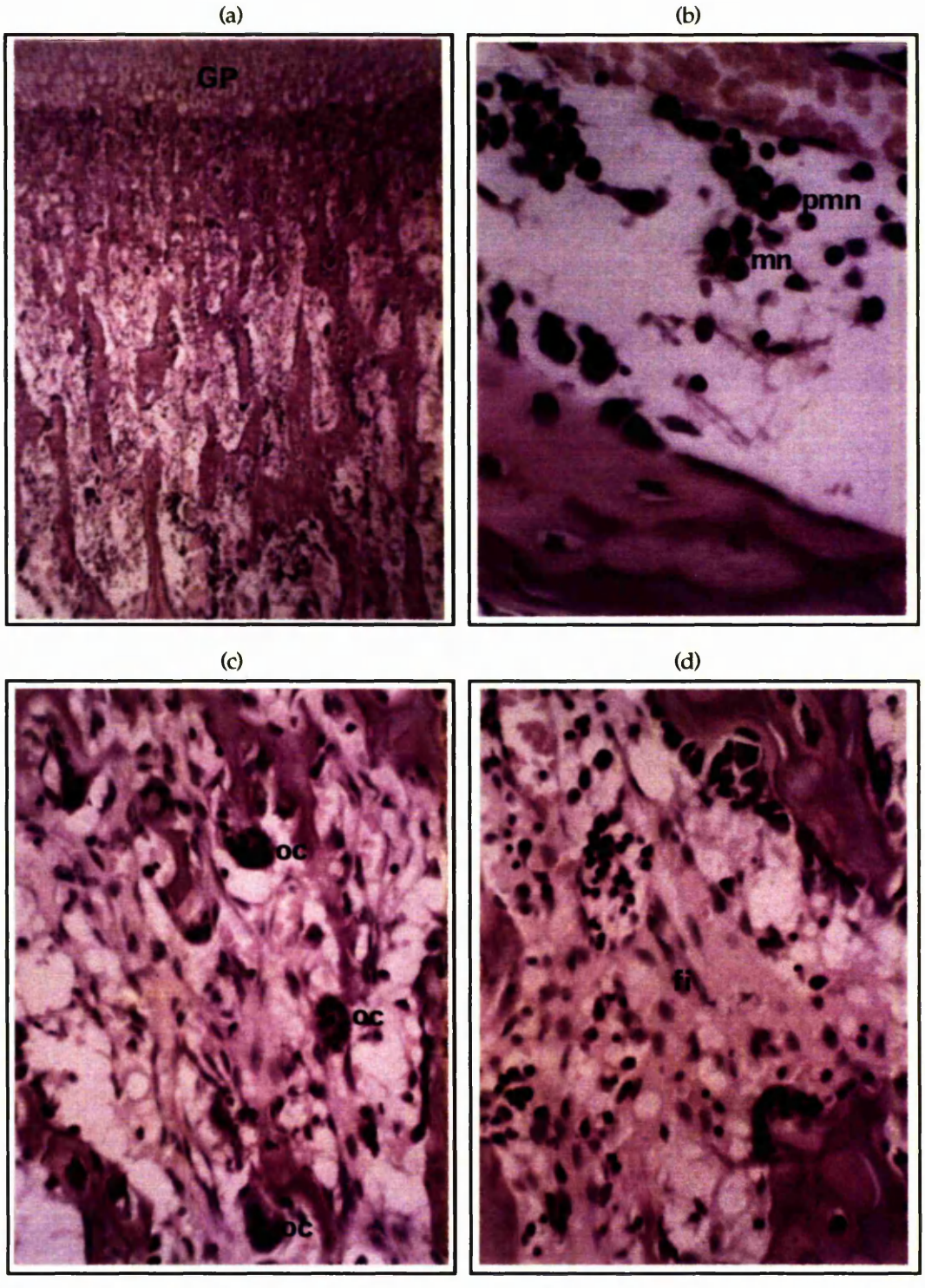
5.1.2.1 Histology

The diagnosis of metaphyseal osteopathy (based on clinical signs and radiographic examination) was confirmed in all cases by histological examination of the affected metaphyseal areas (Figure 5.4).

The histological changes varied in severity from case to case, and, indeed, between different bones from the same case. Pathology was restricted to the metaphyseal areas of bone.

The most prominent feature in all cases was an inflammatory infiltrate between the bony trabeculae, immediately adjacent to the physes (Figure 5.4 (a) and (b)). The infiltrate included both polymorphonuclear and mononuclear cells (Figure 5.4 (b)). There was also increased vascularity within the metaphyses and occasional thrombus formation; haemorrhage and fibrin deposits were also sometimes seen. Many bony trabeculae were irregular, with loss of their normal orientation and, in some sections, this area of disruption appeared as a discrete band within the metaphyses, running parallel to the physis. Some trabeculae contained necrotic bone cells, and an increased number of osteoclasts was a common feature (Figure 5.4 (c)).

Figure 5.4 Histology of metaphyseal osteopathy samples



(a) Low power (Mx60) and (b) high power (Mx240) views of the metaphyses of Case 8, GP - growth plate. Note the polymorphonuclear (pmn) and mononuclear (mn) cell infiltrate. (c) Large number of osteoclasts (oc) and (d) fibrocellular infiltrate (fi) (Mx240).

Occasionally, the adjacent intertrabecular spaces on the diaphyseal side of this disrupted area were filled with a fibrocellular tissue which had replaced the marrow elements (Figure 5.4 (d)). In some sections, periosteal woven bone was evident, sometimes associated with trabecular organisation at its inner aspect.

5.1.2.2 *In situ* hybridisation experiments

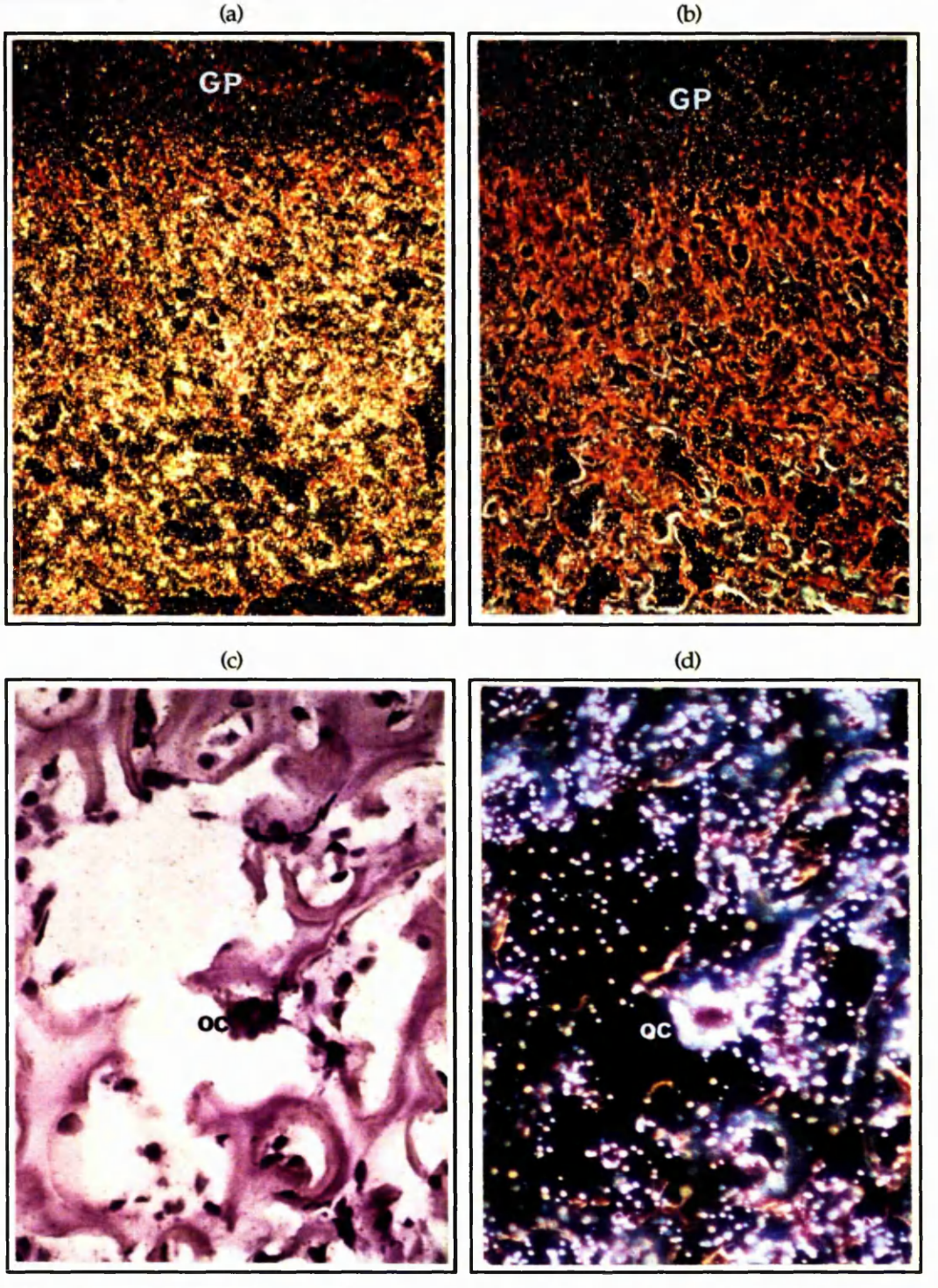
Necrosis of the affected bone was so great in the sample from Case 13 that it was not possible to accurately determine whether the sample showed positive hybridisation or not, hence this sample was ignored.

Positive hybridisation was seen in all other cases with the CDV-N antisense probe, but not with the CDV-N sense, or the MV-N probes (Figure 5.5). Hybridisation was found in osteoclasts and osteoblasts in the diseased areas, but not in adjacent normal bone, in chondrocytes of the growth plate, or in epiphyseal bone.

No hybridisation was seen with the CDV-N or MV-N probes in the bladder and spleen samples from Case 9 (Figure 5.6).

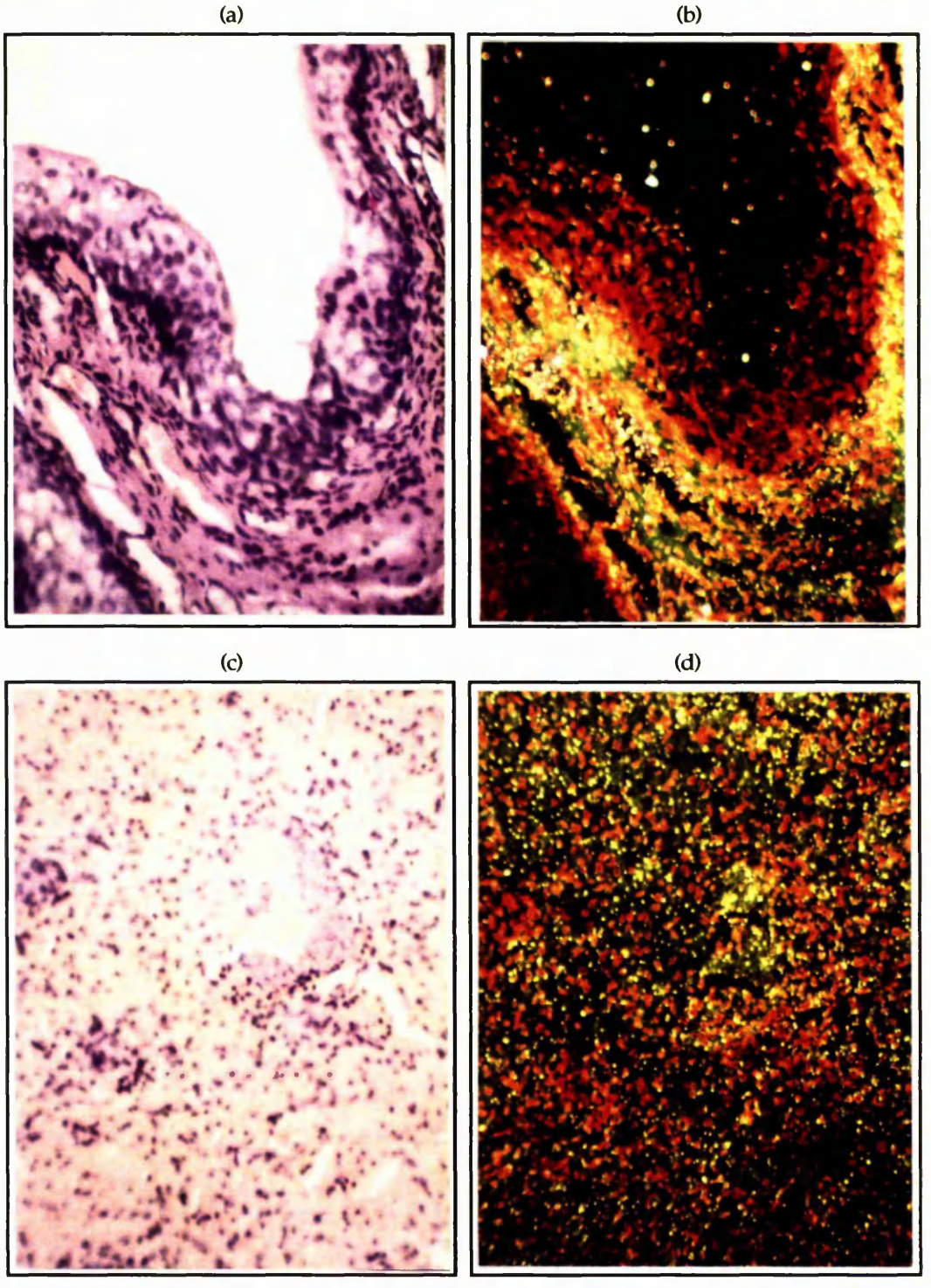
Accessibility to hybridisation was confirmed in all samples using the ß-actin probe and no hybridisation was seen with the random SP6 vector sequence probe, or with any of the probes following RNase pre-treatment (not shown).

Figure 5.5 *In situ* hybridisation results – metaphyseal osteopathy bone



Low power dark-field views of the metaphyseal areas showing (a) positive hybridisation with the CDV-N antisense probe, and (b) no hybridisation (background level of silver grains) with the MV-N probe. Growth plate (GP) (Mx60). High power (c) light-field and (d) dark-field views of the metaphyseal area showing positive hybridisation with the CDV-N antisense probe. Osteoclast (oc) (Mx240).

Figure 5.6 *In situ* hybridisation results – metaphyseal osteopathy bladder and spleen



No hybridisation (background level of silver grains) with the CDV-N antisense probe in the bladder viewed by (a) light-field and (b) dark-field microscopy. No hybridisation with the CDV-N antisense probe in the spleen viewed by (c) light-field and (d) dark-field microscopy (Mx90).

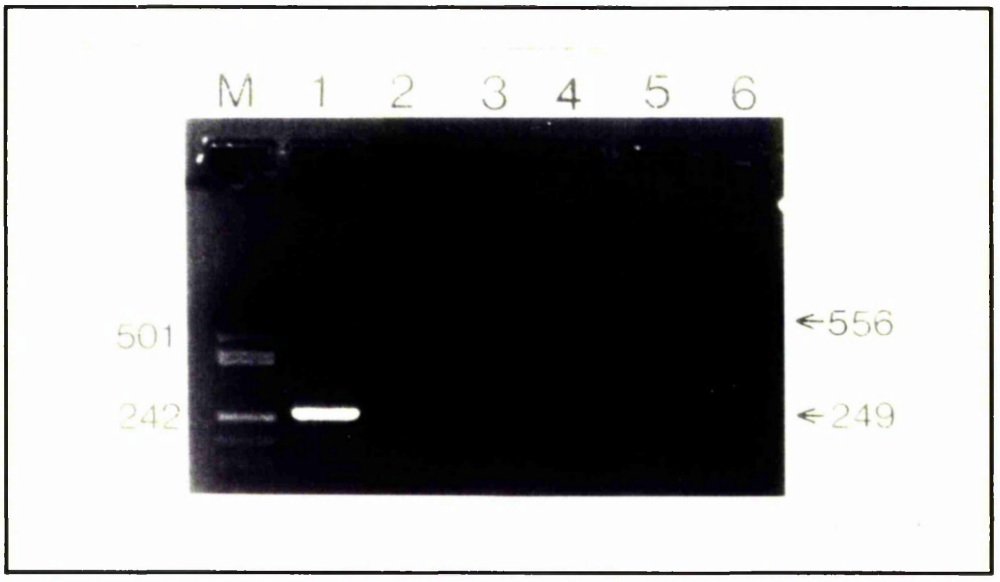
5.2 Polymerase Chain Reaction and Southern Blotting

The specificity and efficiency of the primers was confirmed previously using CDV, MV or mock infected vero cells (Gordon et al 1992) (results not shown here). With the CDV-infected cells, products of 249bp and 556bp were seen with the CDV-N primers and the primers to MV-N did not amplify. The converse was true for the MV-infected cells, where a product of 1019bp was seen with the MV-N primers, and none were seen with the CDV-N primers. All primers were negative against uninfected vero cells.

5.2.1 Distemper-infected and Uninfected Dogs

With the sample from the distemper-infected dog (Case 18), bands of the expected size for the CDV-N gene (249bp and 556bp) were visualised by ultraviolet illumination of the agarose gel (Figure 5.7). No band was seen with the MV-N primers (Figure 5.7).

Figure 5.7 PCR results – distemper-infected and uninfected dogs



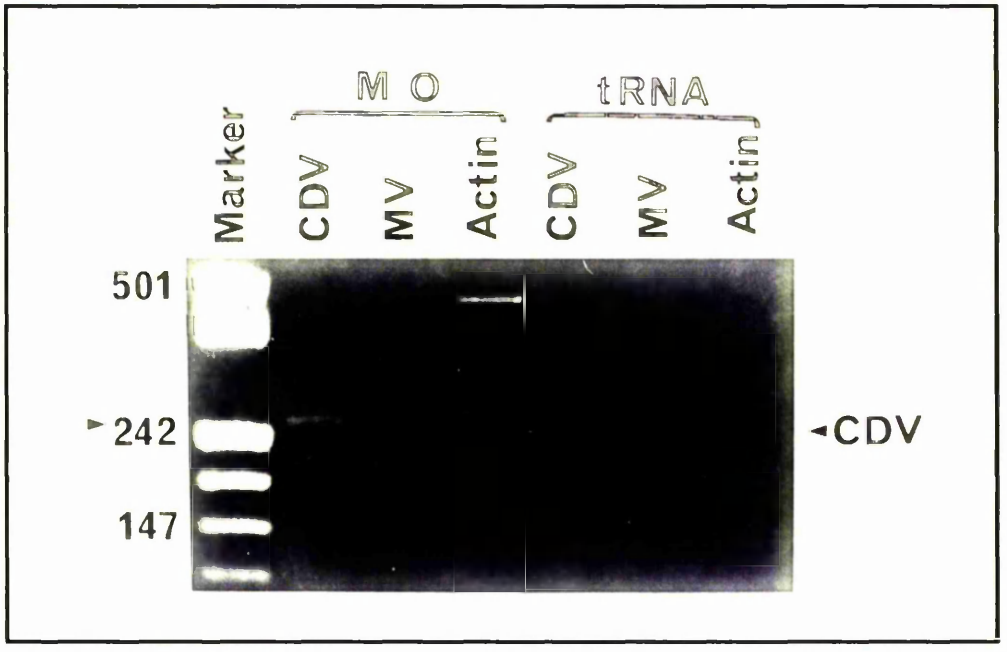
Note bands of the expected size for CDV-N (249bp and 556bp) using the cDNA from the distemper-infected dog (Case 18) (lanes 1 and 2). No band is seen with the primers for MV-N (lane 3). No bands are seen with any of the primers using the cDNA from the uninfected dog (Case 17) (lanes 4, 5 and 6). Marker – pSP72 cut with Hpa II.

With the samples from uninfected dogs (the figures shown are representative of all of the samples examined), no bands were seen with either the CDV or MV primers (Figure 5.7).

5.2.2 Dog with Metaphyseal Osteopathy

With the cDNA obtained from the MO tissue (Case 8), bands of the expected size for the CDV-N gene (249bp) and ß-actin (434bp) were visualised by ultraviolet illumination of the agarose gel (Figure 5.8). Using the primers for the 556bp fragment of CDV-N, no band was visible on the agarose gel, however, subsequent Southern blotting and probing did reveal a band in the correct position (not shown). With the MO cDNA, no band was seen with the primers to MV (Figure 5.8). No bands were seen with any of the primers using the cDNA from the reverse transcribed tRNA (Figure 5.8).

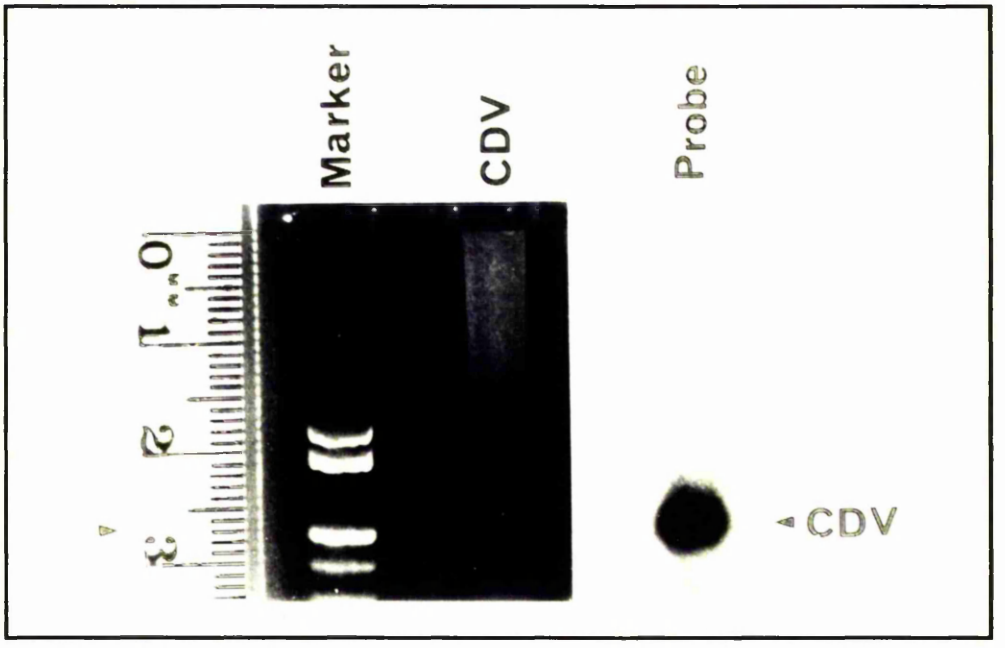
Figure 5.8 PCR results – dog with metaphyseal osteopathy



Note bands of the expected size for CDV-N (249bp) (arrows) and ß-actin (434bp) using the cDNA from the dog with metaphyseal osteopathy (MO). No band is seen with the primers for MV-N. No bands are seen with any of the primers using the cDNA from reverse transcribed tRNA. Primers were to the CDV-N gene (CDV), the MV-N gene (MV) and exons 4 and 5 of the ß-actin gene (Actin). Marker – pSP72 cut with Hpa II.

Subsequent Southern blotting and probing confirmed the identity of the 249bp fragment as CDV-N (Figure 5.9).

Figure 5.9 Southern blot results – dog with metaphyseal osteopathy



Positive hybridisation with the cDNA probe to CDV-N, showing as a dark band on the X-ray film (Probe), in the expected position (arrows). Primers were to the CDV-N gene (CDV). Marker – pSP72 cut with Hpa II.

5.3 Marrow Cultures

Results from the uninfected dogs are represented as an average of the five dogs (Cases 14, 15, 16, 17 and 19). The distemper-infected sample was from Case 18.

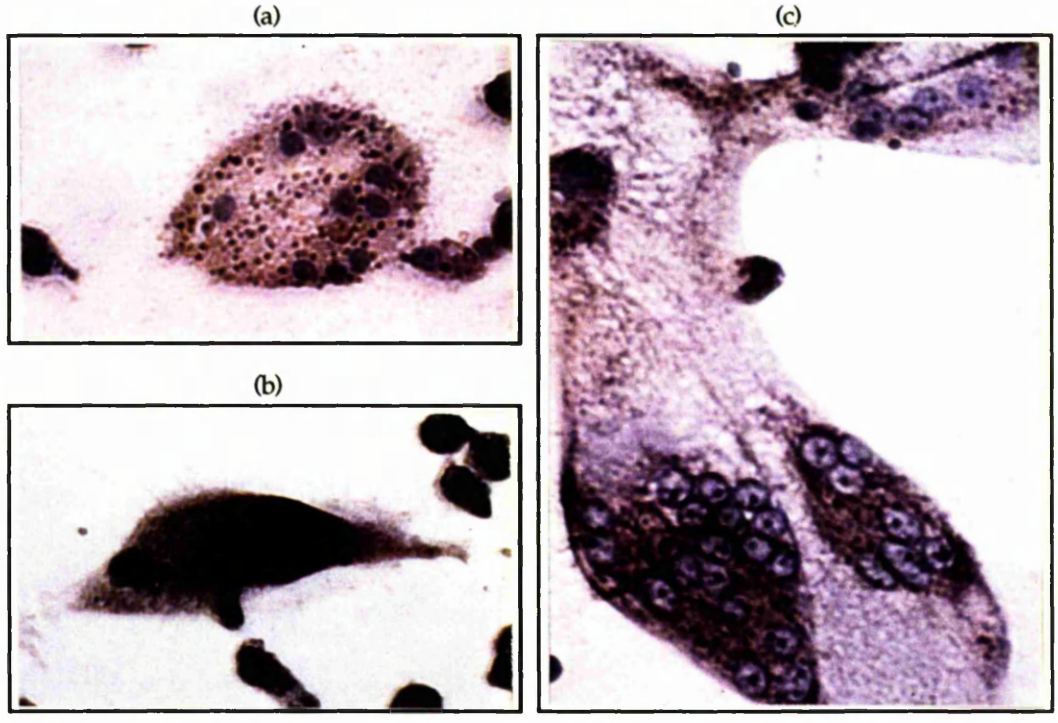
Differences between samples were compared using the unpaired Student's t test.

5.3.1 Multinucleated Cell Formation and TRAP Activity

TRAP positive multinucleated cells (MNCs) were formed at all concentrations of $1,25(OH)_2$ vitamin D_3 (10^{-7}–10^{-11}M). The intensity of TRAP staining was variable from cell to cell. Some cells showed a diffuse granular staining pattern, whilst others were more intensely

stained (Figure 5.10 (a)). Some very large MNCs appeared to have been formed by fusion of several smaller ones (Figure 5.10 (b)).

Figure 5.10 TRAP positive multinucleated cell formation



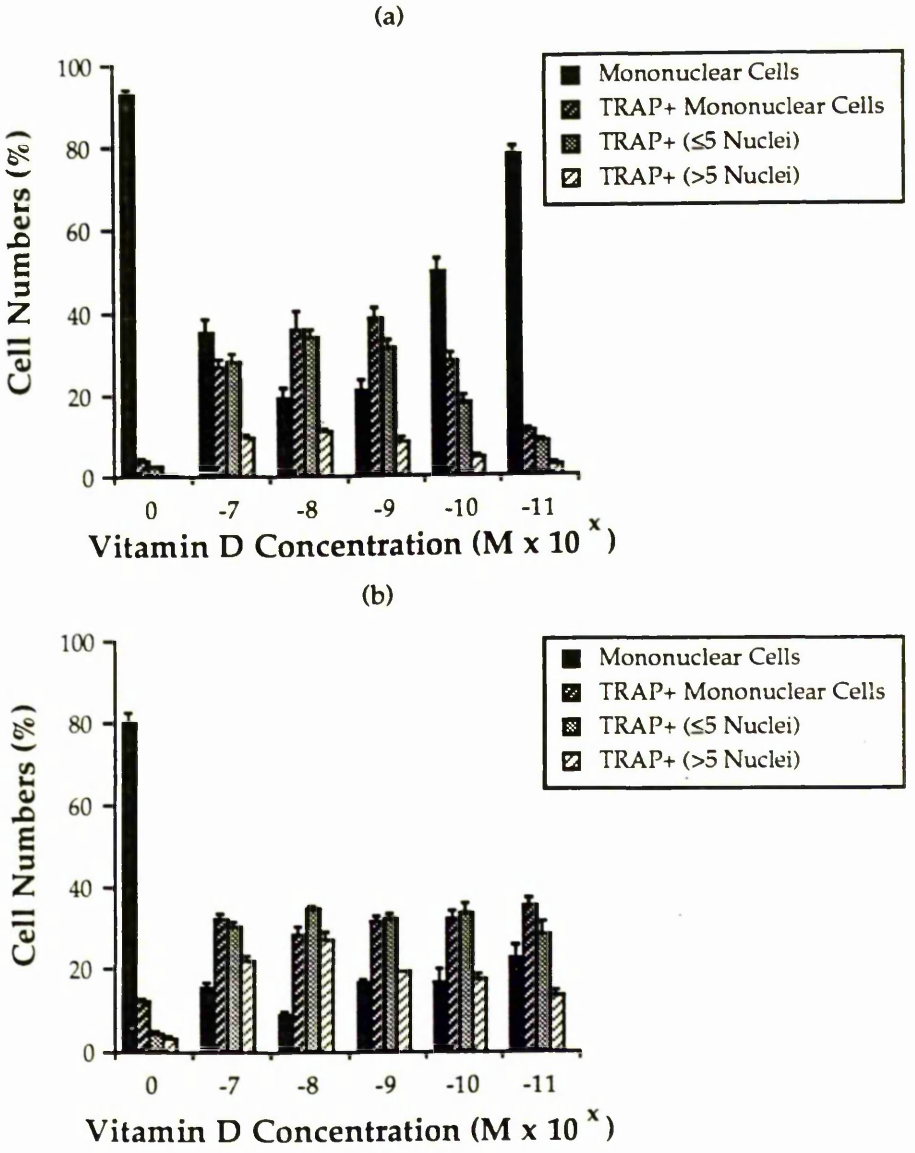
TRAP activity is seen as a brown stain. (a) Multinucleated cell showing diffuse staining pattern. (b) Multinucleated cell showing more intense staining pattern. (c) Very large multinucleated cell formed by apparent fusion of several multinucleated cells (Mx125).

Maximum stimulation was seen, in both distemper-infected and normal samples, at 10^{-8}M $1,25(OH)_2$ vitamin D_3 (Figure 5.11). Cultures from the distemper-infected dog contained a significantly higher percentage of MNCs (both small and large) than those from normal dogs (Figure 5.11 (b)). Even at low concentrations of vitamin D, there were more MNCs in the cultures from the distemper-infected dog than the maximum number seen in the cultures from the normal dogs.

Addition of the Onderstepoort strain of CDV (10–10^5 pfu/ml) to the cultures caused a dose dependent (up to 10^3 pfu/ml) significant increase in the percentage of MNCs formed, with a

corresponding decrease in the number of mononuclear cells (Figure 5.12). At higher doses (10^4–10^5 pfu/ml) many of the cells were killed, hence the relative decrease in MNCs seen at these concentrations.

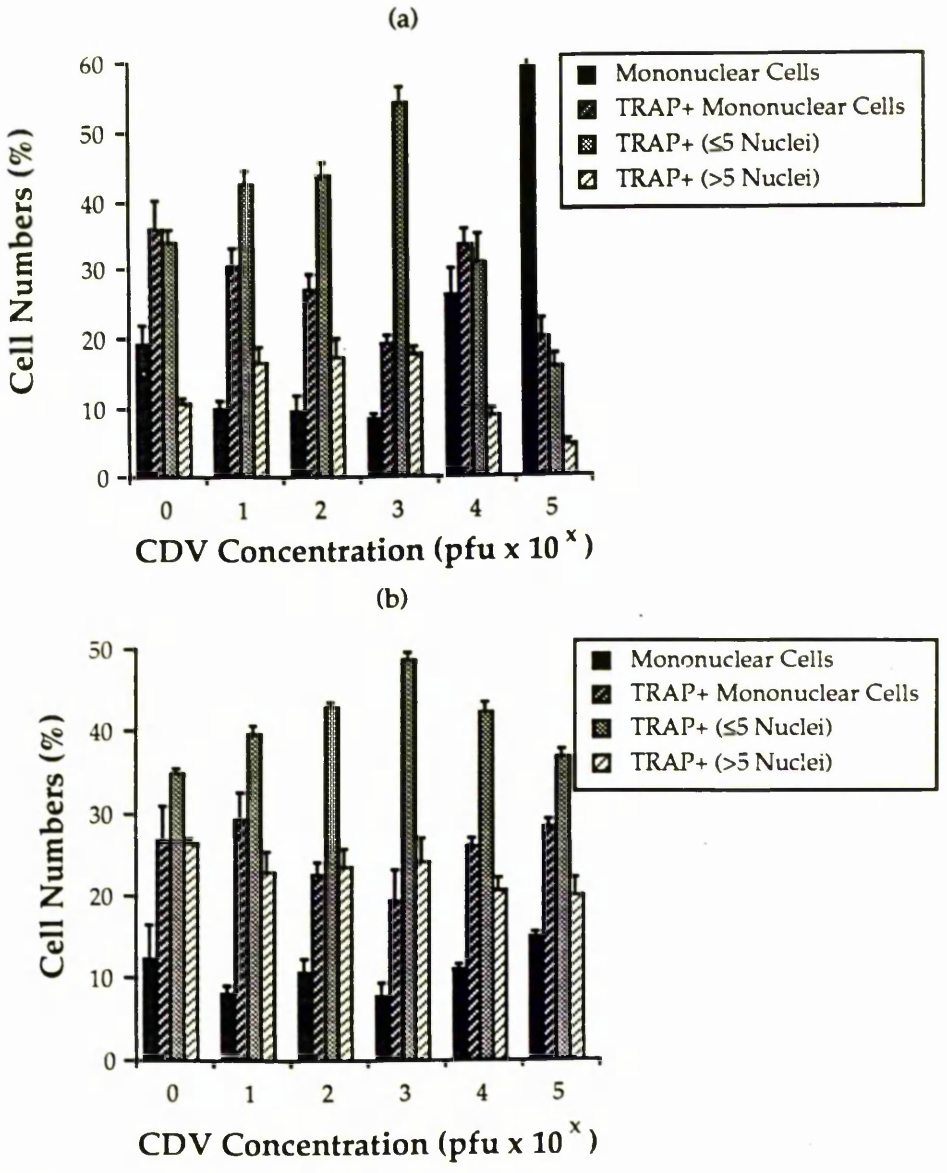
Figure 5.11 Effects of varying doses of $1,25(OH)_2$ vitamin D_3 on cell populations



(a) Normal dogs. (b) Distemper-infected dog. Note that, even at low concentrations of vitamin D, more multinucleated cells were formed than the maximum seen in (a). Bars represent standard error.

The dose dependent effect differed slightly between the samples from infected and normal dogs: in the cultures from normal dogs, the increase was seen in both the small and large MNC populations (Figure 5.12 (a)), whereas, in the cultures from the distemper-infected dog, only the small MNCs were increased in number (Figure 5.12 (b)).

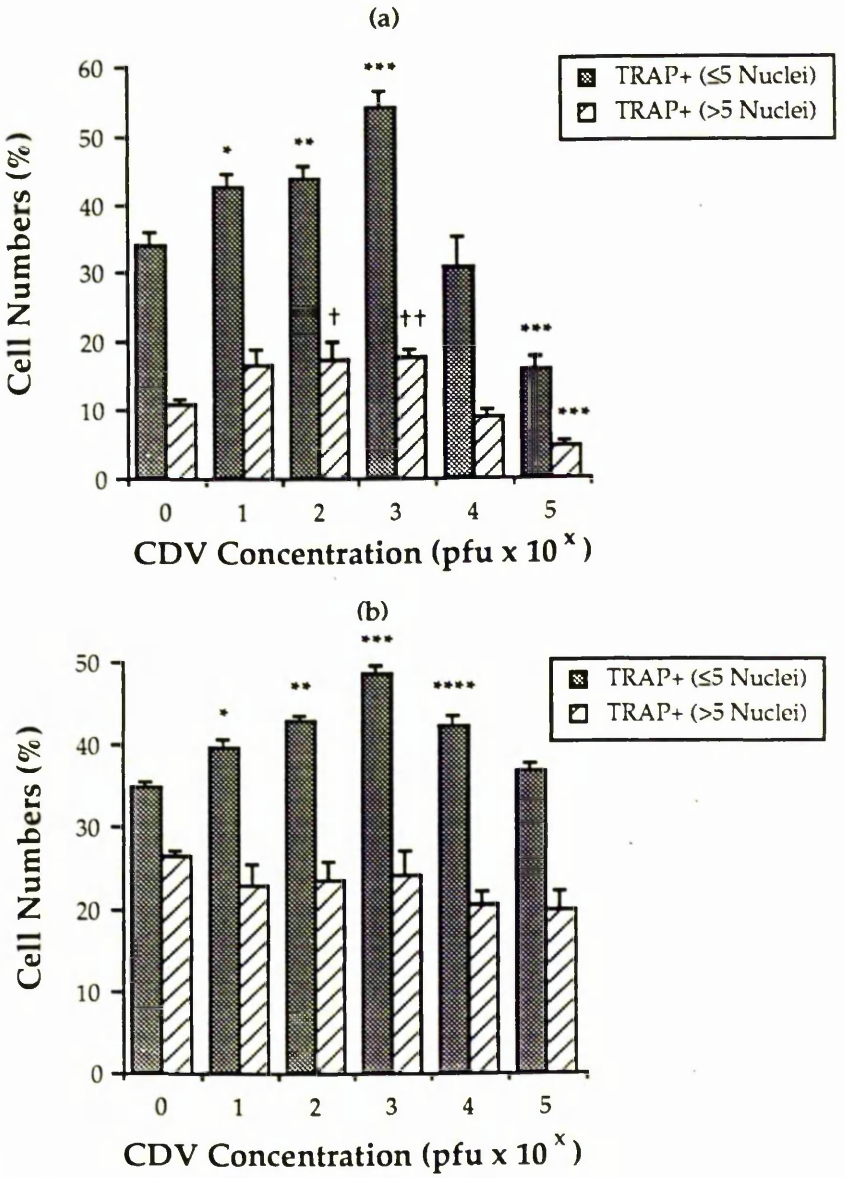
Figure 5.12 Effects of *in vitro* addition of CDV on all cell populations



CDV was added on the first day of culture. (a) Normal dogs. (b) Distemper-infected dog. Note a dose dependent (up to 10^3pfu/ml) increase in the number of multinucleated cells. Bars represent standard error.

The changes in the MNC populations are more easily seen in Figure 5.13.

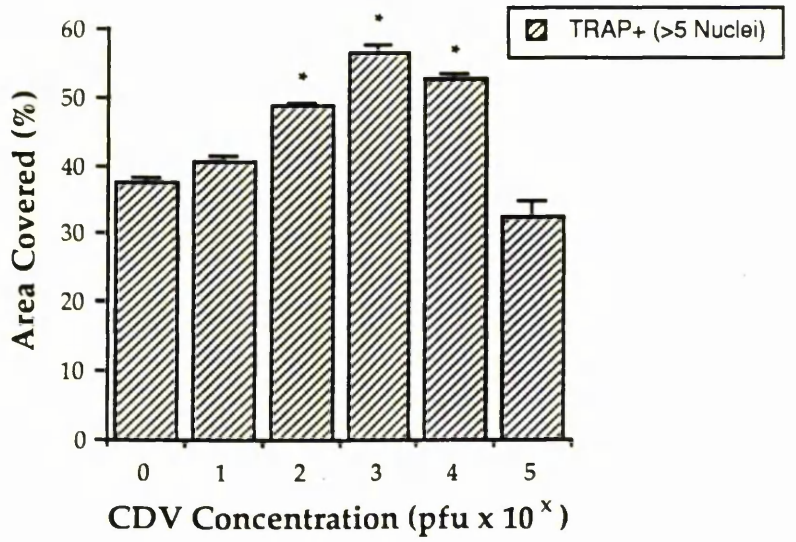
Figure 5.13 Effects of *in vitro* addition of CDV on the multinucleated cells



(a) Normal dogs. Note a dose dependent (up to 10^3pfu/ml) increase in small and large multinucleated cells. *p=0.02, **p=0.006, ***p=0.001, †p=0.05, ††p=0.002. (b) Distemper-infected dog. Note a dose dependent (up to 10^3pfu/ml) increase in the number of small multinucleated cells. *p=0.01, **p=0.001, ***p=<0.001, ****p=0.005. Note also, that the total number of multinucleated cells in the cultures from the normal dogs infected with 10^3pfu/ml CDV, are similar (approximately 65%) to the number in the original cultures from the distemper-infected dog. Bars represent standard error.

The average surface area of the large MNCs in the cultures from the distemper-infected dog was, however, increased in these cultures (Figure 5.14).

Figure 5.14 Effects of CDV on the surface area of large multinucleated cells



Note a dose dependent increase in area of the large multinucleated cells in the cultures from the distemper-infected dog (expressed as an average percentage). *p=<0.001. Bars represent standard error.

This increase in size was also reflected by an increase in the number of nuclei in these large MNCs (Table 5.1).

Table 5.1 Effects of CDV on the number of nuclei in the large multinucleated cells

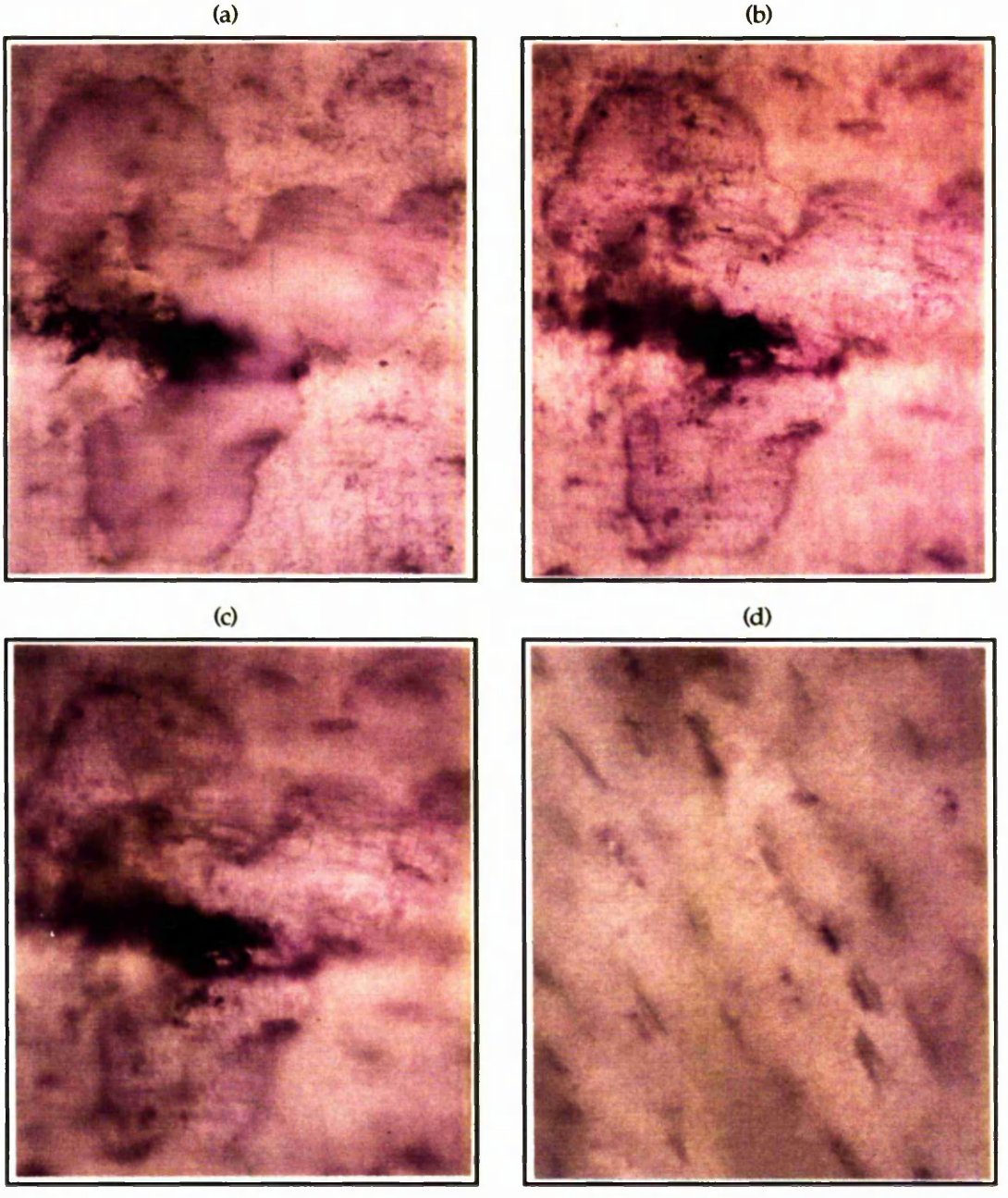
	CDV Dose (pfu/ml)					
Sample	0	10	10^2	10^3	10^4	10^5
Normal	7.8 ±1.9	9.6 ± 3.8*	12.0 ± 4.4**	16.1 ± 6.5**	10.4 ± 3.7**	7.4 ± 1.3
	I		II	III	IIII	
Infected	9.3 ± 2.1	10.6 ± 2.9†	15.1 ± 6.4††	20.5 ± 9.0††	12.9 ± 4.6††	7.8 ± 1.7††

Values represent the mean ± standard deviation. * p values compare infected samples from the normal dogs with the basal number (7.8); † p values compare additionally infected samples from the naturally infected dog with the basal number (9.3); I p values compare samples from infected and normal dogs.
*p=0.02, **p=<0.001; †p=0.003, ††p=<0.001; Ip=<0.001, IIp=0.006, IIIp=0.007, IIIIp=0.004.

5.3.2 Bone resorption

Resorption pits were formed on devitalised bovine cortical bone slices (Figure 5.15).

Figure 5.15 Formation of resorption pits



The cells were cultured on bone slices in the presence or absence of 10^{-8}M vitamin D for two weeks . The cells were removed and the slices were briefly counterstained with toluidine blue. (a) – (c) Resorption pit formed on bone slice (photographed at different depths of the pit). (d) Bone slice from control sample cultured without vitamin D. No resorption pits are visible (Mx125).

However, resorption pits were only formed in cultures containing 10^{-8}M 1,25$(OH)_2$ vitamin D_3, and there was only a small number of pits on each bone slice (ranging from one to twelve pits per slice).

Although the number of pits was slightly increased in the distemper-infected cultures, there was no statistically significant difference between normal and infected (either *in vivo* or *in vitro*) samples.

5.3.3 *In Situ* Hybridisation

5.3.3.1 CDV

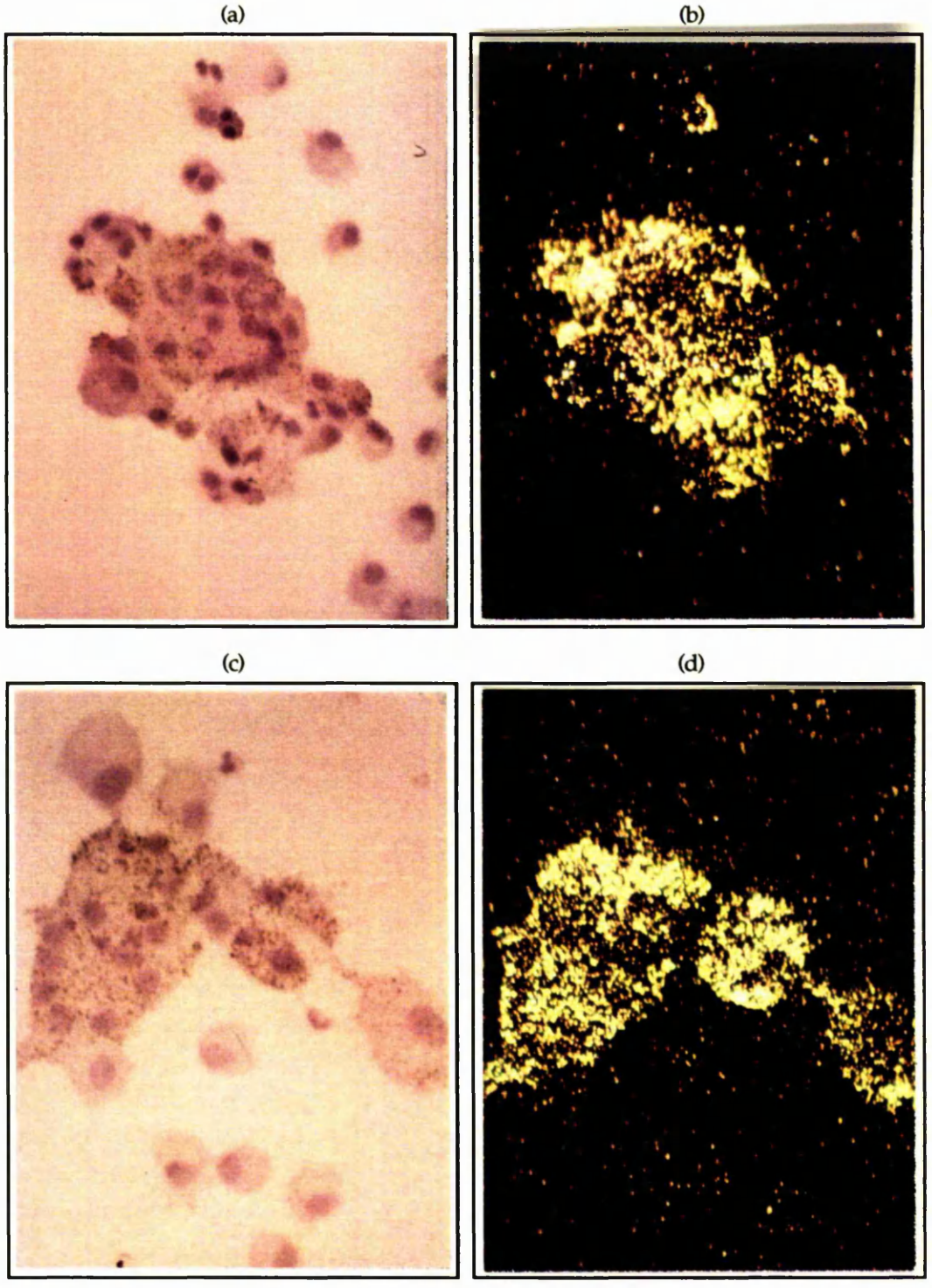
Positive hybridisation was seen using the CDV-N probe in both the original and the *in vitro* CDV-infected cultures from the distemper-infected dog (Figure 5.16).

No hybridisation was seen in any of the original samples from the normal dogs (Figure 5.17 (a) and (b)). However, positive hybridisation was seen in the samples from these dogs that had been infected *in vitro* with the Onderstepoort strain of CDV (10^3 pfu/ml) (Figure 5.17 (c) and (d)).

In all samples that were positive, the levels of hybridisation were similar with both the CDV-N sense and antisense probes.

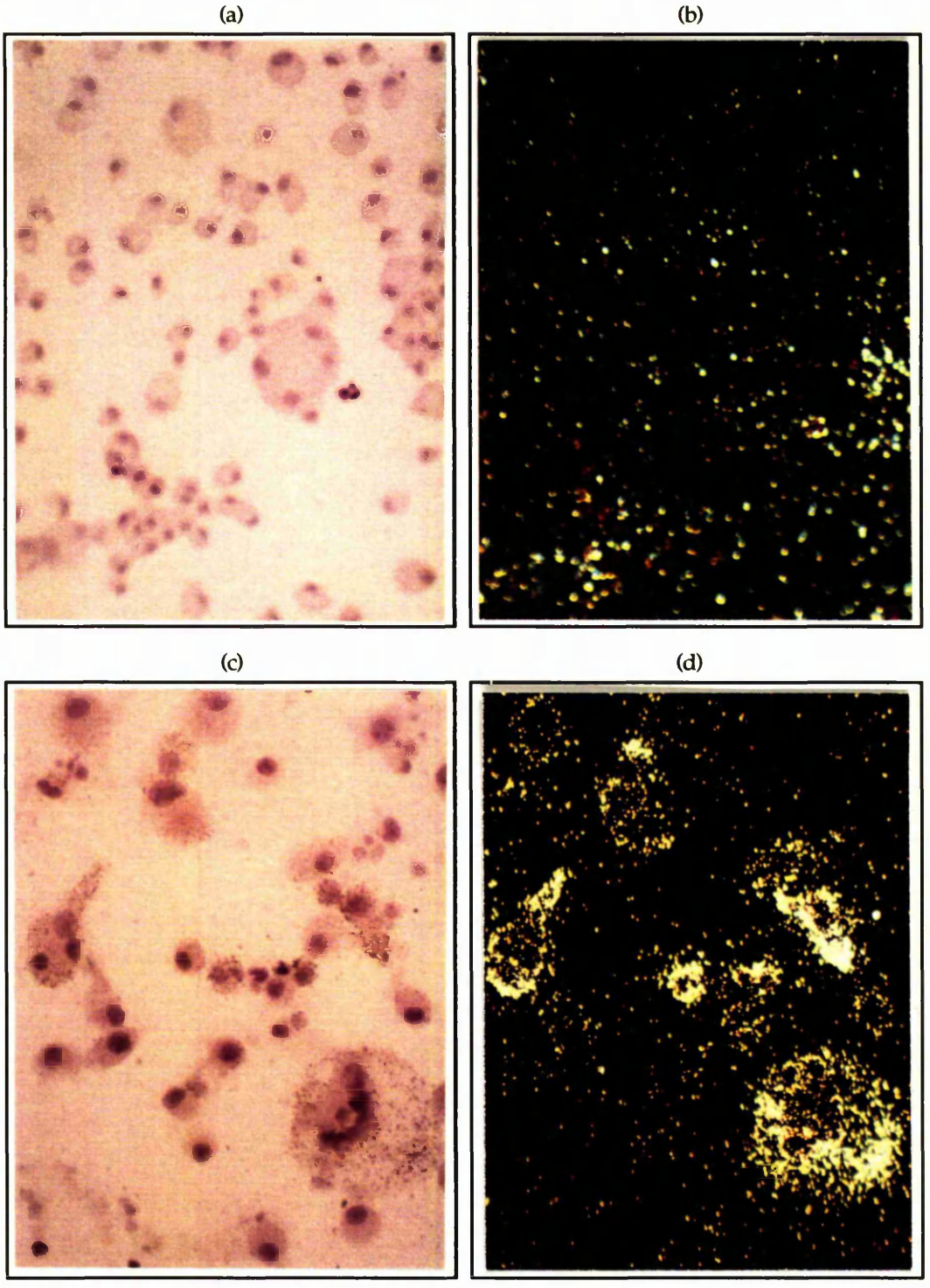
No hybridisation was seen in any of the samples that had been RNase treated, and positive hybridisation was seen in all the samples with the probe to ß-actin (not shown).

Figure 5.16 *In situ* hybridisation results – distemper-infected dog marrow cultures



Positive hybridisation with the CDV-N sense probe. (a) Light-field and (b) dark-field views of the initial sample from the distemper-infected dog (Case 18). (c) light-field and (d) dark-field views of the sample from this dog following the addition of 10^3pfu/ml CDV (Mx125).

Figure 5.17 *In situ* hybridisation results – uninfected dog marrow cultures



(a) Light-field and (b) dark-field views of the initial sample from the uninfected greyhound(Case 19) showing no hybridisation (background level of silver grains) with the CDV-N sense probe. (c) light-field and (d) dark-field views of the sample from this dog, showing positive hybridisation with the CDV-N sense probe following the addition of 10^3pfu/ml CDV (Mx125).

The average percentage positivity to CDV-N for each cell type in each sample is shown in Table 5.2.

Table 5.2 Comparison of the number of distemper-positive cells in each culture

Sample	Mononuclear cells	Small multinucleated cells (3-5 nuclei)	Large multinucleated cells (>5 nuclei)
Normal	0	0	0
Normal + CDV	6.9 ± 1.8	49.1 ± 5.7	47.3 ± 7.4
Infected	3.7 ± 1.3	47.4 ± 4.4	46.4 ± 3.5
Infected + CDV	10.9 ± 1.9*	64.4 ± 5.3**	66.0 ± 4.9*

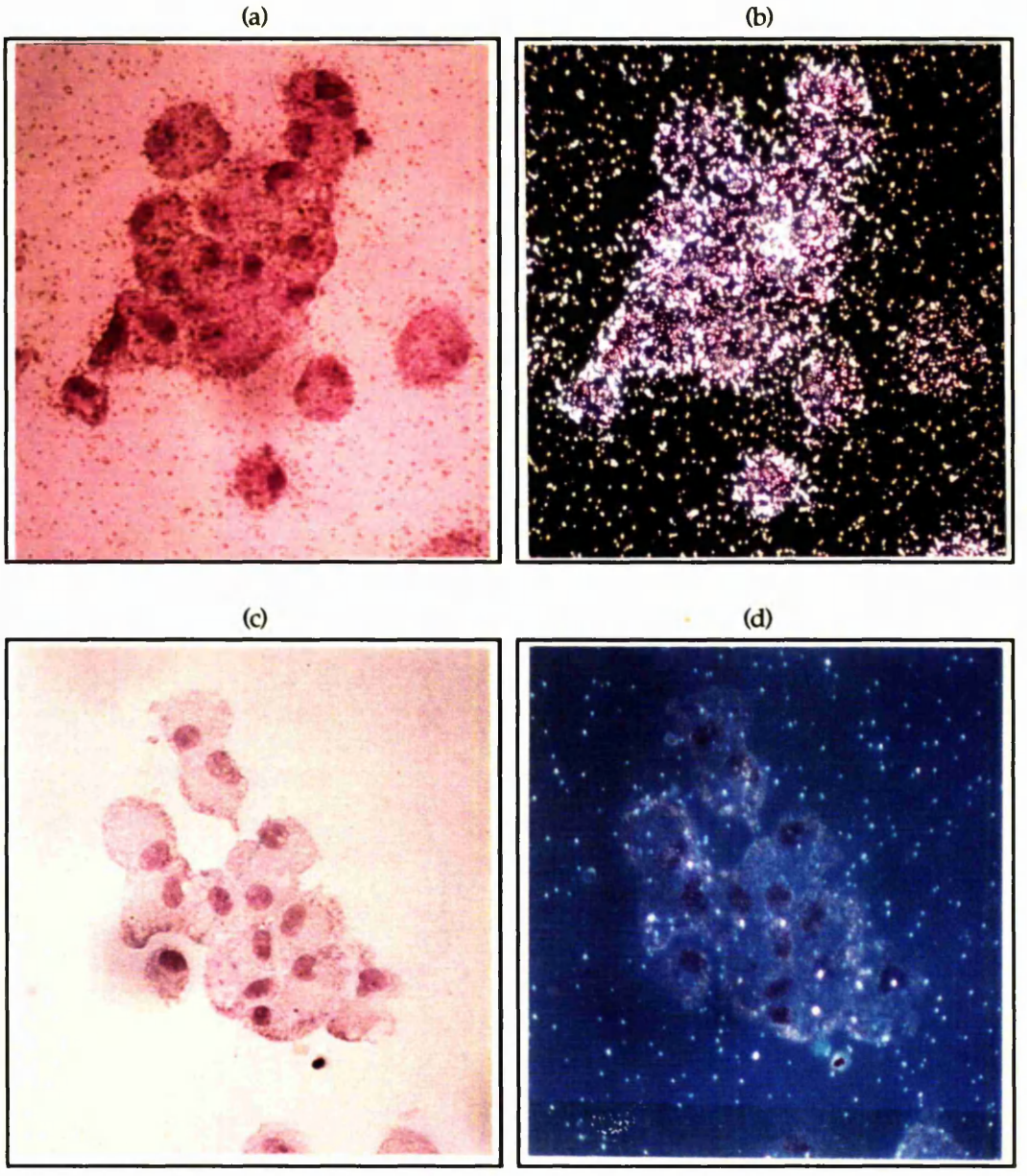
Results are expressed as mean ± standard deviation. CDV (10^3pfu/ml) was added to samples from both infected and normal dogs (+CDV). p values compare the additionally infected samples from the naturally infected dog with the original samples from this dog. *p<0.001, **p=0.001.

The original samples from the distemper-infected dog showed a similar level of infection to those from the normal dogs following *in vitro* infection. However, there was more variation in the levels of infection in the latter samples (larger standard deviations). Also, the number of positive mononuclear cells in the samples from the normal dogs was greater. The samples from the distemper-infected dog that were additionally infected with CDV *in vitro* showed the highest level of infection in all cell types, and these levels were significantly greater than those in the original samples from this dog. There was no obvious difference between the levels of infection in small and large MNCs in any of the samples studied.

5.3.3.2 IL-6 and c-Fos

Positive hybridisation was seen with the IL-6 cDNA probe in the original cultures from the distemper-infected dog and following *in vitro* infection of the normal cultures with CDV (Figure 5.18). No hybridisation was seen with this probe in any of the original cultures from the normal dogs (Figure 5.18).

Figure 5.18 *In situ* hybridisation results – IL-6

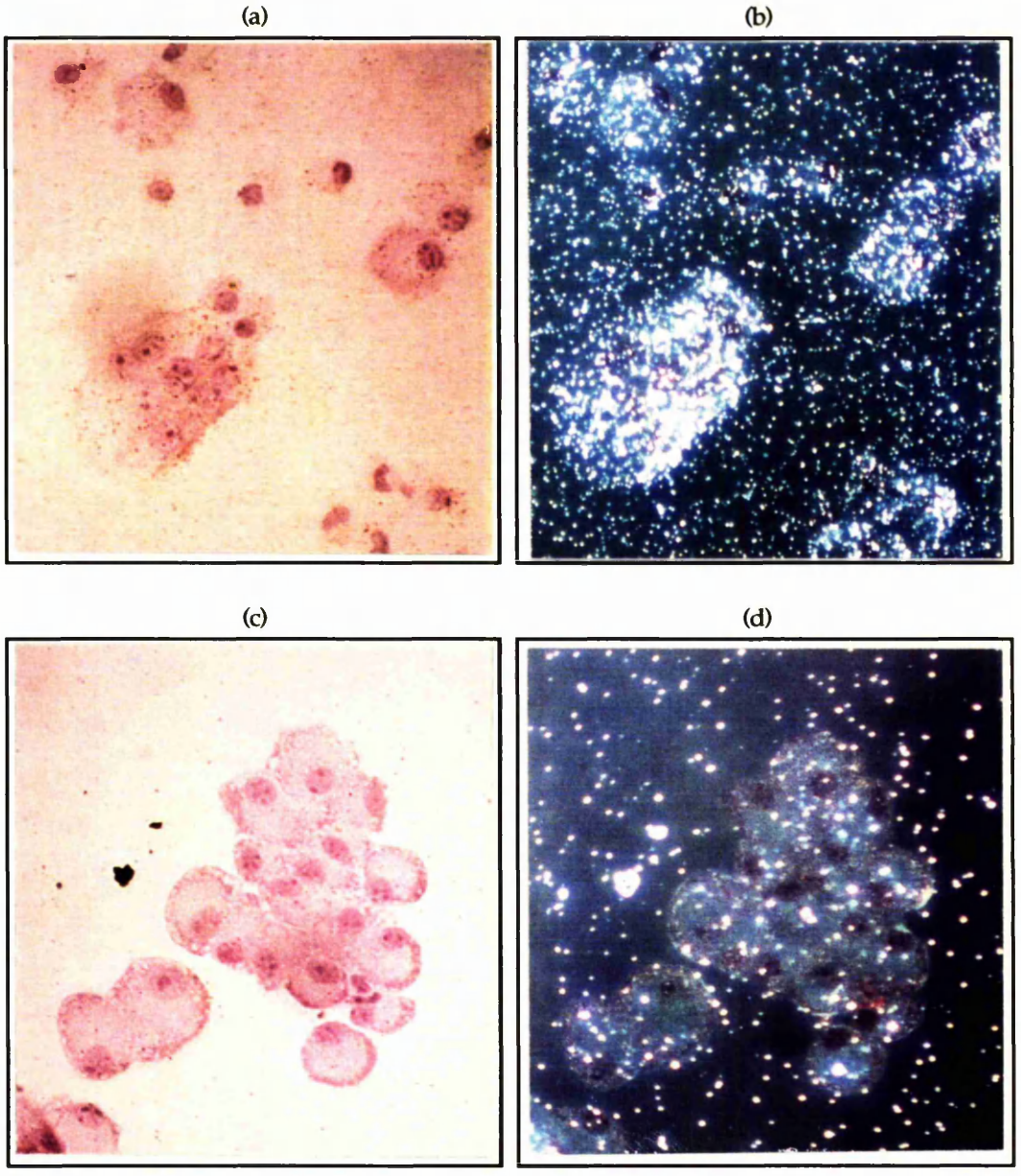


Positive hybridisation with the IL-6 cDNA probe, shown in (a) light-field and (b) dark-field views of the initial sample from the distemper-infected dog. No hybridisation shown in (c) light-field and (d) dark-field views of the initial sample from the GSD (Case 17) (Mx125).

Positive hybridisation was seen with the c-Fos cDNA probe in the cells from the distemper-infected dog that had been further *in vitro* infected with CDV (Figure 5.19). No

hybridisation was seen with the c-Fosprobe in any of the other marrow samples examined (Figure 5.19).

Figure 5.19 *In situ* hybridisation results – c-Fos

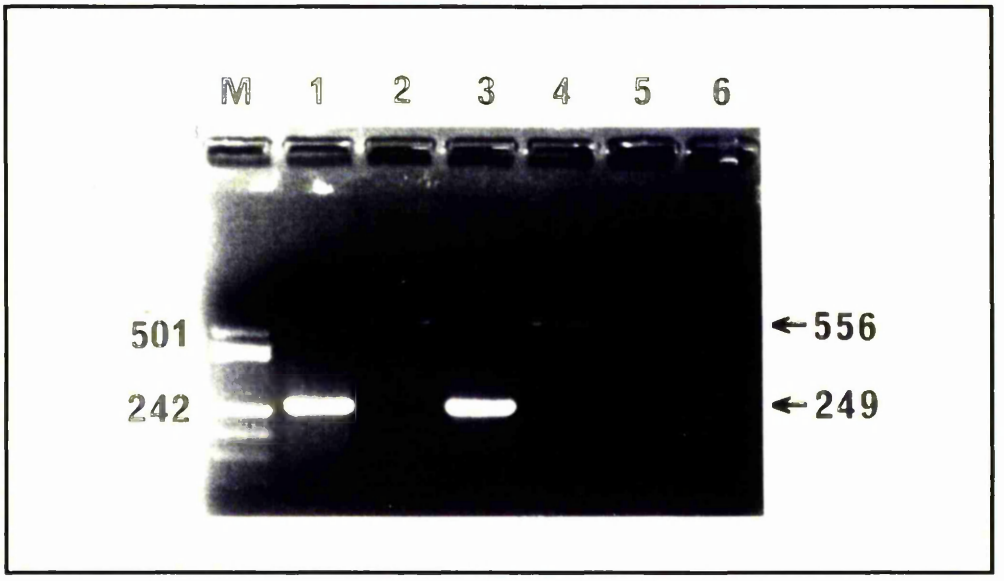


Positive hybridisation with the c-Fos cDNA probe, shown in (a) light-field and (b) dark-field views of the sample from the distemper-infected dog (Case 18) following further *in vitro* infection with CDV. No hybridisation with the c-Fos probe, shown in (c) light-field and (d) dark-field views of the sample from the greyhound (Case 19).

5.3.4 Polymerase Chain Reaction

Bands of the expected size for the CDV-N gene (249bp and 556bp) were seen in the samples from the distemper-infected dog using both sets of primers (Figure 5.20). No bands were seen with either set of primers in the samples from the normal dogs (Figure 5.20).

Figure 5.20 PCR results – marrow cultures



Lanes 1 and 2 are the original samples from the distemper-infected dog. Lanes 3 and 4 are from the same dog following addition of CDV to the cultures. Lanes 5 and 6 are the original samples from the normal dog. Expected band sizes for CDV were 249 (lanes 1, 3 and 5) and 556 (lanes 2, 4 and 6). Note bands confirming the presence of distemper in both samples from the distemper-infected dog (arrows). No bands are visible with either set of primers in the samples from the normal dog. Marker – pSP72 cut with Hpa II.

5.4 Summary of Results

The results are summarised in Table 5.3.

Of the five distemper-infected dog bone samples, three showed positive hybridisation with both sense and antisense riboprobes to the CDV-N gene (Cases 1, 2 and 18). The presence of distemper was confirmed in the bone samples from Case 18 by RT-PCR.

Table 5.3 Summary of results

Case Number	Diagnosis	Samples	ISH results CDV	IL-6	c-Fos	PCR results
1	CDV+	L proximal femur	+	nd	nd	nd
		Bladder	+	nd	nd	nd
		Spleen	+	nd	nd	nd
2	CDV+	L proximaltibia	+	nd	nd	nd
		Bladder	+	nd	nd	nd
3	CDV+	R proximal tibia	-	nd	nd	nd
		Bladder	+	nd	nd	nd
4	CDV+	L distal radius	-	nd	nd	nd
		Bladder	+	nd	nd	nd
5	CDV-	R distal radius	-	nd	nd	nd
6	CDV-	L distal femur	-	nd	nd	nd
		Bladder	-	nd	nd	nd
7	CDV-	R proximal tibia	-	nd	nd	nd
8	MO	L proximal tibia	+	nd	nd	+
		R proximal tibia	+	nd	nd	+
		L distal tibia	-	nd	nd	nd
		R distal radius	+	nd	nd	nd
		R distal ulna	-	nd	nd	nd
9	MO	R distal radius	+	nd	nd	nd
		R distal ulna	+	nd	nd	nd
		Bladder	-	nd	nd	nd
		Spleen	-	nd	nd	nd
10	MO	L distal radius	+	nd	nd	nd
11	MO	R distal radius	+	nd	nd	nd
12	MO	R distal radius	+	nd	nd	nd
13	MO	L distal radius	nd**	nd	nd	nd
14	CDV-	L distal radius	-	nd	nd	-
		Bladder	-	nd	nd	nd
		Original marrow	-	nd	nd	-
		Marrow + CDV	+	nd	nd	+
15	CDV-	R distal radius	-	nd	nd	-
		Bladder	-	nd	nd	nd
		Original marrow	-	-	-	-
		Marrow + CDV	+	+	-	+
16	CDV-	L distal radius	-	nd	nd	-
		Bladder	-	nd	nd	nd
		Original marrow	-	-	-	-
		Marrow + CDV	+	+	-	nd
17	CDV-	L distal radius	-	nd	nd	-
		Bladder	-	nd	nd	nd
		Original marrow	-	-	-	-
		Marrow + CDV	+	+	-	nd
18	CDV+	L distal radius	+	nd	nd	+
		Bladder	+	nd	nd	nd
		Original marrow	+	+	-	+
		Marrow + further CDV	+	+	+	+
19	CDV-	R distal radius	-	nd	nd	-
		Bladder	-	nd	nd	nd
		Original marrow	-	-	-	-
		Marrow + CDV	+	+	-	nd

Diagnosis – Based on clinical signs: CDV+ distemper infection, CDV- no signs of distemper infection, MO Metaphyseal osteopathy, Samples: L – left, R – right. Results: + positive for CDV (IL-6 or c-Fos), - negative for CDV (IL-6 or c-Fos), nd not determined, ** unable to determine due to necrosis of sample.

The presence of distemper was confirmed in the five cases by positive hybridisation to the CDV-N gene in bladder and/or spleen samples from these cases.

Of the six metaphyseal osteopathy samples, five showed positive hybridisation with the antisense CDV-N probe. It was not possible to accurately determine the presence or absence of CDV transcripts in the sample from Case 13, due to extreme necrosis of the sample. The presence of distemper was confirmed in Case 8 by RT-PCR.
Bladder and spleen samples from one of the MO cases (Case 9) were negative for CDV.

None of the samples from the uninfected dogs (bone, bladder or spleen) showed positive hybridisation with the CDV probes. None of the samples from any dog examined showed positive hybridisation with the MV-N or SP6 probes, or following RNase pre-treatment. Accessibility to hybridisation was confirmed in all samples using a probe to ß-actin.

In bone marrow cultures, both *in vivo* and *in vitro* infection with CDV produced an increase in the number of osteoclast-like cells formed. The increase seen following *in vitro* infection was dose dependent. *In vitro* infection of samples from a naturally distemper-infected dog (Case 18) also produced an increase in size of the osteoclast-like cells. The presence or absence of CDV was confirmed using *in situ* hybridisation and RT-PCR.

CDV infection (both *in vitro* and *in vivo*) induced IL-6 production in the marrow cells, and c-Fos was only seen in the marrow cells from the distemper-infected dog that had been further infected *in vitro*.

Chapter 6

Discussion

6. Discussion

6.1 Introduction

Canine distemper is a highly contagious disease of dogs and other carnivores, which primarily causes respiratory and gastrointestinal signs. Neurological signs develop later in some cases (Appel 1987). In previous studies, it has been shown, by *in situ* hybridisation and RT-PCR, that canine distemper virus (CDV) is present in the bone cells of over 60% of the pagetic bone biopsies so far tested, suggesting that the virus may be involved in the aetiology of this bone disorder (Gordon et al 1991, 1992; Cartwright et al 1993). In the present work, the same techniques have been used to test for the presence of CDV RNA in canine bone cells in dogs with naturally occurring distemper and in dogs with metaphyseal osteopathy (MO). To further examine the possible role of the virus in canine bone disorders, the effects of *in vivo* and *in vitro* infection with CDV on multinucleated osteoclast-like cell formation have also been studied.

6.2 CDV in Naturally Infected Dogs

The presence of CDV in bone cells was confirmed in three of the five distemper infected bone samples, but not in any of the uninfected controls. CDV sequences were detected in the bladder and spleen samples from all five infected dogs, so the dogs were clearly infected. When present in the bone cells, the virus was concentrated in the growth plate region of the long bones and was particularly prominent in the multinucleated osteoclasts. The dramatic accumulation of this virus in osteoclasts of the natural host is consistent with the hypothesis that Paget's disease is associated with CDV infection of the bone resorbing cells. However, in contrast to the previous findings in human pagetic bone, in which only the anti-sense probe (hybridising with mRNA) was positive (Gordon et al 1991, Cartwright et al 1993), both the sense and anti-sense CDV probes were found to bind strongly to the canine bone sections. This suggests that large amounts of viral genome are present within this tissue, and may represent the difference between disease in the

"natural" versus the "unnatural" host, or between a chronic (Paget's) and acute (distemper) disease.

The inability to detect the virus in the bone samples from two of the dogs with active distemper may indicate variability between different types of dogs in susceptibility of bone cells, or, more likely, indicate differences in stage and severity of the disease, i.e. the virus had not reached the bone cells at the time of death, or the bone cells were only transiently infected. Although there were only five distemper-infected dogs examined, it is interesting that two of the dogs which showed positive hybridisation in their bone cells were only several weeks old (compared with the dogs which showed no hybridisation in their bone cells, which were several months old), and this may reflect some reduced ability of the virus to infect bone cells in older dogs, or may be due to the gradual decrease in the vascular supply to the metaphyses that occurs as dogs mature (Rhinelander and Wilson 1982). Alternatively, the disease in the older dogs may have been present for longer than in the young dogs, and the virus might have cleared from the bone cells. This latter hypothesis is supported by studies using experimental dogs (Krakowka S, personal communication regarding the work of Boyce et al (1983)). In these studies, CDV antigen was initially detectable (by immunocytochemistry) in marrow cells between 5 and 7 days post-infection (pi). Between 8 and 27 days pi, viral antigen was found in marrow cells, osteoblasts, osteoclasts and osteocytes. This was accompanied by mild osseous changes (primarily osteoclast necrosis, associated with subsequent persistence of the primary spongiosa). The numbers of immunopositive cells then declined, until they were undetectable at 41 days pi. It is possible that the two dogs which showed no hybridisation in their bone cells had been infected for longer than 41 days, and this would explain the negative findings.

There may also have been differences in infectivity between different strains of virus (Appel 1987). It is also possible that CDV was present in the two dogs, but that *in situ* hybridisation was not sensitive enough to detect the virus. Unfortunately, samples were not available from these dogs for RT-PCR. This more sensitive technique might have revealed the presence of virus if it was only present in very small amounts.

The distinct pattern of hybridisation seen in the bladder epithelium was comparable with previous immunocytochemical studies (Appel 1987) and confirmed the specificity of the probes, which had previously been shown using measles infected human brain (SSPE), and on distemper-infected dog brain (Gordon et al 1991). The CDV probe used has only approximately 40% homology with MV (Rozenblatt et al 1985), and the levels of stringency used were sufficient to prevent any cross reaction with MV.

6.3 CDV in Dogs with Metaphyseal Osteopathy

Bone samples obtained from five young dogs with MO were examined by RT-PCR (one case) and *in situ* hybridisation (all cases) for the presence of CDV RNA. A further sample was obtained from a six month old German Shepherd, but the sample was too necrotic to be reliably examined. A band of the expected size for the CDV-N gene was detected using RT-PCR and this was confirmed as CDV by subsequent Southern blotting and probing. Using *in situ* hybridisation, positive hybridisation was seen within osteoblasts and osteoclasts of all the dogs examined, confirming the presence of the virus within the affected areas. There is always a risk of contamination using PCR, hence the use of *in situ* hybridisation as a further test for the presence of CDV.

The negative findings in the bladder and spleen samples from Case 9 suggest that the localisation of CDV within the metaphyses was specific, and not an incidental finding consequent of viraemia.

In contrast to the findings in the distemper-infected dogs (see Section 6.2), and in keeping with the findings in Paget's disease (Gordon et al 1991; Cartwright et al 1993), only the antisense probe (hybridising with mRNA) was positive in the dogs with MO. This suggests that, as in Paget's disease, viral genome is not present in large amounts in MO. This might be due to mutations within the genome that, whilst favouring transcription of the virus genome within the bone, might preclude active replication of the virus (see Section 2.5). Metaphyseal osteopathy has been reported in both vaccinated and

unvaccinated dogs (Meier et al 1957; Grøndalen 1976; Rendano et al 1977; Vaananen and Wikman 1979; Woodard 1982). Most of the dogs in this study were vaccinated with a polyvalent commercial vaccine (which included live, attenuated CDV) prior to the onset of clinical signs of MO (Cases 11 and 12 were unvaccinated, Case 10 was vaccinated one week after the initial signs of MO). However, it is not certain that the CDV RNA detected was from the vaccine, since the dogs could have had concurrent wild strain infections. If the vaccine strain does infect bone cells, it is possible that attenuation of the virus during manufacture of the vaccine causes mutations, or acts in some other way to prevent replication of the virus within the bone cells. In MV isolates from cases of SSPE, mutations of the viral RNA are seen (Cattaneo et al 1988), and similar mutations in CDV transcripts obtained from pagetic bone have been described (Gordon et al 1992; Mee and Sharpe 1993, 1994). It is possible that the failure of *in situ* hybridisation to detect genomic RNA of CDV in bone from both MO and Paget's disease patients is a result of these mutations. If this is the case, it may be that vaccination protects against the natural infection, by preventing active replication of the virus, but still allows transcription into mRNA.

The failure to detect genomic RNA of CDV could also be due to one or more virus or host factors, which include differences in infectivity of different strains of CDV (Appel 1987). It may even be that the probes bind to a previously unknown Morbillivirus, that has high homology with CDV, although this is unlikely due to the high levels of stringency used.

Certain breed predispositions occur with MO, particularly Boxers and Weimeraners (Grøndalen 1976; Woodard 1982), and it may be that genetic factors are involved in the disease. This might also be the case in Paget's disease, where associations have been found between the disease and certain types of major histocompatibility complex (see Section 3.3.3.2).

Experimental studies in Great Dane dogs have suggested that overnutrition might be involved in the pathogenesis of MO (see Section 3.4.3.4). However, the lesions produced differed both histologically and pathologically from the naturally occurring disease, and the pyrexia seen with MO was not reproducible. Furthermore, not all dogs suffering MO

have a history of oversupplementation and, although nutritional supplementation is very common in the large breeds of dog, very few develop MO. Overnutrition and supplementation with vitamins and minerals might play a role, but it is probable that some other factors are also involved. Large breeds of dog are subjected to more stresses and strains in their bones than smaller breeds, and the damage produced may provide the focus for an infective process to occur. Bacterial osteomyelitis in young children occurs most commonly in the metaphyses, due to the hair-pin loops of the metaphyseal vessels trapping bacterial emboli (Trueta 1959) (see Section 3.2.1). This metaphyseal localisation of osteomyelitis has also been reported, although less commonly, in the dog (see Section 3.2.1). It is therefore possible that viruses, or virus immune complexes, might also be deposited in this area. Immune complexes containing CDV are known to be infective for leucocytes (Appel et al 1984) and it may be that they are also infective for bone cells.

Previous attempts to isolate an organism from bone affected with MO and to transmit the disease to other dogs have proved unsuccessful (Grøndalen 1979; Woodard 1982), although Grøndalen reported that, of 7 dogs which were given blood from dogs with MO, 3 developed symptoms of distemper (Grøndalen 1979). At the time of her initial report of several cases of MO (Grøndalen 1976), there was a distemper epidemic in the Netherlands, and the prevalence of MO reduced as distemper became less common (Grøndalen J, personal communication). Reports in the literature provide further, circumstantial, evidence that CDV might be involved in MO. The initial clinical signs of pyrexia, pain and swelling and the histological evidence of suppurative inflammation all support the theory that some infective agent is partly, or wholly, responsible for the disease. Several authors report that the clinical manifestations of MO are often accompanied by, or preceded a few days earlier by, respiratory or gastrointestinal symptoms (Meier et al 1957; Grøndalen 1976; Rendano et al 1977; Vaananen and Wikman 1979; Woodard 1982), both of which are seen with CDV infection (see Section 2.7.3). Meier et al (1957) even went as far as to describe "distemper-like" symptoms that appeared during their observations. Tooth enamel hypoplasia occurs as a sequel to CDV infection (Bodingbauer 1960; Dubielzig et al 1981) and

has also been reported as a post mortem finding in 3 littermates that died with MO (Woodard 1982).

Dogs, especially large breed pedigree dogs, are usually vaccinated against distemper (and several other canine diseases, including parvovirus and ICHV), between 6 and 16 weeks of age. Hence, most dogs are usually exposed to distemper, whether vaccine or wild type, at an early age (i.e. a similar age as dogs affected with MO).

There are no previous clinical reports of viral osteomyelitis in the dog, although Boyce et al (1983) (and Krakowka S, personal communication) reported metaphyseal lesions associated with both experimental and natural CDV infection. The disease is also rare in humans, although several viruses have been shown to produce bone lesions (see Section 3.2.2). Most reports of rubella infection suggest that these lesions are a result of metabolic or nutritional insufficiency during gestation, although inflammatory infiltrates have been reported within the affected areas (Sekeles and Ornoy 1975). The lesions caused by variola are usually symmetrical and inflammation is commonly seen. Relapses frequently occur, periosteal new bone often forms around the metaphyses and this usually extends to involve the diaphyses. Experimental herpes virus infection in cats was also found to produce necrosis of metaphyseal bone (Hoover and Griesemer 1971).

The circumstantial evidence from previous reports and the similarities between the lesions reported in human and feline viral osteomyelitis (see Sections 3.2.2 and 3.2.3) and those seen in MO are suggestive of a viral aetiology for this canine disease.

6.4 The Effects of CDV on Osteoclast-like Cell Formation

Marrow mononuclear cells from a dog with distemper and from five normal dogs were cultured in varying concentrations of $1,25(OH)_2 D_3$. Tartrate resistant acid phosphatase (TRAP) positive multinucleated cells (MNCs) were formed at all concentrations of $1,25(OH)_2 D_3$ used. The variations in TRAP staining patterns observed were similar to those reported in previous marrow culture experiments (Testa et al 1981; Bird et al 1992) and in isolated osteoclasts (Addison 1978). The maximum number of MNCs was seen at a

concentration of 10^{-8}M 1,25$(OH)_2$ D_3, and hence all *in vitro* viral effects were examined at this concentration.

In situ hybridisation and RT-PCR studies confirmed the presence of CDV in the original cells (i.e. prior to the *in vitro* addition of virus) from the dog with clinical signs of distemper. Cells cultured from the normal dogs were shown to be initially free of virus using the same techniques. Following *in vitro* addition of CDV to the cultures, the cells from all dogs were shown to have been infected by the virus.

The *in situ* hybridisation results also provided a method of determining the number of infected cells in each sample. There was no obvious difference between the levels of infection in the original cultures from the distemper-infected dog and those from the normal dogs following *in vitro* addition of 10^3pfu/ml CDV. This was reflected in the similar total numbers of MNCs (approximately 65%) seen in these samples. Following *in vitro* infection of the cultures from the distemper-infected dog, the number of positive cells (both mono- and multinucleated) was significantly increased, suggesting that additional cells had been infected by the virus.

Owing to the sensitivity of the technique and hence, the possibility of contamination, RT-PCR was not used to confirm the presence of virus within the cells from the normal dogs following *in vitro* infection. However, the *in situ* hybridisation findings were conclusive, and, hence, the effects seen are presumed to be the result of viral infection of the cells.

Previous reports of canine osteoclast-like cells derived from marrow cultures have either failed to show bone resorption (Bird et al 1992), or shown that the cells could attach to bone spicules (Seed et al 1988). However, in the latter case, resorption was not convincingly demonstrated. In neither of these reports was 1,25$(OH)_2$ D_3 added to the cultures.

Bone resorption was assayed in these experiments by culturing cells on devitalised bovine cortical bone slices. A small number of resorption pits was seen with cells cultured in 10^{-8}M 1,25$(OH)_2$ D_3, suggesting that, whilst most of the cells did not resorb bone, at least some of the cells were behaving as authentic osteoclasts. It is possible that a small number of preformed osteoclasts might have been present in the original marrow samples. However,

if this were the case, it is reasonable to assume that some resorption would have been seen in most of the samples at all concentrations of $1,25(OH)_2 D_3$, and this was not the case. Hence, it is assumed that the pits seen were produced by newly formed cells in the cultures.

Failure to resorb bone appears to be one of the major problems with osteoclast-like cells cultured from marrow cells, and it has been suggested that the cells behave more like macrophage polykaryons (see Section 1.5.2). Another possibility is that the limits of the culture system prevent extensive resorption. The cells might be too immature to resorb bone (after two weeks, the cultures became rapidly overgrown with fibroblasts, hence it was not possible to assay resorption after longer periods of time), or, more likely, the presence of other factors, particularly those derived from osteoblasts (Burger et al 1984) or marrow stromal cells (Flanagan et al 1994), may be necessary to induce bone resorption by the osteoclast-like cells. Despite these drawbacks, there was a slight increase in numbers of resorption pits following distemper infection, although, owing to the small numbers seen, this increase was not statistically significant.

This failure to produce bone resorption might be overcome by using isolated canine osteoclasts to examine the effects of CDV on their activity. However, isolation of osteoclasts would be more difficult than using bone marrow, and this system would not allow the examination of the effects of the virus on precursor recruitment and fusion.

Whilst the effects of CDV on bone resorption could not be satisfactorily determined in these cultures, there were obvious changes in the cell populations as a result of viral infection. As in previous reports (Takahashi et al 1988; MacDonald et al 1987; Testa et al 1981; Seed et al 1988; Bird et al 1992) only cells with three or more nuclei were classified as multinucleated. There appeared to be a distinct population of small MNCs in all cultures (3-5 nuclei) and, hence, these were counted as a separate group. Comparative cultures from the dog with natural distemper infection contained a significantly higher level of both small and large MNCs than those from normal dogs. Addition of virus to cultures from the normal dogs produced a dose dependent significant increase in the MNC population. This increase was seen in both small and large MNCs, particularly in the former. Addition of

virus to the cultures from the dog with distemper produced a further increase in the number of MNCs, although this increase was only seen in the small MNC population. However, the large MNCs did increase in size. The increase in number of cells was only seen up to a dose of 10^3pfu/ml. At higher doses, cell clumping and death occurred, hence, the relative number of MNCs was reduced. This suggests that the cells were incapable of supporting infection of high levels of virus. This effect was much less noticeable in the cultures from the distemper-infected dog, suggesting that previous exposure of the cells to the virus rendered them capable of surviving higher levels of additional infection.

It is possible that breed and age differences may play a role in the number of osteoclast-like cells formed in these cultures. However, the uninfected cases were of several different breeds (Gordon Setter, Beagle, crossbreed and Greyhound) and of ages varying from 9 to 24 weeks, and similar results were obtained from them all. The distemper-infected dog studied was a 12 week old German Shepherd. Hence, whilst it is possible that breed differences may be important (though unlikely in view of the results from the uninfected dogs), it appears that, in dogs less than 6 months old, that age is less important with regard to the number of MNCs formed.

Paget's disease is characterised by an increased number of abnormally large osteoclasts (Kanis 1991), and increased numbers of osteoclasts are also seen in the early stages of MO (Grøndalen 1976; Woodard 1982). The *in vitro* findings detailed here are therefore supportive of the hypothesis that CDV might be involved in these diseases. The virus appears to enhance the production of MNCs, in a dose dependent manner, by fusion of mononuclear precursors and also by fusion of several MNCs into one. The cultures from the dog with natural distemper infection were particularly interesting. In the original cultures from this dog, the cells appeared to be hyper-responsive to $1,25(OH)_2 D_3$, as, even at low doses of $1,25(OH)_2 D_3$, the number of MNCs was greater than the maximum number seen in the cultures from normal dogs. Marrow cells from patients with Paget's disease in long term cultures have also been shown to be hyper-responsive to $1,25(OH)_2 D_3$ (Kukita et al 1990). More recently, pagetic CFU-GM cells have been shown to be similarly hyper-responsive

(Demulder et al 1993). In a recent editorial, Kahn (1990) postulated that Paget's disease might be caused by repeated infection with the same, or a different, paramyxovirus. The fact that the cells from the distemper-infected dog were capable of supporting further higher levels of CDV infection, and the significant increase in size seen in the osteoclast-like cells from this dog following additional infection by CDV would support this hypothesis.

The induction of *IL-6* and c-*Fos* in the marrow cells following CDV infection is also interesting as both of these factors have recently been shown to be up-regulated in Paget's disease (Hoyland et al 1994; Hoyland and Sharpe 1994).

6.5 General Discussion and Conclusions

Canine distemper is a highly contagious disease of the respiratory, gastrointestinal and nervous systems, and there are no clinical reports of any bone involvement in this condition, although metaphyseal lesions have been described in both naturally and experimentally infected dogs (Boyce et al 1983). The same group has provided further immunocytochemical evidence of staining of osteoclasts in distemper infected tissue, although no mention was made of its significance (Axthelm and Krakowka 1986).

The findings from both *in situ* hybridisation and RT-PCR, that CDV RNA was present in bone cells from cases of natural distemper infection confirm these previous reports. The detection of CDV in the bone cells of distemper-infected dogs and cases of MO suggests that CDV might be involved in canine bone disorders and further supports the hypothesis that the virus is involved in the pathogenesis of Paget's disease of bone in humans.

The results from canine bone marrow cultures suggest that these cultures will be useful for examining the effect of viral infection on osteoclast-like cell formation, and could serve as a useful *in vitro* model for both Paget's disease and MO. The formation of MNCs following *in vitro* infection with paramyxoviruses has long been recognised (see Chapter 2), but these experiments show that the virus can infect and cause fusion of osteoclast precursor cells, and

that CDV might be involved in their formation *in vivo*. This latter suggestion is supported by the fact that natural *in vivo* infection with CDV also caused an increase in the number of osteoclast-like cells cultured *in vitro*. This would argue against the possibility that the increase seen *in vitro* is merely a reflection of the standard *in vitro* cytopathic effects of the virus.

The findings from *in situ* hybridisation, that CDV induces *IL-6* and c-*Fos* mRNA expression in the marrow cells further support the hypothesis that the virus is involved in Paget's disease. The up-regulation of c-*Fos* in both Paget's disease and CDV-infected marrow cells may also be important with respect to the development of neoplasia. c-*fos* is associated with osteosarcomas in mice (Rüther et al 1987, 1989), and sarcomatous change is seen in Paget's disease (Kanis 1991). Osteosarcomas in dogs are most commonly found involving the metaphyses of large breed dogs (Liu 1990), hence, the localisation of CDV to the metaphyses of infected dogs might be involved in the later development of osteosarcomas at these sites.

Unfortunately, all of these results must be taken in the context of the number of samples examined. Canine distemper used to be a very widespread and highly fatal disease. However, with the advent of widespread vaccination programmes, the disease has become much rarer, and it was extremely difficult to obtain samples for evaluation. There was a similar difficulty in obtaining samples from MO cases. However, the problem with these was not only that the disease is rare, but that it can be relatively easily diagnosed on clinical and radiographic signs. Hence, taking a biopsy from these cases is usually unjustifiable (and unethical in the UK). The samples used in this study were obtained following euthanasia, or at surgery to correct complications of the disease. Three of the MO samples came from Utrecht, where the legislation regarding biopsy material is less stringent than in the UK. However, import restrictions prevented the taking of samples for RT-PCR and marrow cultures. It would have been interesting to examine cases of panosteitis and craniomandibular osteopathy. However, no samples from dogs with either of these

disorders became available, reflecting the rarity of these diseases, and the ability to diagnose these conditions on the basis of clinical and radiographic examinations.

The results obtained from the marrow cultures were particularly interesting. The samples from uninfected dogs gave reproducible dose dependent results, and the results from the naturally infected dog were significantly different from those of the uninfected dogs. The increased response to $1,25(OH)_2 D_3$ following CDV infection is similar to that seen with marrow cells cultured from Paget's patients, and the IL-6 and c-FOS results are also similar. However, once again, the number of samples studied makes these results difficult to confidently interpret, especially regarding the naturally distemper-infected case. If these initial results can be expanded upon, and found to be consistent, then marrow cultures could prove extremely useful for examining the effects of viral infection on many aspects of osteoclast biology (see Section 6.5).

6.6 Proposed Future Work

The failure to detect distemper in the bone cells of two of the distemper-infected cases examined requires further investigation. Particularly, the age at infection and sampling, and correlation of bone involvement with severity of clinical signs should be examined. Unfortunately, the first four distemper samples were obtained without thorough clinical details (apart from the presumptive diagnosis of distemper), prior to the onset of the study. Hence, it was not possible to make these comparisons in these cases. The last case of distemper (Case 18) was a stray, and the duration and extent of clinical signs, and exact age were not known. The results obtained from immunocytochemistry on bones from experimentally infected dogs (Boyce et al 1983; Krakowka S, personal communication) provide some answers, but it would be useful to use more sensitive molecular techniques, as this might show whether the virus can persist in the bone cells for longer than 41 days without actively replicating.

A fully controlled experimental study would be required to accurately determine the factors involved in bone infection by CDV. However, in view of the scarcity of the disease in the UK, and the high morbidity and mortality caused by the virus, this would be difficult to justify at the moment. None of the UK drug companies were testing CDV vaccines during the period of this work, but if studies are carried out in the future, then examination of the bone involvement would be both justifiable and interesting.

Obtaining samples from dogs with bone disorders will remain a problem until it is more widely recognised that further research is needed to understand these diseases more fully. More interest from the general veterinary profession would also help to overcome this problem (a letter to the Veterinary Record requesting samples from distemper and MO cases only produced one case of distemper (no cases of MO) in eighteen months).

If further cases of MO and other bone disorders are examined, it would be of interest to sequence any viral transcripts, to compare them with natural and vaccine strains of CDV, and with sequences obtained from pagetic bone. Attempts were made to sequence the products of RT-PCR from Case 9, but these attempts were unsuccessful.

The possible role of CDV in sarcomatous change could be investigated by examining bone from dogs with osteosarcomas for the presence of the virus, and for the presence of c-Fos.

If samples do become available (which should be possible at least from normal dogs, to examine the *in vitro* effects of CDV), it is hoped that the marrow cultures could be used to further document the effects of CDV on IL-6 and c-Fos production in the marrow cells. The effect of exogenous addition of these factors, and of potential therapeutic agents, such as the bisphosphonates, could also be examined. ROS are known to stimulate osteoclast formation (Suda 1991) and activity (Garrett et al 1990) and have been demonstrated in distemper-infected brain cell cultures (Bürge et al 1989). It would also be of interest to measure the production of ROS (and NF-κB) in these marrow cultures (see Section 3.3.5).

One explanation for the small amount of bone resorption seen in these cultures is the absence of osteoblasts and other cells of the normal bone environment. The possible role of

osteoblasts and marrow stromal cells could be examined by co-culturing them with the osteoclast-like cells.

The possibility that infection by more than one of the paramyxoviruses might be involved in Paget's disease could also be examined further. It would be possible to determine whether the osteoclast-like cells can simultaneously support infection by more than one paramyxovirus (such as both CDV and MV), and, if so, what effects this might have.

Chapter 7

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7. References

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