STERILIZATION OF HIV INFECTED BONE ALLOGRAFTS

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This thesis is dedicated to my wife Melissa for her continued support, encouragement and assistance during its compilation.

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ABSTRACT

Human Immunodeficiency Virus (HIV) transmission from bone allografts has occurred despite screening. Bone allografts are commonly treated with 25 kGy irradiation. This thesis examines the hypothesis that *HIV infected bone allografts can be sterilized with 25 kGy of gamma irradiation.*

An HIV infected bone allograft model was created to simulate bone transplantation from an acutely infected donor who eludes screening. Because of limitations with this non-quantitative model alternative methods were examined. Further studies were done to determine the amount of bone HIV contamination (bioburden) and to calculate the irradiation dose-response curve for HIV.

A semi-quantitative polymerase chain reaction (PCR) technique was developed. Bone, cartilage and blood from ten HIV infected autopsy specimens were analysed. Bone samples demonstrated HIV DNA in all samples but the quantitative component of the PCR analysis was unreliable. HIV infection of human cartilage was demonstrated in 9 samples. HIV infection of human cartilage has not previously been reported *in vivo.*

In vitro infection of human bone derived cells was examined. Human bone derived cells were not susceptible to cell-free infection. Cells co-cultivated with HIV infected lymphocytes were not susceptible to productive HIV infection and PCR analysis demonstrated less than 0.1% HIV infected cells. It is possible that latent infection was established in the bone derived cells but the bioburden of bone is not likely to exceed the bioburden of blood.

Human Immunodeficiency Virus was exposed to a Cobalt 60 source. The inactivation of virus was assessed by calculation of the virus titre in a lymphocyte culture system and confirmed with immunofluorescence. The decimal reduction value was 8.82 kGy.

The irradiation dose required to inactivate the anticipated bioburden and achieve a sterility assurance level of 10^{-6} probability of virus surviving is 89 kGy. This dosage exceeds current recommendations for radiation sterilization of bone allografts. The hypothesis that *HIV infected bone allografts can be sterilized with 25 kGy of gamma irradiation* was not true.

The risk of transplanting untreated bone from an HIV infected donor despite current screening techniques was calculated. The estimated risk in Australian bone allograft banks is 2.5 per 10^8 living donors and 2 per 10^7 cadaver donors.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except with due reference made in the text of the thesis.

The author consents to the thesis being made available for photocopying and loan if applicable, if accepted for the award of the Doctor of Philosophy.

David G. Campbell

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
bp	base pair
CPE	cytopathic effect
CPM	radiation counts per minute
D_{10}	decimal reduction value
dATP	deoxyadenosine triphosphate
dCTP	deoxcytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DDT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
gm	gram(s)
HIV	human immunodeficiency virus
kb	kilobase
kGy	kilogray(s) (10 kGy = 1 megarads)
Mab	monoclonal antibody

ml	mililitre(s)
ng	nanogram(s)
mM	millimol(s)
OD	optical density
^{32}P	a phosphorus radioisotope
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate (also called sodium lauryl sulphate)
TAE	40mM Tris-HCl, 1mM EDTA, pH 8.0
TBE	50mM Tris-HCl pH 8.0, 50mM boric acid, 1mM WDTA
TCA	trichloro acetic acid
$TCID_{50}$	tissue culture infectious dose 50
ug	microgram(s)

PUBLICATIONS ARISING

Sterilization of HIV Infected Bone Allografts with Gamma Irradiation

D.G. Campbell, P. Li, A. Stevenson, R.D. Oakeshott

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HIV Infection of Human Bone. (abstract)

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PRIZES AND AWARDS

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Sterilization of Human Allografts.

Winner, 1994 prize for best presentation for Fellowship Trainees

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HIV Infection of Human Bone.

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1. INTRODUCTION

1.1 Preface

There have been two reports of Human Immunodeficiency Virus (HIV) transmission to bone allograft recipients (Centres for Disease Control 1988b); (Simonds et al 1992). HIV infection in bone allograft recipients is a topical but potentially preventable problem. Bone allograft banks try to minimize the problem of HIV transmission by excluding HIV infected donors and by treating allograft bones, frequently with gamma irradiation. This thesis examines the magnitude of the problem in Australian bone allograft banks and examines the efficacy of sterilizing bone allografts with gamma irradiation. A priority of this research has been to determine if virus inactivation by gamma irradiation can combine a high reduction of virus activity with sufficient preservation of the biological and biomechanical activity of bones.

HIV infection in allograft recipients is an infrequent clinical problem because of donor screening. Viral diseases which cannot be detected and those which are unknown may present a greater problem than HIV. The studies of HIV in this thesis may be used as a model for other bone allograft virus infections which cannot be examined with contemporary methods.

1.2 The Human Immunodeficiency Virus

Acquired Immune Deficiency Syndrome (AIDS) is a universally fatal disease that is caused by infection with the Human Immunodeficiency Virus (HIV). The virus was isolated from the lymphocytes of AIDS patients in 1983 by both Barre-Sinoussi *et al* (1983) and by Gallo *et al* (1983).

AIDS was first described in 1981 (Gottlieb et al 1981); (Masur et al 1981); (Siegal et al 1981). It probably originated from an animal virus among monkeys and crossed into humans in Central Africa (Emau et al 1991); (Shibata et al 1990). HIV spread from Africa to Haiti among guest workers in the 1970's and then to the U.S., Carrabean and South America among homosexual men and intravenous drug users (Gallo 1987); (Piot et al 1988); (Crofts 1992). The World Health Organization estimates that over 2.5 million cumulative AIDS cases had occurred since June 1993 (World Health Organization 1993).

HIV is a retrovirus which are RNA containing viruses that replicate through a double stranded DNA intermediate using an enzyme known as reverse transcriptase. HIV is classified as genus of the retroviridea family called lentiviruses which include a large number of different viruses that infect a diverse group of animal species (Coffin 1992); (Haase 1986); (Levy 1986). The first lentivirus, the Equine Infectious Anaemia Virus, was discovered in 1904 (Vallee et al 1904). Since the discovery of HIV other lentiviruses have been discovered including the Simian Immunodeficiency Virus (SIV) and Feline Immunodeficiency Virus (Benveniste et al 1986); (Daniel et al 1985); (Murphey Corb et al 1986); (Pedersen et al 1987).

A second and less virulent human immunodeficiency lentivirus that causes AIDS, and may cross react with HIV screening, was named HIV-2 and the first HIV-1 (Clavel et al 1986). In this thesis all experiments and discussions refer only to HIV-1 and are abbreviated to 'HIV'.

1.2.1 Pathology of HIV infection

An acutely infected individual can present with a virus-like illness termed stage I disease. (Centers for Disease Control 1986) and 40% will give a history of a mononucleosis like illness (Tindall et al 1988); (Tindall et al 1991). These

symptoms usually last from one to three weeks followed by an asymptomatic period of months to years (stage II disease). The asymptomatic period lasts an average of ten years (Taylor et al 1991) but may be as long as fourteen years (Lifson et al 1991); (Rutherford et al 1990). Clinical disease develops with persistent generalized lymphadenopathy (stage III) and AIDS (stage IV). AIDS is subgrouped into constitutional disease (fever, weight loss, or diarrhea), neurological disease, secondary infectious disease (moderately indicative of a defect of cell-mediated immunity), secondary cancers, and other conditions of HIV infection (suggestive of impaired cell-mediated immunity). AIDS is universally fatal.

High titres of HIV are found in blood during the acute infection but within weeks there is a strong cellular and humoral response to the virus with the production of detectable anti-HIV antibodies and a marked reduction in the titre of virus (Clark et al 1991); (Daar et al 1991). As the disease progresses to stage III and IV the concentration of HIV in blood rises substantially. The period of time following infection prior to the development of detectable anti-HIV antibodies is known as the 'window period'.

A major feature of AIDS is the reduction of circulating CD4+ T-lymphocytes. This may partly explain the immune system deficits of AIDS. During the early viraemic period there is a selective depletion of CD4+ helper T-lymphocytes. During stage II disease the CD4+ cell numbers recover to within normal and slowly decrease over time until clinically apparent disease and a characteristic loss of CD4+ lymphocytes.

The US Centre for Disease Control and Prevention have revised their classification system for HIV-infected adolescents and adults to include clinical conditions associated with HIV infection and CD4+ T lymphocyte counts (Centers for Disease Control 1992). This classification system establishes the following

subgroups for CD4+ counts; stage 1 ≥ 500/ul, stage 2 200 – 499/ul, stage 3 ≤ 200/ul. The revised clinical categories of HIV infection are as follows; category A asymptomatic, acute or persistent generalized lymphadenopathy, category B symptomatic, not (A) or (C) conditions, and category C which includes AIDS indicator conditions. Because the contemporary epidemiological literature relating to HIV infection mostly includes the previous classification (stage 1 to 4 disease) this previous classification system is still widely used in clinical practice in Australia (Dr David Shaw - personal communication). The previous classification system is used in this thesis.

1.2.2 Cells and tissues infected with HIV

During the initial infection the lymphoid organs become HIV infected. They provide a persistent source of virus replication (Pantaleo et al 1991); (Pantaleo et al 1993); (Embretson et al 1993). Within the lymph nodes, and perhaps other tissues, infection may rapidly spread by cell-to-cell transfer rather than cell-free infection and other cell types are potentially infected (Dietzschold et al 1985); (Gupta et al 1989); (Hooks et al 1976); (Pearce Pratt et al 1993). Infected cells may be actively infected or persist in a latent state (Hoxie et al 1985); (Chapel et al 1992). These latent infected cells are a potentially large and persistent bioburden of virus.

CD4+ helper T cells are the major target for HIV infection (Klatzmann et al 1984). However, other cells of the haematopoetic system, particularly macrophages, are the target cells of other lentiviruses (Haase 1986). Other haematopoetic cells known to be infected with HIV include stem cells, monocytes, macrophages, B lymphocytes, natural killer cells, eosinophils, thymic epithelial cells and dendritic cells (Castro et al 1988); (Chehimi et al 1991); (Freedman et al 1991); (Numazaki et al 1989); (Patterson et al 1987); (Sakaguchi et al 1991); (Zucker Franklin et al 1989).

Direct infection of other cell types is suggested by pathologic findings in HIV infected individuals. HIV has been detected in synovial fluid and tissue of AIDS patients (Espinoza et al 1990); (Hughes et al 1990); (Withrington et al 1987). Fibroblasts and Langerhans cells in the skin can be HIV infected (Ikeuchi et al 1990); (Mellert et al 1990); (Rappersberger et al 1988); (Stingl et al 1990); (Tschachler et al 1987); (Zambruno et al 1991). In the brain macrophages and microglia are the major targets of HIV infection but other cell types are HIV susceptible (Michaels et al 1988); (Price et al 1988). In the bowel HIV has been detected in mucosal cells (Gill et al 1992); (Heise et al 1991); (Kotler et al 1991); (Mathijs et al 1988); (Nelson et al 1988). Cultured bowel explants and bowel carcinoma cell lines can be infected *in vitro* (Adachi et al 1987b); (Barnett et al 1991); (Fantini et al 1992); (Fantini et al 1991); (Fleming et al 1992); (Moyer et al 1990). HIV infection of other cells has been described including thymus, liver, kidney, heart, lung, salivary glands, prostate, testes, adrenal and muscle (Lipshultz et al 1990); (Cohen et al 1989); and are reviewed by Levy (1993). Cells known to be susceptible to HIV infection are summarized in Table 1.1.

*In vivo o*bservations of bone marrow tissue has demonstrated changes in stromal cells including epithelial cells, reticular cells, blood cells and immature myeloid cells (Sun et al 1989). Bone marrow may contribute to the dissemination of HIV throughout the lymphomyeloid complex (Yoffey 1990) and haematopoietic progenitor cells isolated from HIV positive patients have been infected (Busch et al 1990). Cells of the myeloid lineage in addition to those of lymphoid origin serve as reservoirs for HIV but the susceptibility of bone forming cells to HIV infection is unknown.

Table 1.1 Human cells susceptible to HIV infection determined by *in vivo* or *in vitro* studies.

Haematopoietic
stem cells
lymphocytes
megakaryocytes
promyelocytes
some epithelial and dendritic cells
Brain
some neural cells
choroid plexus
capillary endothelial cells
Skin
Langerhans cells
fibroblasts
Bowel
columnar and goblet cells
enterochromaffin cells
colon carcinoma cells
Other
adrenal carcinoma cells
cervix (? epithelium)
?chondrocytes
fetal adrenal cells
fetal chorionic villi
hepatic carcinoma cells
hepatic sinusoid epithelium
Kupfer cells
myocardium
osteosarcoma cells
placental trophoblast cells
prostate
pulmonary fibroblasts
renal tubular cells
retina
rhabdomyosarcoma cells
synovial membrane
testes

Adapted from Levy (1993).

1.2.3 HIV infection of bone

In vitro studies of mesenchymal cells have suggested that these cells may be susceptible to HIV infection but differ in the mechanism of virus entry compared with haematopoietic cells such as T-lymphocytes (Mellert et al 1990); (Ikeuchi et al 1990). Chondrocytes and osteosarcoma cell lines can be infected suggesting these cells may be potential targets for HIV *in vivo* and possibly contributing to the establishment of local HIV reservoirs (Clapham et al 1983); (Ikeuchi et al 1990); (Mellert et al 1990).

Direct cell-to-cell spread of viruses is a common mode of transmission of many enveloped virus such as Herpes, Rhabdo viruses and retro-viruses including HIV type I (Dietzschold et al 1985); (Gupta et al 1989); (Hooks et al 1976). *In vitro* studies have demonstrated HIV infection of cells not expressing the CD4 receptor including neuroglial cells (Chiodi et al 1987), primary human synovial cells, chondrocytes, foreskin fibroblasts (Mellert et al 1990); (Adachi et al 1987a); (Tateno et al 1989), and myeloid progenitor cells (Folks et al 1988).

HIV can be detected in bone by co-cultivation techniques (Buck et al 1990); (Merz et al 1991); (Nyberg et al 1990) and analysis of genetic material (Roder et al 1992), but the infected cell type is yet to be identified and the virus load in bone has not yet been determined.

Merz *et al* (1991) have examined *in vitro* infection of human bone pieces with HIV and reported that bone could be infected. This observation is consistent with findings from HIV infected patients where the virus was detected by tissue culture or polymerase chain reaction analysis (Roder et al 1992); (Buck et al 1990); (Merz et al 1991); (Nyberg et al 1990). It is not known whether bone cells, marrow cells, other cells in bone, or blood contaminating the samples have been infected.

1.2.4 HIV bioburden of blood and tissues

The temporal relation of the HIV bioburden in serum of infected patients is well described. Virus titration methods ($TCID_{50}$) have demonstrated the virus bioburden in serum is at a maximum during the initial period prior to antibody formation (window period) and again when the patient becomes symptomatic. The *in vitro* titre of serum is maximally 10^4 $TCID_{50}$/ml during the window period and falls to 1-2 $TCID_{50}$/ml during asymptomatic antibody positive infection (Daar et al 1991). Ho *et al*(1989) reported a mean serum titre of 30 $TCID_{50}$/ml for asymptomatic patients and 3500 $TCID_{50}$/ml for acquired immunodeficiency syndrome (AIDS) patients.

Virus infected cells in blood are in greater concentration than free infectious virus. The average free virus in asymptomatic individuals is 100 $TCID_{50}$/ml (Ho et al 1989); (Pan et al 1993). The number of virus-infected cells is approximately one per 10^3 peripheral blood mononuclear cells (Bagasra et al 1992); (Brinchmann et al 1991); (Harper et al 1986); (Hsia et al 1991); (Psallidopoulos et al 1989); (Schmidtmayerova et al 1992); (Schnittman et al 1989). There are at least 5 x 10^6 leukocytes per milliliter of blood which means approximately 5 x 10^3 infected cells per milliliter of blood are HIV infected.

Massive infection of tissues in HIV infected patients has been identified in the lymphoid system (Embretson et al 1993) and brain (Pang et al 1990a). The HIV bioburden in bone from HIV infected patients is unknown and is explored in this thesis.

1.3 Indications and complications of bone allografts

Transplantation of bone allografts has an established role in orthopaedic surgery. Where there are centralized tissue banks, bone is the most frequently

transplanted allograft apart from blood and blood products. In 1989 there was an estimated 200,000 bone allograft recipients in the United States (Kately 1987) and 6,000 allografts performed in German surgical clinics (Knaepler et al 1990). Although it has been suggested that the most frequently transplanted tissue in Australia is cornea (Chapman 1992) a review of the South Australian Bone Bank as part of this thesis indicated that bone was the most frequently transplanted allograft excluding blood and blood products.

There are many indications for bone allografts and there are several types of allograft bone (Table 1.2). The allografts have differing mechanical and biological characteristics. Satisfactory results have been reported with cancellous allografts and morsellized grafts in contained acetabular defects (Harris et al 1988); (Hirst et al 1987); (Tranick et al 1986) and spinal fusion (McCarthy et al 1986). Successful structural grafts have been used for proximal femoral revision hip arthroplasty (Oakeshott et al 1987); (Oakeshott et al 1987); (Jofe et al 1988) and massive allografts following tumour resection (Gitelis et al 1988); (Mankin et al 1983); (Enneking et al 1980); (Enneking et al 1991).

The clinical success of a bone graft ultimately depends upon its ability to provide mechanical support for the skeleton. It is believed that bone allografts have some biological activity which gives them an advantage over inorganic materials because they have the potential for replacement and integration with the host (Prolo et al 1985); (Goldberg et al 1987). Bone grafts have two major functions: enhancing osteogenesis and providing mechanical support (Goldberg et al 1992).

Table 1.2 Types of bone allografts

structural allografts
massive structural grafts
small structural grafts (corticocancellous)
strut grafts
non structural allografts
morsellized grafts

Massive structural allografts are different to small structural grafts and morsellized bone. They are the least biologically active and replacement of the donor bone may be incomplete over prolonged time periods. Enneking and Mindell (1991) described only 20% replacement of massive allografts by five years but effective attachment of soft tissues with some new bone on the graft surface.

Cancellous allografts are used to provide filler material for cavitary skeletal defects, to augment the quantity of autograft bone and to provide a buttress for skeletal structures (Czitrom 1992). They are not indicated in situations requiring the stimulation of osteogenesis such as non-unions and arthrodesis (Czitrom et al 1988). Cancellous allograft bone is increasingly being used as an alternative to structural cortical bone since the development of impaction grafting techniques (Gie et al 1993); (Sloof et al 1982)

Cortical grafts and corticocancellous grafts may be used for mechanical support immediately after grafting. However, the continued structural integrity of the graft depends upon the interaction of the mechanical environment and the biological response. Cancellous allografts are usually completely resorbed and replaced by new bone but massive cortical grafts may never be completely resorbed and remain a

mixture of necrotic graft bone and viable host bone (Goldberg et al 1987). Allograft cortical bone has a reduced biological effect, ingrowing host vessels become occluded and degenerate (Goldberg et al 1987). There could be an advantage to the impaired biological response as resorption of autograft cortical bone results in a 40 - 50% decrease in structural strength (Burchardt et al 1975).

Allografts implanted adjacent to host bone appear to cause bone to react by vascular invasion and gradual replacement of the graft with simultaneous destruction of dead bone and synthesis of new bone (Friedlaender 1987b). The replacement of allograft bone by resorption and replacement is termed osteoconduction. In osteoconduction, the bone graft serves as a scaffold for the ingrowth of new host bone (Burwell 1961). Osteoconduction occurs in humans but is not confined to bone; it occurs with xenografts, glass tubes, ceramics and plastics (Salama 1983); (Urist 1980); (Ohgushi et al 1989); (Bieniek et al 1991).

Autologous bone transplants will induce new bone formation but few if any of the cells of an autograft remain viable (Heslop et al 1960). Autograft bone appears to induce bone formation by some humoral process, which may involve chemical mediators as suggested by Urist *et al* (1965); 1980); 1979). The formation of new bone in response to bone allografts is called osteoinduction. Osteoinduction is the recruitment from the surrounding host bed of pluripotential cells that differentiate into bone-forming cells (Reddi et al 1987).

The role of osteoinduction in humans is uncertain. Demineralized human bone allografts and human bone extracts will induce bone formation in rats (Aspenberg et al 1989); (Sampath et al 1983). Bone induction occurs when treated allograft bone is transplanted into baboon muscle (Ripamonti 1991). However, allograft bone and demineralised allograft bone transplanted into dogs and primates does not induce new bone formation (Aspenberg et al 1988); (Schwarz et al 1991).

1.3.1 Complications of allograft surgery.

Complications of bone allograft surgery that may be directly attributable to the allograft include stress fractures of large cortical grafts, delayed-union and non-union at the host-graft junction, graft resorption and infection.

Stress fractures are reported frequently when large unsupported diaphyseal grafts are used but they usually heal with non-operative management such as external splinting (Enneking et al 1980). This complication rarely jeopardizes the long term outcome of the grafting procedure.

Non-union and delayed union may have a less satisfactory outcome and may require additional surgery such as autologous bone grafting. Non-union occurs in 33% to 50% of grafts greater than 7.5 cm but the long term outcome is usually favorable with retention of the graft in most instances (Regel et al 1992); (Enneking et al 1980).

Graft resorption is seen frequently in non-weight bearing grafts such as the lateral component of acetabular shelf grafts (Gerber et al 1986); (Sanzen et al 1988). Some graft resorption is part of the normal repair response, which is characterized by resorption followed by graft replacement (Friedlaender 1987b). Allografts induce an immunological response in the host but this rarely leads to rapid graft resorption that compromises the clinical result (Friedlaender 1983). It is believed that pretreatment of the graft by freezing or sterilization lessens the immunological response (Friedlaender 1984); (Pellet et al 1983) in contrast to earlier work by Langer *et al* (1975) who questioned the alteration of immunogenicity by freezing the graft.

Infection is the major cause of graft failure and may extract a high cost to the patient. In Mankin's early series (1983) 14% of patients had an infection of which 36% led to amputation, 29% required graft removal and only one of the fourteen patients who had an infection had a good result.

Graft infection was found in four to twelve percent of patients in the reported series and has been described in detail (Regel et al 1992); (Loty et al 1990); (Mankin et al 1983); (Zasacki 1991); (Hernigou et al 1991); (Lord et al 1988); (Tomford et al 1990). Lord *et al* (1988) analyzed 283 patients who had a massive allograft and attempted to identify factors that may contribute to infection. They were able to demonstrate a correlation between postoperative infection and more extensive surgery, adjunctive radiation, chemotherapy, local wound related problems or multiple operations. However, thirty percent of patients did not have any co-morbid or predisposing factors that could be correlated with an increased risk of infection.

A lower infection rate has been reported by authors utilizing smaller grafts (Zasacki 1991); (Regel et al 1992), which may support the hypothesis of more extensive surgery being associated with an increased infection rate. Other graft related factors may contribute to infection because the infection rate following massive allograft surgery is more than other operative procedures within the same reconstructive surgery units (Lord et al 1988).

Infection from a contaminated bone graft has rarely been reported where the causative organism has been isolated from either the transplanted bone or retrospectively found in samples taken from the donor (Tomford et al 1990); (Tomford et al 1981); (Lord et al 1988). For the majority of allograft infections there is no proven association with graft contamination. It has been suggested that some allograft infections could be due to contamination not detected by routine microbiological methods (Gristina et al 1985). This suggests a need to sterilize all

bone allografts to prevent bacterial infection. The theoretical need to sterilize bone allografts is not supported by clinical results from the Boston bone allograft bank (Tomford et al 1990) . This allograft bank does not use secondary sterilization and has an infection rate similar to that of banks that routinely sterilize all allografts.

1.3.2 Human Immunodeficiency Virus infection in allograft recipients

Donor to recipient transmission of viruses can be demonstrated with almost certainty when there is a low incidence of pre-morbid exposure to the virus and other factors such as pre-operative blood transfusion have been excluded. Transmission of human immunodeficiency virus (HIV) following bone transplantation has been proven on two occasions. The first case was reported in 1988 following transplantation of a femoral allograft to a woman undergoing an elective spinal fusion prior to the licensing of HIV antibody screening tests (Centres for Disease Control 1988b). The donor subsequently developed lymphadenopathy and died as a result of his HIV infection, the patient also developed a proven HIV infection and died as a result.

The second report of HIV infection from bone transplantation described the case of a donor who had three unprocessed bone segments and three organs transplanted with subsequent HIV infection in the six recipients (Simonds et al 1992). The donor was HIV antibody negative but was confirmed to have HIV infection retrospectively by nucleic acid analysis of his stored blood and tissue. Recipients of treated bone and other tissues from this same donor escaped infection. These tissues included 25 ethanol treated bones, one marrow-evacuated fresh frozen bone, two untreated corneas, three lyophylized soft tissues and three irradiated dura allografts (30 - 34 kGy).

HIV donor to host transmission is well documented in many tissues including blood, kidney, cardiac, pancreas and bone marrow transplants (Dummer et al 1989); (Schwarz et al 1987); (Rubin et al 1988); (Irani et al 1991); (Carbone et al 1988); (Kumar et al 1987); (Rubin et al 1987); (Neumayer et al 1987). Recipient HIV infection has also resulted from transplantation of HIV infected blood and solid allografts from donors that have screened negative for HIV antibodies (Irani et al 1991); (Quarto et al 1989); (Bowen et al 1988); (Centers for Disease Control 1987b); (Centers for Disease Control 1987c).

1.3.3 Hepatitis virus

There have been no confirmed reports of Hepatitis B virus infection in a bone graft recipient. Shutkin (1954) reported a case of postoperative jaundice which was suggestive of acute hepatitis following bone allograft surgery. The aetiology of this postoperative hepatitis remains unknown. Other factors such as blood transfusion and/or inhalational anaesthesia are also possible explanations.

Only one case of allograft Hepatitis B transmission is recorded in the literature, Berry *et al* (1987) reported a case of acute Hepatitis B infection in a woman following artificial insemination from a donor subsequently found to be Hepatitis B surface antigen positive. At that time screening of semen donors for Hepatitis B virus infection was not the standard practice and it is inferred that the donor was not tested prior to semen collection.

Since the introduction of donor interview and laboratory screening the risk of blood transfusion transmitted hepatitis B is extremely low (Wylie 1993).

Hepatitis C virus infection from a bone allograft has been documented in one case by Egan and Nordbo (1992). They described Hepatitis C infection from a

femoral head allograft donor to an allograft/total hip replacement recipient. Transmission of Hepatitis C virus is documented in blood products and organ donors (Pereira et al 1991). Routine screening for Hepatitis C virus was recommended in 1991 (Ho 1991) and has been adopted by most blood and tissue banks.

Since the introduction of hepatitis C testing the risk from blood transfusion was thought to be so low it could not be established (Wylie 1993). This conclusion is not correct. Conrad *et al* (1995) reported hepatitis C infection in seven bone and tissue allograft recipients including two with sequence identity to donor isolates confirming the source of infection. The recipients had received allografts from first-generation hepatitis C antibody-negative donors.

During the last two decades hepatitis C, D, and E virus have been discovered and there is evidence for at least two additional viruses (Purcell 1994); (Bowden et al 1996). Bone and blood banks screen for hepatitis C but not for these newer hepatitis viruses. Transfusion or allograft related infection has not been reported from the newer hepatitis viruses (Wylie 1993).

1.3.4 Other viruses

Rapidly progressive dementia possibly from Creutzfeldt-Jakob disease has occurred in a bone allograft recipient from a donor who had received human pituitary derived growth factor (Musclow 1992). Creutzfeldt-Jakob disease has been implicated in cadaveric dura transplantation (Centers for Disease Control 1987a); (Centres for Disease Control. 1989); (Thadani et al 1988); (Centres for Disease Control. 1987) and cornea transplantation (Duffy et al 1974). The estimated risk of Creutzfeldt-Jakob disease in cadaver pituitary growth hormone recipients is 1/300 (Billette de Villemeur et al 1992). Creutzfeldt-Jakob disease transmission from

pituitary-derived gonadotropins has recently been reported (Cochius et al 1990); (Cochius et al 1992).

Creutzfeldt-Jakob disease can be transmitted during surgery suggesting blood as a vector (Masters et al 1978) and it has been found widely disseminated in animal and human tissues including liver, kidney and lung but there are no reports of its relation to bone (Asher et al 1976); (Gajdusek et al 1977).

As a consequence of Creutzfeldt-Jakob disease transmission cadaveric growth hormone replacement has been replaced by recombinant DNA growth hormone (Ellis et al 1992). Scientists may be charged by criminal law following a cadaver growth hormone related Creutzfeldt-Jakob disease outbreak (Balter 1993). Because of these concerns the use of imported dura allografts are banned in Australia.

Cytomegalovirus (CMV) can be transmitted from sero-positive donors to recipients of heart, kidney and bone marrow despite previous immunity to the virus (Gottesdiener 1989). Cytomegalovirus infection is a frequent life threatening complication of solid organ recipients due to their postoperative immunosuppression (Betts 1982). The frequency of CMV in the general community has made it difficult to establish a causal relationship between recipient infection and transplantation. There are no reported cases of CMV infection arising from bone allografts.

Herpes virus transmission appears to be rare (Gottesdiener 1989). There has been one case report that described infection in two immunosuppressed recipients of a kidney transplant from the same donor (Dummer et al 1987). Epstein-Barr virus transmission has been reported following bone marrow transplantation with subsequent development of a carcinoma (Schubach et al 1982). It has not been reported in a solid organ or bone allograft recipient.

1.4 Bone Allograft Screening

Donor demographic data and medical history remain the most important factors to decrease the risk of donor to host virus transmission (Buck et al 1989). Bone allograft banks aim to exclude donors at risk of HIV and hepatitis transmission, neurological slow virus transmission, current or previous malignancies, and other conditions of unproven aetiology such as connective tissue disorders, diabetes and Pagets disease.

Allograft banks also test serum for transmissible viruses. Screening for HIV and hepatitis in Australian bone banks was introduced in 1985 and Hepatitis C antibody screening was introduced in May 1990. Protracted licensing procedures in the USA delayed the introduction of routine HIV screening and will slow the introduction of Hepatitis C antibody screening (Gowans 1992).

Creutzfeldt-Jakob disease is not routinely tested for in any of the major bone banks which are members of either the American Association of Tissue Banks or the European Association of Tissue Banks (personal communication). Some bone allograft banks have started screening for Creutzfeldt-Jakob disease in cadaveric bone with brain biopsies. Hepatitis D, E, and F are not excluded by serum screening.

For an infected donor to go unrecognized the sociomedical screening and serum screening methods would have to fail. Early in the course of infection with HIV a potential donor is HIV-infected but antibody-negative and this period is called the 'window period'. The problem of not detecting an HIV infected donor (Figure 1.1, Table 1.3) relates to the detection of asymptomatic donors that are either antibody test failures or in the window period. Screening live and cadaver donors differs in the screening tests available and with the prevalence of the donor population.

All donors are screened (Figure 1.1) by social screening and surrogate tests. To remain undetected, live donors must fail either the antibody test or the be in the window period. The window period risk is reduced by quarantine. Cadaver donors are additionally screened by autopsy and organ recipient reporting, but are not subjected to graft quarantine.

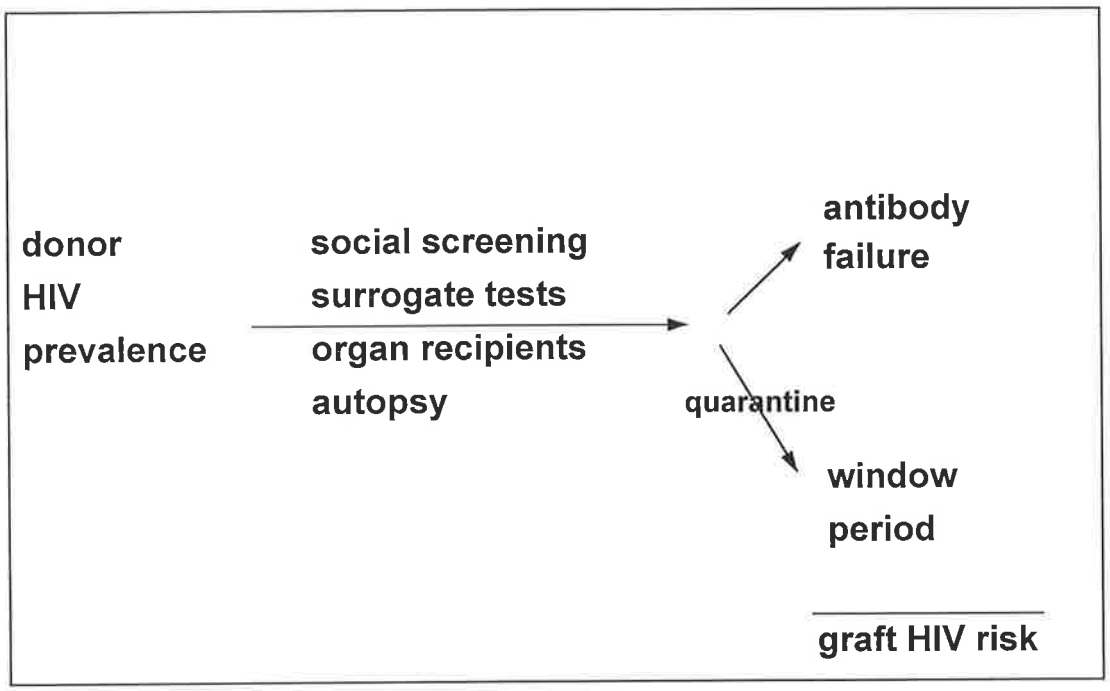


Figure 1.1 Risk of HIV infection from allograft bone. The risk is determined by the donor prevalence of HIV multiplied by the probability of screening test failure.

1.4.1 Social screening

Bone allograft banks screen donors for risk factors of transmissible disease including HIV (American Association of Tissue Banks 1989). Ninety five percent of new Australian HIV infections had an exposure category that would exclude them from bone donation and 5% were from unspecified heterosexual contact (National Centre in HIV Epidemiology and Clinical Research. 1994). Retrospective reviews of HIV infected but antibody-negative blood donors demonstrated risk factors in six of seven cases (Ward et al 1988a). In the United States of America 77% of male

antibody-positive blood donors and 44% of female antibody-positive blood donors (Ward et al 1988b) had risk factors. In Australia, 19 of Australia's 46 antibody-positive blood donors reported no known or potential exposure to HIV other than heterosexual contact (Kaldor et al 1991).

The majority of donors that are HIV infected have risk factors which should be identified by sociomedical screening. The likelihood of missing risk factors, from a potential donor, has been estimated as one in twenty (Buck et al 1989).

1.4.2 Surrogate tests

HIV risk factors are also common to other transmissible diseases which are part of routine bank screening including syphilis, Hepatitis B and C. Approximately 25% of American blood donors with HIV antibodies had a second reason for exclusion (Buck et al 1989). The recent development of Hepatitis C antibody screening is estimated to reduce the HIV risk by one third (Le Pont et al 1990).

1.4.3 Cadaver autopsy

Cadaveric bone and organ donors are autopsied and there is the potential to identify histological changes of HIV that compliment serum screening tests. Following the appearance of HIV antibody, morphologic changes of HIV can be identified (Murchadha et al 1987) but clinically evident lymphadenopathy and fever precedes antibody production during the acute phase of the infection (Daar et al 1991); (Clark et al 1991); (Cooper et al 1985). The estimated sensitivity of detecting acutely HIV infected donors by autopsy is 12.5% (Buck et al 1989). Buck *et al* (1989) recommended a lymph node biopsy which was calculated to have an additional sensitivity of 0.5 but the lymph node biopsy is not included in recommended screening tests for bone and tissue banks (La Prairie et al 1991).

1.4.4 Organ recipient infection

Cadaveric bone donation comes from two sources; multi-organ donors or recently dead non-organ harvests. Bone from multi-organ donors is usually used several months after harvesting and during this time the donated organs will have been transplanted to immunosuppressed recipients. The organ recipients become a biological test for the HIV virus. Immunosuppressed organ recipients who have inadvertently received HIV contaminated allografts have developed HIV related illnesses quickly and are usually readily identifiable (L'Age-Stehr J. et al 1985); (Schwarz et al 1987). Allowing for the logistics of detection of HIV infection in the recipient and reporting back to the organ donation program the estimated sensitivity of this test is 0.5 (Buck et al 1989).

1.4.5 HIV testing

The detection of antibody depends upon both the production of antibody by the host and the sensitivity of detection methods. HIV produces a classic immunological response with the production of IgM followed by IgG anti-HIV antibodies. The effectiveness of screening is dependent upon the sensitivity of the IgG antibody test which is 99.41% (Dax.E 1992) to 99.69% (Dax et al 1992). It is unlikely that the HIV antibody screening tests will be improved although incorporating anti-HIV IgM antibody tests may include weakly reactive IgM antibody responses and may antecede IgG in some cases (Epstein 1991).

The addition of an HIV antigen test remains controversial. Early events of acute HIV infection are associated with the release of HIV antigens including the p24 antigen which can be detected by commercially available licensed tests. A case of HIV transmission from antigen-positive antibody-negative blood has been reported (Irani et al 1991). Stramer *et al* (1989) screened 35,000 plasma donors and identified two antigen-positive antibody-negative donors. However, the peak

viraemic period may only be several days (Stramer et al 1989) and applying this technology to allograft donors has been disappointing. Large numbers of blood donations, including those from HIV endemic areas, have been screened with the p24 antigen assay but antigen-positive antibody-negative donors were not detected (Baecker et al 1988); (Alter et al 1990); (Busch et al 1990).

HIV antigen testing is only in clinical application in Thailand which is an area of extreme risk. However, only one antigen positive but seronegative donor has been detected by this method (Chiewslip et al 1991).

The polymerase chain reaction method has identified infection in antibody-negative high risk patients (Pezzella et al 1989); (Redfield et al 1988); (Daar et al 1991); (Clark et al 1991); (Imagawa et al 1989); (Laure et al 1988) and it has been suggested that this technology can be applied to the detection of seronegative at risk individuals (Cassol et al 1991) or as a routine screening test (Loche et al 1988). In the future it may be possible to apply the polymerase chain reaction to dried blood spots for diagnostic purposes (Cassol et al 1991). At the moment however, using PCR for diagnosis is only possible in sophisticated reference laboratories, the results of which must still be viewed with caution (Dax.E 1992); (Sheppard et al 1991).

1.4.6 Window period

During the window period HIV infection in donors will not be detected by HIV antibody screening. The acute hyperviraemic phase of HIV infection disappears with the production of detectable antibodies (Daar et al 1991); (Clark et al 1991); (L'Age-Stehr J. et al 1985). There have been reports of patients remaining seronegative for prolonged periods (Ranki et al 1987); (Horsburgh, Jr. et al 1989) but this pattern of disease appears unusual with the vast majority of patients seroconverting within a short period of time. First generation antibody tests detected most donors within nine

months (Goudsmit et al 1986); (Gaines 1989); (Albert et al 1987) however more recent tests have reduced the window period to three to six weeks (Haseltine 1988); (Redfield et al 1988); (Gaines et al 1990). Dax et al (1992) calculated the window period, of four weeks in a ten year infection (Taylor et al 1991), to be 0.8% of the time course of infection.

The window period estimate is validated by observation of areas with a high background prevalence of HIV infection. In the Bangkok area 222,611 units of blood were transfused in 1990 (National Blood Centre 1990) from which the anti-HIV rate in previously untested male donors is approximately one in 140 (Chiewslip et al 1991); on this basis an estimated 1,590 HIV positive patients should be encountered. If 0.8% of HIV infected donors are seronegative at the time of donation it is expected that 13 HIV positive seronegative donors would be present. Three cases of HIV transmission from seronegative blood components were reported (Chiewslip et al 1991) which is consistent with the estimated figure of 13 cases.

1.4.7 Graft quarantine

To reduce the chance of not detecting an HIV infected donor in the window period it is recommended that grafts from living donors undergo a quarantine period with an HIV antibody test 90 to 180 days following harvesting (La Prairie et al 1991); (American Association of Tissue Banks 1989); (Chateauvert et al 1990). These recommendations now apply to Australia (Australian National Council on AIDS 1990). By providing a quarantine of 12 or more weeks before testing for HIV antibody the estimated potential to detect seronegative donors is 99% (Centres for Disease Control. 1988a).

Table 1.3 Estimated value of bone allograft screening tests.

Screening Procedure	Sensitivity (%)
social screening	95
surrogate tests	33
autopsy	12.5
organ recipients	50
antibody test **	99.55
quarantine	99

**Antibody sensitivity is the average of reported IgG anti-HIV antibody sensitivities.

1.5 Allograft Sterilization

The risk of virus transmission from bone allografts has not been completely eliminated by donor screening and methods of virus inactivation are frequently employed. In contrast to organ transplantation, bone allografts are preferentially non viable at the time of grafting, and banking methods for these grafts are not dependent on preservation of living cells. Sterilization methods have included both chemical and physical methods including ionizing radiation. In addition, at the time of the allograft surgery chemicals such as ethanol or Povidone-iodine are frequently used to thaw frozen bone allografts and to inactivate bacteria surface contamination.

Sterilization of allografts may reduce the chance of HIV infection if an infected graft escaped screening procedures. The efficacy of bone HIV sterilization remains unknown (Campbell et al 1994). HIV infected tissue has been transplanted from bone and tissue that was sterilized without recipient infection (Simonds et al 1992), suggesting sterilization may have reduced graft HIV infectivity. However, the infectivity of HIV infected bone allografts is unknown and it cannot be assumed that

the absence of HIV infection in recipients of treated bone can be entirely related to the graft treatment. Most recipients of HIV infected blood transfusions will become infected (Ward et al 1989). However other organs have been donated without subsequent infection, including kidneys (Kerman et al 1987); (Dummer et al 1989) and corneas (Pepose et al 1987); (Schwarz et al 1987), suggesting this route is perhaps less infective than receiving infected blood.

1.5.1 Virus inactivation by chemicals

HIV is an enveloped virus with a lipoprotein coating which makes it vulnerable to inactivation by organic solvents and many chemical disinfectants. Other viruses that do not have a lipid coating such as Hepatitis B virus are inactivated only by powerful disinfectants such as chlorine and aldehydes. The effectiveness of chemical processing and sterilization of HIV with chemicals has been reviewed by several authors (Asselmeier et al 1993); (Prince et al 1987); (Angermann et al 1991).

Alcohols are effective germicidal agents and 70% ethanol reduces HIV titres by more than 7 $\log_{10}$ $TCID_{50}$/ml within one minute (Resnick et al 1986); (Quinnan et al 1986). The expected inactivation with a 2-10 minute ethanol treatment may be as high as 18 $\log_{10}$ reductions (Martin et al 1985). Ethanol fractionation is frequently used during the preparation of immunoglobulins and has been extensively researched, during the ethanol fractionation of plasma the process efficiency may inactivate 15 $\log_{10}$ infectious titres (Wells et al 1986).

Alkylating agents including formaldehyde and ethylene oxide effectively inactivate HIV *in vitro*. Organic mercurial antiseptics and hydrochloric acid are also effective *in vitro* but are in limited clinical usage. Povidone-iodine and chlorhexidine formulations are frequently used as surface active agents and both efficiently inactivate HIV *in vitro* (Kaplan et al 1987); (Montefiori et al 1990).

1.5.1.1 Problems of chemical sterilization methods

Alcohols are currently used to treat bone allografts (Tuli et al 1988) but there are concerns regarding the efficiency of chemicals to inactivate HIV. Alcohols are effective germicidal agents *in vitro* but their virucidal activity is variable and impaired in the presence of proteins. Hansen *et al* (1989) examined the efficiency of gluteraldehyde and alcohols with clinically relevant conditions and realistic contact periods. Dried HIV in the absence of additional serum or organic material was not inactivated by 70% ethanol or industrial methylated spirit exposure for 20 minutes.

Dahners and Hoyle (1989) examined ethanol sterilization of bacteria contaminated bone for four and eight hours. They found all samples were sterile at eight hours but 10 - 20% were not sterile after a four hour exposure. Knaepeler *et al* (1992) have reported the failure of ethanol sterilization of HIV contaminated bone. They perfused 70% ethanol through a 3mm and 6mm slice of HIV spiked human cancellous bone for 24 hours and found HIV was not inactivated. Gas chromatography measurements of ethanol diffusion through the bone samples showed 70% ethanol achieved a concentration of only 26% with the 3mm bone sample and 18% with the 6mm sample. Dilution of alcohols and the fixative properties of alcohols both interfere with their action as disinfectants (Sattar et al 1991).

Ethylene oxide treated bone has been successfully used for many years (Cloward 1980). This is despite the observation that ethylene oxide exposure destroys the osteoinduction properties of bone transplantation in rats (Munting et al 1988); (Aspenberg et al 1990). Ethylene oxide treatment has been associated with recipient morbidity and chemical breakdown products (ethylene chlorohydrin) have been implicated (Jackson et al 1990). Other agents which are effective against HIV

have been precluded because of the potential for recipient morbidity. Alkylating agents such as formaldehyde and glutaraldehyde are effective *in vitro* but are not widely used because of this problem (Angermann et al 1991). Merthiolate and Cialit are organic mercurial antiseptics which have been used to treat bone allografts and are effective against HIV *in vitro* (Wilmes et al 1987) but their use for treating bone allografts has been discontinued because of their potential toxicity (Tomford et al 1983).

Detergents and surface active antiseptics including povidone-idoione and chlorhexidine are effective at inactivating HIV *in vitro* but their effectiveness in the presence of organic contaminants and safety of the host after long term graft storage has not been examined.

Various sterilizing methods were examined by Withrow (1990) using an animal retro-virus model. The feline leukemia virus is a retro-virus similar to HIV and these authors examined bone from infected cats that had been treated with chemical and physical methods. Infectious virus was retrieved from all bones exposed to ethylene oxide or demineralized with hydrochloric acid. All cats were heavily infected with virus and the biological amplification method to assess the presence of virus was very sensitive. However, the implication is that standard methods of chemical sterilization of bone do not inactivate the feline leukemia virus and correlation to HIV may be implied.

1.5.2 Virus inactivation by physical methods

Physical methods of graft treatment are commonly used for storing and decontaminating bone allografts; ionizing irradiation with gamma rays or electrons are the most common form of secondary sterilization used by bone banks. Heat and autoclaving have been reported, but are not in common use outside of Europe.

Physical methods of graft treatment have an advantage because the treatment is not inactivated by organic materials such as serum or bone (although the medium, state of hydration and temperature will affect the efficiency of the sterilization process). Furthermore physical methods do not expose the recipient to potential toxicity from chemical sterilising agents or their breakdown products.

1.5.2.1 Sterility assurance level

The inactivation of viruses by irradiation and other physical methods follows exponential law and inevitably this means that there is always a finite probability that an organism may survive regardless of the dose delivered. For a given dose the probability of survival is determined by the number of organisms being irradiated and environment in which the organisms exist during irradiation. The sterility of an item is not absolute but can be defined in terms of the probability of existence of a single non sterile item. The value of this probability is the sterility assurance level achieved by the sterilization process (International Atomic Energy Agency 1990).

The sterility assurance level is arbitrarily determined but in most countries a sterility assurance level of 10^{-6} is applied in the sterilization of medical products (International Atomic Energy Agency 1990); (Gaughran 1985). The sterilising dose to prevent bacterial contamination has been approximated to six or eight times the D_{10} value as a practical standard for sterilization (Christensen et al 1982). The D-6 values for Bacillus pumilus, strain E601 is commonly used as a reference strain for radiation sterilization of medical devices (International Organization for Standardization. 1993). Other bacillus and non spore forming bacteria have a similar radiation dose response curve and the D-6 or D-8 value is 10 - 30 kGy (Kristensen et al 1981); (Gardiner et al 1986). It may be reasonable to accept a D-6 or D-8 value for bacteria because the potential survival of a limited number of organisms may not lead

to the catastrophic sequel that could occur with the transmission of lethal viruses such as HIV, Hepatitis and slow viruses. The bioburden for bacteria may be calculated as the concentration of infectious agent per milliliter but it is more correct to determine the total number of organisms that may be contaminating the entire item (International Organization for Standardization. 1993).

1.5.3 Sterilization of bone allografts by irradiation

Initial reports of HIV inactivation by irradiation suggested the virus was uniquely radio-sensitive and as little as 0.25 - 2.5 kGy may be required to inactivate HIV (Bigee 1988); (Spire et al 1985). These reports have led to the belief that sterilising bone grafts with irradiation would eliminate the risk of recipient HIV infection and reports as recent as 1992 have suggested there may be no risk of HIV or Hepatitis transmission from irradiated bone allografts (Ferrante et al 1992).

Bone allograft banks commonly sterilize frozen bone by irradiation and 15 - 25 kGray is frequently used. Industry guidelines that do not directly relate to bone allografts have suggested bone banks use 25 kGy (Van Winkle, Jr. et al 1967) or calculate the sterility assurance level of irradiated bone (International Organization for Standardization. 1993); (Gaughran 1985). *In vitro* irradiation of virus inoculated bone with 15 kGy caused HIV inactivation (Knaepler et al 1992) but this observation has not been confirmed by others who have suggested 25 kGy may not be sufficient to sterilize frozen bone (Conway et al 1990); (Campbell et al 1994).

Withrow et al (1990) examined the feline leukemia virus model described previously with various treatments including irradiation with 29kGy. Irradiation delayed but did not prevent virus infection of the recipient cats. Fideler et al (1994) have examined gamma irradiation sterilized patella ligament-bone grafts from HIV infected donors with the polymerase chain reaction method. They found doses of 20

or 25 kGy did not destroy the genes of HIV but the DNA was not detectable in grafts treated with 30 or 40 kGy of gamma irradiation. The amount of irradiation required to inactivate the virus that may reside in HIV infected bones remains uncertain and the dose required to achieve a satisfactory sterility assurance level has not been addressed.

Inactivation of viruses is known to occur as a first order reaction and the virucidal effectiveness of gamma irradiation is directly related to the genome size of RNA viruses (Ginoza 1968). It should be possible to determine the initial contamination level (bioburden) and calculate the decimal reduction value (D_{10} - value) to determine the amount of irradiation required to achieve the sterilization assurance level. The sterilising dose of most bacteria clinically relevant to contaminated bone allografts are inactivated by 10 - 30 kGy using an inactivation factor of 10^{8} (eight D_{10} - values) (Gardiner et al 1986); (Kristensen et al 1981). The D_{10}-values of many viruses range from 3.9 - 5.3 kGray (Sullivan et al 1971) but some very small viruses such as poliomyelitis and those associated with the Creutzfeldt-Jakob disease are highly resistant (Gardiner et al 1986); (Gibbs et al 1978).

Unlike chemical agents gamma irradiation and heat treatment are not dependent on physical contact. It is therefore possible to determine the dose related effectiveness of these sterilising agents. The constant nature of thermal treatment and irradiation inactivation of viruses makes it is possible to calculate the dose-response relationships (Gardiner et al 1986).

If the virus bioburden of an infected bone allograft is known, it should be possible to calculate the dose required to achieve a certain probability of the virus surviving the treatment.

1.5.4 Effects of irradiation on allograft biology

Although very high sterilising doses would seem desirable to ensure maximum sterility, increasing the radiation dose is deleterious to the biological and structural integrity of allograft bone. Fresh bone elicits an immune response when transplanted to an unmatched recipient and most authors report that this response is diminished by freezing and freeze drying (Burwell et al 1985). It has also been reported that low levels of irradiation further diminished this response (Friedlaender 1984). It may be that the clinical success of bone allografts is partially attributable to the reduced immune response and low dose irradiation may confer some biological advantages to the healing and incorporation of allografts.

Pellet *et al* (1983) examined the alteration in immunologic response using a rat allograft model and observed enhanced graft incorporation with low dose irradiation up to 10 kGy but this effect is negated when combined with freeze drying or when doses of 50 - 75 kGy are used. Pelker *et al* (1989) used a similar model to evaluate the effect of low dose irradiation on frozen bone allografts and did not demonstrate an advantageous biologic response in graft incorporation as measured by torsional strength.

The biological activity of bone grafts in humans is poorly defined and may relate to the osteoinductive or osteoconductive activity of bone (chapter 1.3). The osteoinductive capacity of bone is significantly reduced when lyophylized bone extracts are irradiated by more than 30 kGy at room temperature (Munting et al 1988); (Schwarz et al 1988); (Buring et al 1967). Dziedzic Goclawska (1991) reported similar findings with complete resorption of allografts that were lyophylized and irradiated at room temperature but they observed no difference from non-irradiated control samples when frozen allografts were irradiated with 35 - 50 kGy. Pellet *et al* (1983) observed incomplete healing with frozen rat segmental femoral allografts that had been irradiated with 50 and 75 kGy. There was incomplete

healing in some grafts irradiated with one and 10 kGy but their small sample size precludes an accurate opinion regarding the lesser doses.

1.5.5 Effect of irradiation on allograft biomechanics

The effects of irradiation sterilization upon the biomechanical properties of allograft bone have been reviewed (Bright et al 1983); (Pelker et al 1987); (Asselmeier et al 1993); (Pelker et al 1983). The conclusion from the reviews was that freezing bone did not significantly alter its mechanical properties and irradiation below 30 kGy had little effect upon bone grafts but doses greater than 30kGy significantly reduced the strength of allograft bone.

More recent studies have examined the biomechanical properties of irradiated frozen bone which simulates the practice of many bone banks. However conclusions are difficult to interpret as there is much variation of results. Lotty *et al* (1990) reported a 20% decrease in bending strength after 27kGy irradiation of frozen human cortical bone subjected to bending tests, but this decreased quite dramatically to 65% of control strength when irradiated with 37kGy. Triantafyllou (1975) reported similar results with frozen bovine tibia examined by three point bending and found a 50 - 75% reduction of bending strength when bone was irradiated with 30kGy. In contrast, Komender (1976) examined machined human femoral cortex and reported minimal mechanical changes in compression, torsion and bending with 10 and 30 kGy, but observed a 20% decrease in compressive strength and a decrease in torsion and bending strength to 65 - 70% of control when irradiated with 60kGy.

Biomechanical studies of cortical bone are relevant to large structural allografts but the mechanical properties of cancellous bone may be more relevant as the majority of bone allograft reconstructions involve cancellous or cortico-cancellous grafts that will be loaded in compression. There have been limited studies and the

effect of irradiation is not clear. Knaepler *et al* (1991) examined frozen trabecular pig bone in compression and reported no mechanical effect when radiated with 10kGy but a reduction to 61 - 69% of controls when irradiated with 25kGy. These results were in contrast to the work of Anderson *et al* (1992) who examined the compressive mechanical properties of human cancellous bone irradiated with 10, 31, 51 and 60kGy. They reported irradiation below 60kGy did not affect the mechanical properties of the cancellous bone, but the compressive failure stress and elastic modulus decreased significantly with 60kGy.

It is unknown what a significant decrease in the mechanical strength of allograft bone is. There is no consensus on the clinically important biomechanical requirements of an allograft bone which relates to the varied applications of allograft bones and their structural importance, and to the variability of the normal mechanical strength of bone. There are regional variations in density, porosity, mechanical strength and stiffness of human cortical and cancellous bone between individuals as well as regional variation in the same individual (Keller et al 1990); (Hansson et al 1980). In addition the biological behavior of bone grafts affects their mechanical properties. As grafts are revascularized, and subsequently replaced by bone ingrowth there is a decrease in strength and increase in graft porosity. Animal segmental autograft models have demonstrated a 40 - 60% decrease in mechanical properties as the graft replacement takes place (Burchardt et al 1975); (Springfield 1987).

1.5.6 Thermal treatment of bone grafts

It has been suggested that freezing may decrease the virus load in grafts harvested from individuals who are infected with HIV (Buck et al 1990); (Salzman et al 1993). Nemzek *et al* (1994); 1993) have used the feline leukemia virus model to examine frozen connective tissue cancellous bone allografts. The frozen allografts resulted in transmission of the retro-virus, even when combined with a water flush to

remove bone marrow. These animal findings have been confirmed in humans with a report of HIV transmission arising from freeze dried factor VIII (Centers for Disease Control.Update. 1984).

Boiling allografts for 10 - 30 minutes before freezing or immediately before use has been used as a method of graft preparation prior to the availability of bone banks. These techniques are used less frequently at present but since the HIV epidemic a renewed interest in thermal treatment is being reported. Successful clinical outcomes after resection and autoclaving massive bone defects from bone tumour excision have been reported (Shimozaki et al 1992); (Harrington et al 1986). Successful short term clinical results have also been reported with xenografts treated with very high temperature dry heating that removes the organic components of bone (Ueno 1988). The main objection to the wide spread use of heat treatment is that heating coagulates protein and at temperatures above 60^0C the bone morphogenic protein is destroyed which could result in a reduced osteogenic response (Angermann et al 1991).

HIV has been demonstrated to be very heat labile under laboratory conditions. HIV is inactivated slowly at 56^0C but more rapidly at 60^0C wet heat (Quinnan et al 1986). Hilfenhaus *et al* (1986) found HIV was rapidly inactivated and was undetectable within 30 - 60 minutes with 60^0C heating of HIV spiked plasma protein preparations. Quinnan *et al* (1986) found an inactivation rate of at least 10^6 *in vitro* infectious units with a two hour incubation at 60^0C. McDougal *et al* (1985b) found the rate of thermal decay was consistent with first order kinetics and the virus was inactivated in 24 seconds at 60^0C but was dramatically more resistant with previous lyophilization as confirmed by others (Tersmette et al 1986); (Lancz et al 1985).

Laboratory findings suggest HIV is sensitive to heating at 60^0C wet heat, but caution must be exercised using dry heat. Epstein and Fricke (1990) have reported

HIV and hepatitis transmission to hemophilia patients after transfusion with clotting factors treated with dry heat less than 68^0C for 72 hours. Vanderberg *et al* (1986) have reported seroconversion from commercially heat treated factor VIII.

Moderate heat treatment of bone allografts has recently been investigated. Heating a femoral allograft bone to 80^0C will achieve an average central temperature of 66^0C after one hour (Staudte et al 1991). Knaepler, Gurrell *et al* (1993); 1993) and Chiron (1993) have examined the effects of moderate heat treated or autoclaved cancellous bone. Chiron *et al* demonstrated virus inactivation of HIV spiked femoral heads treated in an autoclave for 20 minutes. They found no significant difference between the mechanical results of moderate heat treated bone, autoclaved bone, and controls but they observed a delay in autoclaved graft integration. In contrast Knaepler *et al* found a significant reduction in the mechanical strength of autoclaved bone and the rate of complications was almost 20% greater than untreated bone grafts. They reported satisfactory biological results with moderate heat treated grafts.

Other workers have reported satisfactory results of moderate heat treated bone allografts in animal models (Kuhne et al 1992) and Knaepler *et al* have reported successful short term results in humans (Knaepler et al 1993). Longer term studies in humans are not yet available. Moderate heat treatment would seem to be an effective mechanism to inactivate most bacteria and HIV, but it is not effective against Hepatitis viruses and slow viruses (van den Berg et al 1986); (Epstein et al 1990); (Mikhailov et al 1987); (Brown et al 1986).

1.6 Aims and scope of thesis

The aim of this thesis was to determine if gamma irradiation of bone allografts is a satisfactory method to sterilize HIV infected bone allografts that have not been detected by routine screening methods. Irradiation with 25 kGy is the current

practice of many bone allograft banks and a residual chance of one HIV infection per million grafts is the commonly accepted standard of sterility assurance.

Understanding of the epidemiology of HIV has progressed during the period of this study (1990 - 1996) and a contemporary evaluation of bone allograft use and HIV infection risk was undertaken.

Initial reports suggesting HIV is unusually radiosensitive were examined early in the period of study (Campbell et al 1994) and the results suggested the need for further study to accurately quantify the efficacy of allograft sterilization with irradiation. This thesis addresses two specific questions related to allograft sterilization:

1) what is the virus bioburden of HIV infected allografts that have escaped donor screening? The *in vivo* and *in vitro* infection of human bone is examined in the thesis.

2) what is the inactivation rate of HIV?

Having addressed these two questions it was anticipated the efficacy of irradiation sterilization would be resolved mathematically to determine the dose required to achieve a sterility assurance level of one per million HIV infected bone allografts.

Allograft cartilage is used in osteochondral allografts and is an important contaminant of bone allografts. A secondary aim of the thesis was to determine if human cartilage is susceptible to HIV infection.

2. MATERIALS AND METHODS

2.1 Cell culture and virus infection

2.1.1 HIV laboratory safety procedures

All experiments with potentially HIV contaminated material were done in a containment laboratory (safety rating C1) with gowns and gloves worn at all times. Once inside the laboratory re-entry into the air-lock was prohibited until removing gloves, gown and washing hands.

Before using the bio safety cabinet (Gelman Sciences Australia) the work surface was sprayed and wiped with 70% ethanol. A bench coat was placed in the cabinet work area and a small autoclave bag for waste was placed in the cabinet, and removed as soon as work with infected material was completed. In the event of spills in the cabinet the bench coat was immediately placed in the autoclave bag and the floor of the bio safety cabinet thoroughly decontaminated with either 70% ethanol or 0.5% hypochlorite.

Double gloves were used when working with infectious material. Outer gloves were removed, and remaining gloves sprayed with ethanol, whenever movement out of the cabinet was necessary.

Any vessel that contained infectious or potentially infected material was filled with 0.5% hypochlorite before being sealed and placed in an autoclave bag. Washing discards, etc., were poured off into a large paper cup filled with hypochlorite soaked cotton wool and saturated with hypochlorite before being sealed and placed in an autoclave bag. Used pipettes and pipette tips were discarded into an autoclave bag without hypochlorite treatment.

Any material leaving the bio safety cabinet, which was not to be discarded was sprayed thoroughly with 70% ethanol and immediately brought out of the cabinet and wiped dry. After doing precipitation and solubilization of virus in preparation for reverse transcriptase assays, hypochlorite was squeezed into the ice container, the container was sprayed with 70% ethanol and removed from the bio safety cabinet. The container was then dunked in a 0.5% hypochlorite bath and rinsed. The same procedure was used for removal of test tube racks from the cabinet.

Centrifuge buckets were loaded inside the cabinet, sealed and sprayed with 70% ethanol before being taken out of the cabinet. After centrifuging, the buckets were opened only inside the cabinet. Centrifuge buckets were decontaminated by filling them with Cidex, leaving them for 30 minutes and washing extensively with tap water and then distilled water.

After viewing of any flask of cells under the microscope, knobs and mount were wiped with 70% ethanol.

Any item to be removed from the containment laboratory was decontaminated with 70% ethanol, wiped, and placed in the air lock.

2.1.2 Cells and virus

2.1.2.1 H3B cells

H3B cells obtained as a gift from Dr. P. Li (Institute of Medical and Veterinary Science, Adelaide, South Australia) were used as the virus producer cells for virus supernatant and were employed as the virus donor cells in the cell-to-cell transmission format. These cells are a clone derived from HTLV-IIIB infected H9 lymphocytes and are greater than 95% HIV p24 antigen positive as judged by

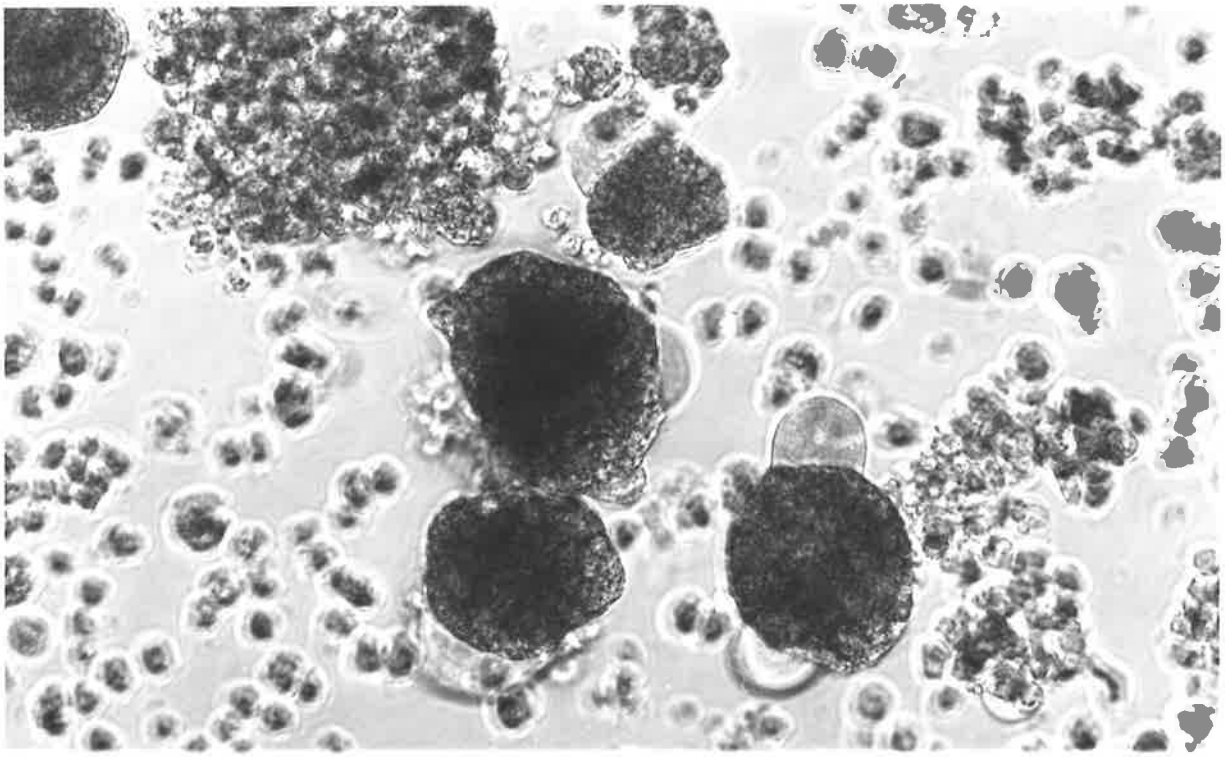
immunofluorescence and contain an average of two copies of unintegrated HTLV-IIIB pro virus DNA per cell (Li et al 1992a).

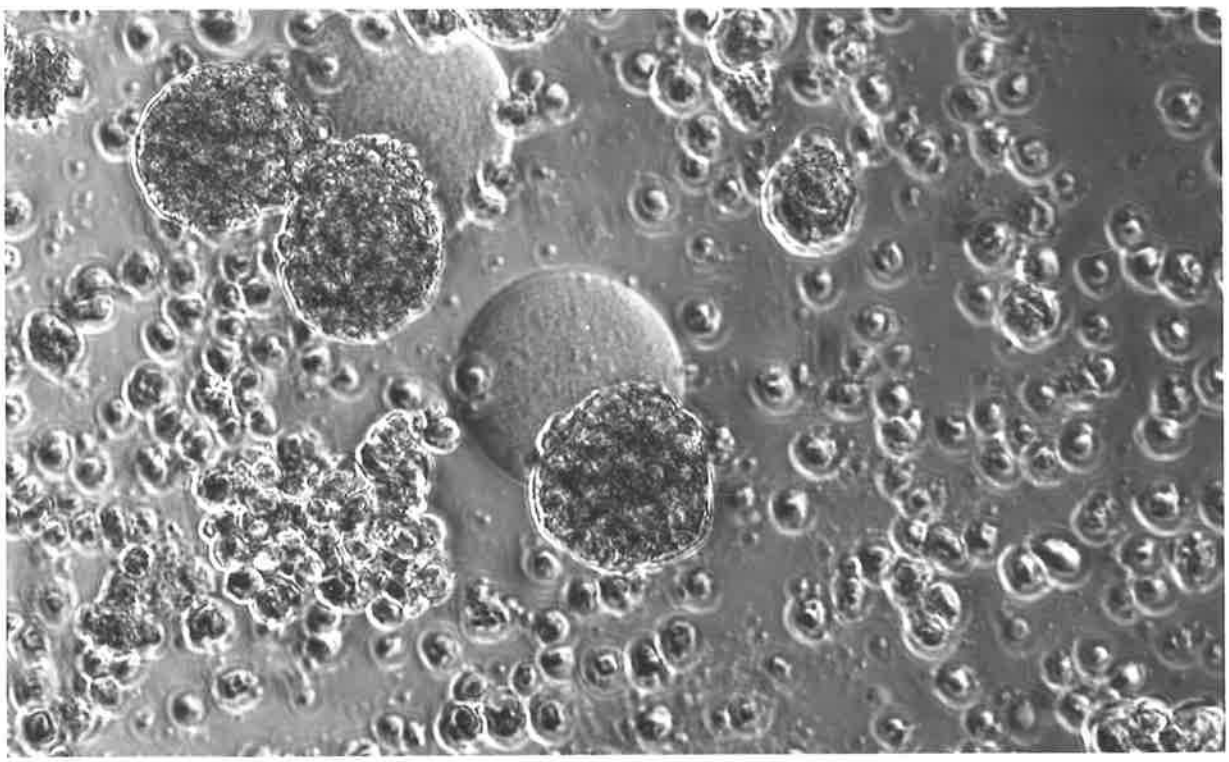
H3B cells were propagated in RPMI-1640 growth medium (Cytosystems) prepared using ultra pure water supplemented with sodium carbonate 0.85 grams/litre, HEPES 20 mM, L-glutamine 0.29 grams/litre, penicillin 25 u/ml, streptomycin 25 u/ml and phenyl red and further supplemented with 10% heat-inactivated foetal calf serum immediately prior to use. Cells were maintained at a density of $5x10^5$ cells/ml in 150 cm^2 flasks (Costar).

2.1.2.2 HUT-78 cells

HUT-78 cells (NIH AIDS Research and Reference Reagent program, ERC Bioservices Corp. Rockville, MD) were employed as indicator cells for co-cultivation after cell-free infection. When infected with HIV or co-cultivated with HIV infected cells they coalesce to form syncytia (Figure 2.1) and immunofluoresce for p24 antigen. HUT-78 cells were maintained in sub-culture in RPMI-1640 growth medium (as above) supplemented with 10% heat-inactivated foetal calf serum at a density of $5x10^5$ cells/ml.

Figure 2.1 Examples of giant cell formation after inoculation of HUT-78 cells with Human Immunodeficiency Virus. **(top)** Five days after inoculation the multinucleated giant cells are easily differentiated from the smaller HUT-78 lymphocytes and have a characteristic ballooning appearance. **(lower)** Nine days after inoculation the giant cells have a darker multinucleated nucleus than immature giant cells. The surrounding HUT-78 lymphocytes demonstrate cytopathic changes, fragmentation and many are beginning to coalesce as the precursor to further giant cell formation. Magnification, x40.





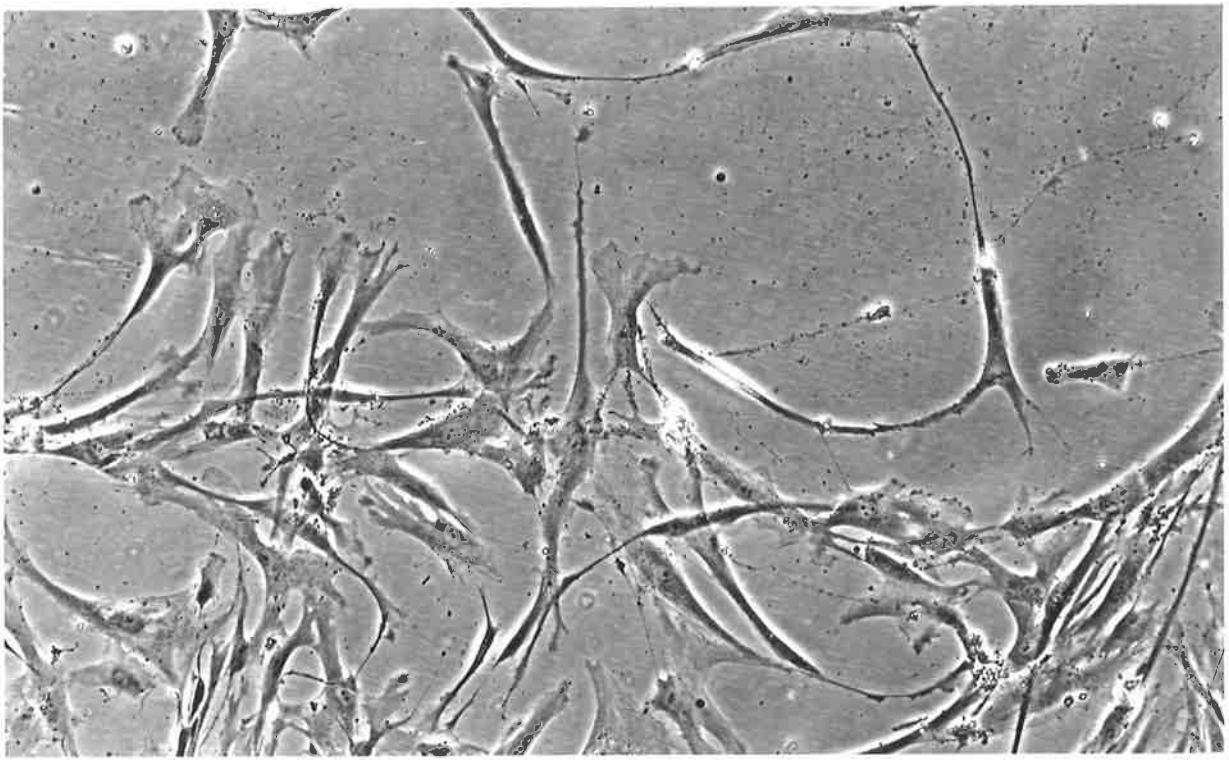
2.1.2.3. Human bone derived cells

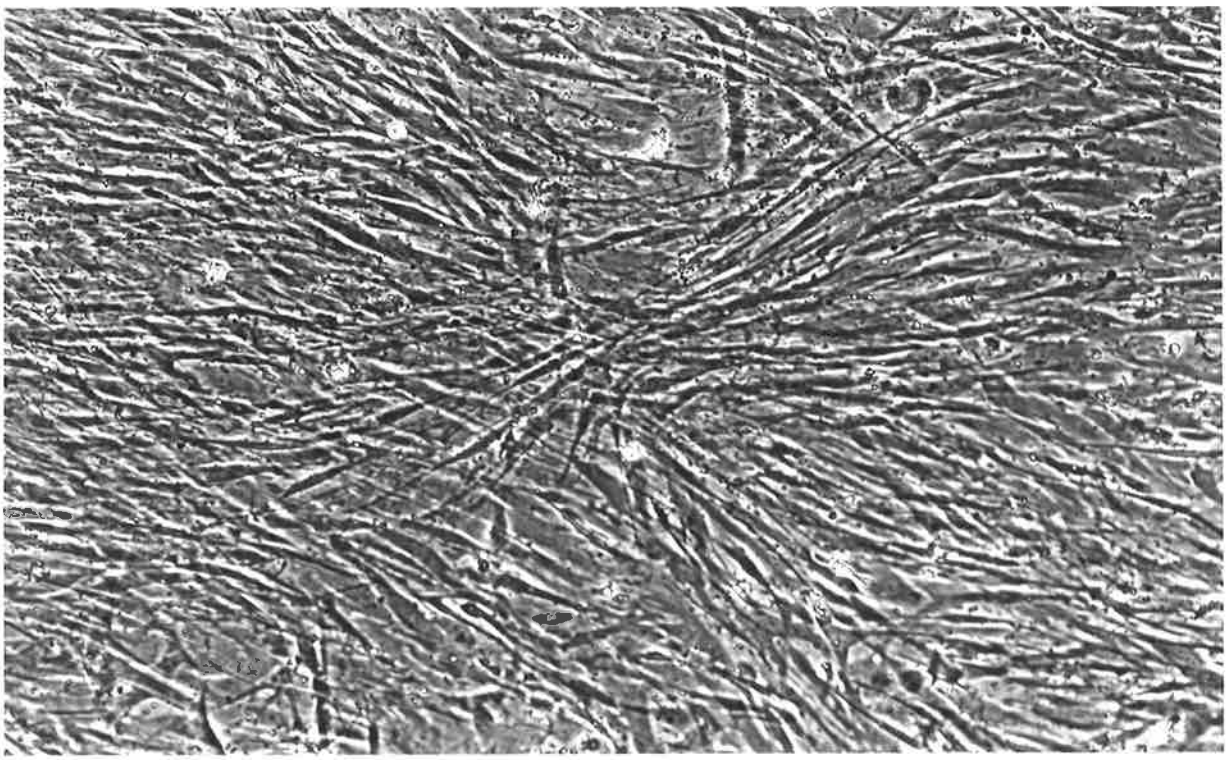
The human bone derived cell method of Beresford *et al* (1983) was used exclusively. The culture medium used for all human bone derived cell culture experiments was Dulbecco's modification of Eagles minimum essential medium (DMEM, Gibco). The DMEM (glucose 4.5 grams/litre) was prepared using ultra pure water supplemented with sodium carbonate 0.85 grams/litre, HEPES 20 mM, L-glutamine 0.29 grams/litre, penicillin 25 u/ml, streptomycin 25 u/ml and phenyl red. The medium was supplemented with 10% heat-inactivated foetal calf serum and ascorbate-2 phosphate 100 uM immediately prior to usage.

First or second passaged cells were obtained as a gift from Dr. Stephen Graves and Mrs. Shelley Hay, University of Adelaide, South Australia. Bone explants obtained from elective orthopaedic surgery patients were placed in sterile saline. Explants were washed three times in phosphate buffered saline using a vortex mixer to remove contaminated blood cells. Using aseptic technique, the tissue was cut into small pieces approximately 2-3 mm and placed into a 75 cm flask (Costar). Ten milliliters of culture medium was added with the flask cap loosened and incubated at 37° in 5% carbon dioxide. The medium was changed at week one and two, then three times per week.

A confluent cell layer (Figure 2.2) was collagenized and trypsinized (section 2.1.3), adjusted to a density of $5x10^4$ cells/ml, then re-seeded onto either 25 cm flasks (Costar) or 24 well trays (Costar, cat. no. 3524) to which a sterile cover slip was added. Flasks were incubated with the lid loosened until virus inoculation (after which an air tight seal was mandatory for laboratory safety), 24 well plates had water added to the remaining unused wells and surrounds, then taped and sealed with plastic food wrap and incubated at 37°C, 5% carbon dioxide. Half volume medium changes were continued three times per week. Confluent cells were removed and adjusted to approximately $5x10^4$ cells/ml to encourage continued culture growth

Figure 2.2 Human Bone Derived Cells grown to confluence. The cells coalesce to form a monolayer and adhere to the floor of the culture flask. These monolayer cells are easily distinguished from H3B and HUT-78 cells which are spherical and remain in suspension throughout the media. Magnification; top x 10, lower x 30.





2.1.3. Collagenase 1 and Trypsin digestion

Flasks were washed twice with calcium and magnesium free phosphate buffered saline for five minutes. Two hundred and fifty units of Collagenase 1 (Sigma Collagenase 1, C-0130) was added, incubated at 37° and checked every 15 minutes to a maximum of two hours. When the majority of cells had become rounded (usually after 30 minutes) the supernatant was removed.

Five milliliters of 0.05% trypsin (Sigma Trypsin Type X1, T-1005) was added and incubated at 37° for five minutes. The cell sheet was lifted with a sterile disposable pipette to enhance cell separation. Fresh medium was added (neutralizing further trypsin enzyme action) and the supernatant was removed and centrifuged at 15,000 rpm for 10-15 minutes at 4°C. The cell pellet was dispersed into fresh medium and counted.

2.1.4. Virus supernatant preparation

To prepare virus supernatant H3B cells were maintained at a density of $5x10^7$ cells/ml with an hourly medium change as described by Li *et al* (1992b). Cells were centrifuged 930rpm for 30 minutes, the supernatant was harvested and the cells resuspended in medium and incubated at 37^0 C supplemented with 5% carbon dioxide. The supernatant was pooled, chilled on ice and centrifuged at 3,500 rpm for ten minutes at 4^0 C, divided into aliquots of 5ml and stored at -70° C. One vial was thawed for virus titre (greater than 10^5 tissue culture infective doses ($TCID_{50}$) per milliliter) and reverse transcriptase activity (greater than 40,000 counts/ml).

2.2. Assessment of HIV infection

2.2.1. Microscopy

Light microscopy observations were made for cytopathic changes and the formation of multinucleated giant cells, or syncytia (Figure 2.1), as described by Sodroski *et al* (1986) and Lifson *et al* (1986). Cell fusion is thought to indicate the fusion of virus infected cells with uninfected cells and is associated with virus replication. HUT-78 cells incubated with virus or H3B cells as positive controls consistently demonstrated giant cell formation and cytopathic changes.

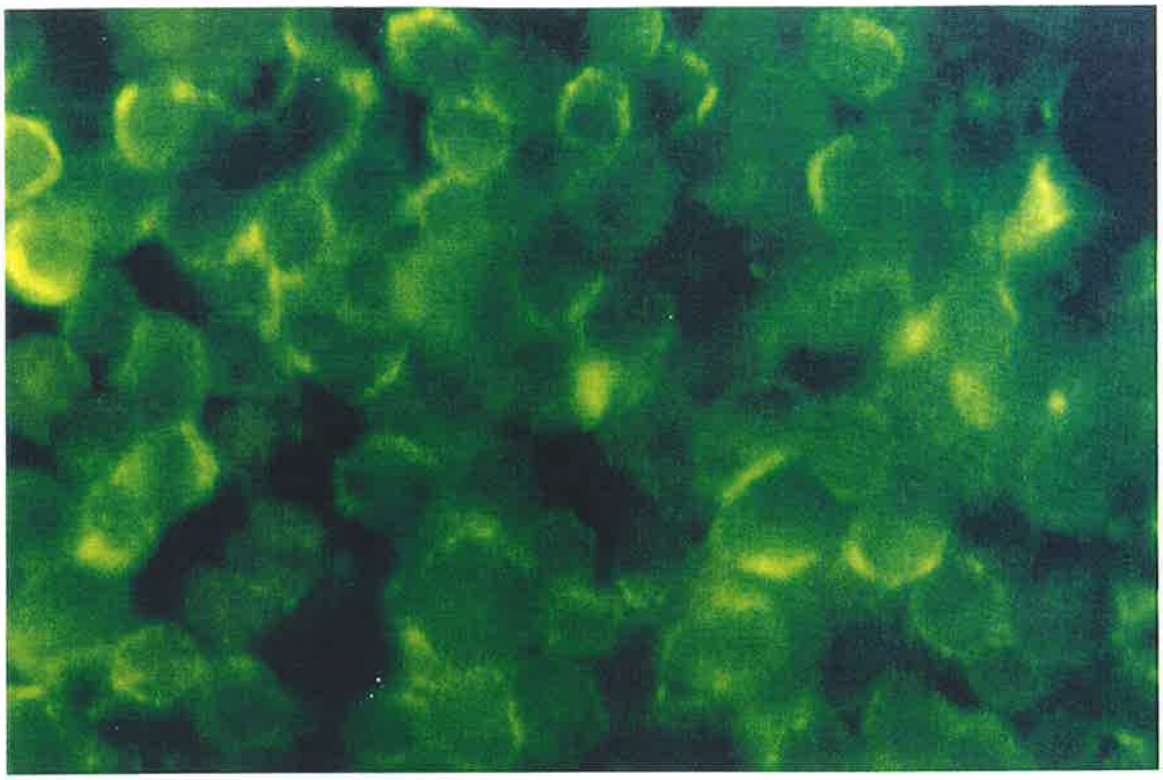
2.2.2. p24 antigen immunofluorescence

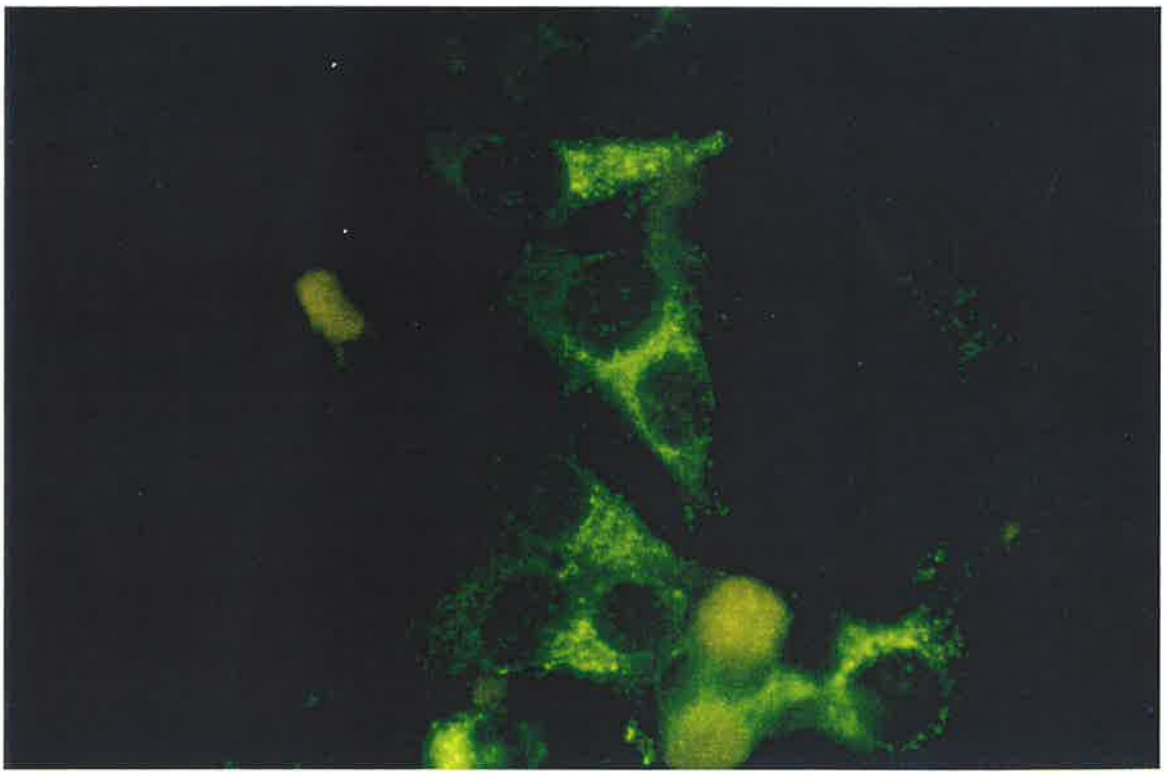
Suspension cells were washed twice with phosphate buffered saline (PBS) and resuspended at 10^6 cells/ml in PBS, spotted onto immunofluorescence slides (PH17, Wellcome) and allowed to air dry. Coverslips with adherent cells were removed from the culture plate, washed three times in 1 x PBS and allowed to air dry before being mounted onto glass slides. Slides were then fixed in cold 1% formalin (in PBS) for 30 minutes, followed by 70% ethanol for one minute.

Slides were washed for five minutes in 1x PBS followed by a 20 minute wash in 0.05% Nonidet P40 (Boehringer, Mannheim). The slides were then washed a further two times in 1x PBS followed by incubation with monoclonal antibody to HTLV-III p24 (cat. no. MAB 880-A,Chemicon, Single Oak Drive, Temecula, CA, USA), diluted 1/100 in 1 x PBS, for 45 minutes in a humid chamber at room temperature. After washing twice with 1 x PBS, FITC-conjugated sheep anti-mouse immmunoglobulin (Silenus Laboratories, NW 58th Street, Miami, Florida, USA), diluted 1/20 in 1x PBS, was added and incubated for 30 minutes in a humid chamber at room temperature. The cells were washed twice in 1x PBS before coverslip mounting with glycerol.

p24 antigen positive cells were evaluated using a Zeiss fluorescence microscope and graded negative, indeterminate or positive immunofluorescence. Non-infected HUT-78 cells were the negative controls and H3B cells were the positive controls which consistently immunofluoresced.

Figure 2.3 p24 Antigen Immunofluorescence of HIV infected cells. Chronically infected H3B lymphocytes **(top)** are small round cells in suspension and are macroscopically distinct from the larger human bone derived cells that adhere to the floor of the cell culture flask. HIV infected epithelial cells included as an example of HIV infected cell explants **(lower)**. Magnification, x60.





2.2.3. Virus titres

HUT-78 cells were incubated with 0.001% Diethyaminoethyl-Dextran (DEAE-Dextran) at 37^0 C for 30 minutes to enhance virus absorption (Duc-Nguyen 1968); (Levy et al 1985). Cells were pelleted (930 rpm for three minutes) and resuspended in RPMI-1640 medium and aliquoted one milliliter ($1.5x10^5$ cells) in eppendorf tubes. Cells were pelleted (6500 rpm for three minutes), the supernatant was removed and cells were resuspended in virus dilution.

Samples were thawed to room temperature and serially diluted ten fold to 10^{-7} in serum-free RPMI-1640 medium.

HUT-78 cell pellets ($1.5x10^5$ cells) were resuspended in 600ul of virus dilution or 600ul RPMI-1640 for controls and incubated for two hours. Cells were then washed three times in serum-free medium, resuspended in 1200ul of RPMI-1640 supplemented with 10% foetal calf serum, and aliquots of 200ul (2.5×10^4 cells) were placed into six wells of a forty eight well plate (Costar, cat. no. 3548). The plates were sealed and incubated at 37^0 Celsius.

A further 200ul of foetal calf serum supplemented medium was added to each well the following day. Half volume medium changes were made as required.

Observations for cytopathic effect were made on day five and seven, when smears for immunofluorescence were made for conformation of microscopic findings.

2.2.3.1. Calculation of $TCID_{50}$

Virus titres were calculated by the Spearman-Karber method (Dougherty 1964) for determination of 50% end points using the equation;

$$\text{Log } TCID_{50} = L - d\ (S - 0.5).$$

L = negative log lowest dilution

d = difference between log dilution steps

S = sum of proportions of cytopathic effect from virus.

The following example of $TCID_{50}$ calculation is included

virus dilution	proportion of infected cultures
10^{-1}	6/6 = 1
10^{-2}	6/6 = 1
10^{-3}	6/6 = 1
10^{-4}	3/6 = 0.5
10^{-5}	1/6 = 0.167
10^{-6}	0/6 = 0

$\text{Log } TCID_{50} = 1 - d(S - 0.5)$

$= -1 - 1(3.667 - 0.5)$

$= -4.167$

and is expressed as $10^{4.167} = 4.167\ \log_{10}$ units

2.2.4. Reverse transcriptase activity

The reverse transcriptase assay was essentially as described (Hoffman et al 1985). Five hundred microlitres of 30% polyethylene glycol (PEG) was added to 250ul of cell supernatant, vortexed and iced for 30 minutes followed by 10 minutes at 13000rpm. The supernatant was removed and the pellet resuspended by vortex in 100ul of virus solubilization buffer (0.5% Triton X-100, 800mM NaCl, 20% glycerol, 50mM tromethamine (hydroxymethyl)-aminomethane (Tris) hydrochloride pH 7.8) and incubated on ice for 10 minutes.

20ul of each sample was added to 180ul of reaction mix (Tris-hydrochloride pH 7.8, 10mM $MgCl_2$, 5mM dithiothreitol (DTT), 30ul/ml p(rA).p$(dT)_{10}$, 40ul/ml dATP, 20ul/ml, (^{3}H)TTP (aqueous) and incubated for two hours at 37°C. Samples were precipitated with 50ul of 50% trichloro acetic acid (TCA) at 4^0C.

The samples were harvested onto 2.5cm 3MM paper discs using a millipore manifold filter system. Tubes and filters were washed three times with 10% TCA, three times with 5% TCA and once with 70% ethanol. Filters were dried and added to 5ml scintillation fluid (OptiPhase 'HiSafe', LKB Scintillation Products) and counted in a Packard 1900 TR Liquid Scintillation Analyzer.

H3B cell virus supernatant was the positive control and fresh media or Human Bone Derived Cells cultured in non infective media were the negative controls.

2.3 Irradiation

2.3.1 Infected bone allograft model

The infected bone allograft model (chapter 4) was irradiated in a commercial gamma chamber (Steriteck Pty. Ltd. Dandenong, Victoria, Australia) using a

Cobolt-60 source. Irradiation dose rate was estimated from the dose measured in commercial samples.

Radiation exposure was measured after irradiation of samples with an optical dosimeter (Nordion International, Kanata, Ontario, Canada). Dosimeters were extracted and the dose of irradiation established by spectrophotometric analysis of dosimeters and calibrated using reference ceric-serous sulphate solutions (Steriteck Pty. Ltd. Dandenong, Victoria, Australia).

2.3.2 Inactivation of HIV with gamma irradiation experiments

Vials of frozen virus maintained on dry ice (approximately $^{-}70^{o}C$) were exposed to a cobalt 60 source at a commercial facility (Australia Nuclear Science and Technology Organisation, Lucas Heights Research Laboratories, Lucas Heights NSW). Samples were maintained in a polystyrene lined metal can loaded into a gamma pond canister and processed in the cobalt 60 reactor (LC2) for intervals calculated to deliver 0 - 40 kGy irradiation at 5kGy intervals.

The irradiation dose delivered to virus samples was calculated by suspending ceric/cerous dosimeters in a receptacle specially prepared for the virus. Dosimeters were irradiated and measured at 20^{o} C which is the preferred temperature range for the dosimeters. Three dosimeters were examined for two hours 15 minutes each and the average dose rate measures was 6.39 kGy per hour, range 6.344 - 6.4414. The dose rate was used to determine the exposure time of samples.

2.4 DNA preparation

2.4.1. Chromosomal DNA extraction

Genomic DNA was extracted by standard methods (Maniatis et al 1982). Cells were washed three times in phosphate buffered saline, monolayer cell cultures were collagenased and trypsinised (section 2.1.3). Cells were spun at 1500 rpm for three minutes and resuspended in digestion buffer (100 mM NaCl, 10mM tromethamine (hydroxymethyl)-aminomethane (tris) Ph8, 25 mM ethylenediaminetetra-acetic acid (EDTA) Ph8, 0.5% Sodium Dodecylsulphate (SDS), and 0.1 mg/ml proteinase-K (Merck) for approximately 18 hours.

Digested cells were extracted two or three times with phenyl-chloroform-isoamylalcohol (25: 24: 1) and the DNA was precipitated by ethanol sodium acetate. The pellet was rinsed with 70% ethanol, dried and resuspended in 50 microlitres TE (10mM Tris.HCl pH 8.0, 1mM EDTA pH8.0). The DNA yield was usually 2ug from 10^6 cells.

2.4.2 DNA extraction from bone

The method used to extract DNA from bone was a modification from Maniatis *et al* (Sambrook et al 1989) and is examined in chapter five; the following method was used for clinical material. One gram of bone or cartilage was crushed with mortar and pestle and incubated for 72-78 hours at 37^0 C in a lysis solution consisting of 100mM NaCl, 10mM Tris pH 8, 25mM EDTA pH 8, 0.5% SDS and 0.1 mg/ml proteinase-K (Merck). The solution was extracted three times with phenyl-chloroform-isoamylalcohol (25:24:1) and the DNA was precipitated by Sodium Acetate and ethanol. The pellet was rinsed with 70% ethanol, dried overnight and resuspended in 50ul TE. The DNA yield was usually 1-10ug from 1-1.5 grams of bone.

2.4.3 DNA extraction from blood.

Ten milliliters of blood was lysed in 50mls of lysis buffer (0.144M NH_4Cl and 0.01M NH_4HCO_3) until the sample darkened and cleared. Samples were spun at 2,000 rpm for five to ten minutes, the supernatant was removed and the pellet resuspended in another 50mls of lysis buffer.

The sample was spun again and the pellet resuspended in seven milliliters of buffer 1 (0.01M Tris pH 7.4, 0.1M NaCl, 0.01M EDTA). An equal volume of buffer 2 (0.01M Tris pH 7.4, 0.1M NaCl, 0.01M EDTA, 0.5% SDS) was added. Two milligrams of proteinase-K (Merck) was added and incubated at 37° for 18-24 hours.

The sample was extracted twice with phenyl-chloroform-isoamylalcohol, DNA precipitated in Sodium Acetate and ethanol, rinsed, ethanol dried and resuspended in 50ul of TE. The DNA content was measured spectrophotometrically (OD=260, (Sambrook et al 1989). The DNA yield was usually 200ug to 1,000ug per 10mls whole blood.

2.4.4 Phenol extraction and ethanol precipitation.

Nucleic acid samples were digested as above. Samples were added to 2.5 volumes of phenyl-chloroform-isoamylalcohol (25:24:1). The mixture was vortexed for three to five minutes and centrifuged 15 minutes at 4,000 rpm. The sample separated into three layers with the aqueous supernatant containing the DNA, the discolored lower layer containing phenol with a small intermediate layer of undissolved proteins. The aqueous layer was removed to a separate tube and the extraction repeated a second time.

If the phases did not resolve sufficiently another volume of digestion buffer was added, omitting proteinase-K and the centrifugation was repeated. If there was a thick layer of white material at the interface the organic extraction was repeated.

The aqueous layer was removed to a new tube. Nucleic acids were then precipitated by the addition of 1/10 volume 3M Sodium Acetate and 2-3 volumes 100% refrigerated ethanol. Samples were refrigerated for two hours at -20°C or 30 minutes at -70°C and centrifuged for ten minutes at 4,000 rpm. The pellet was rinsed with 70% refrigerated ethanol and air dried overnight in an incubation hood at 37°. The DNA pellet was dissolved in TE to a final volume of 50-100ul.

To determine the DNA concentration samples were measured with 1/500 or 1/50 distilled water dilutions in a spectrophotometer as before.

2.5 Nucleic acid analysis

2.5.1 Oligonucleotides

Primer pairs used for polymerase chain reactions (PCR) were manufactured by the Department of Molecular Biology, Institute of Medical And Veterinary Science, Adelaide, and adjusted to 100 ng/ul. A 115 base pair region of the HIV-1-*gag* gene was amplified using SK38 and SK39 primers (Kellogg et al 1990). A 242 base pair region of HLA-DQ-α gene was amplified using primers GH26 and GH27 (Saiki et al 1985).

SK38 5'- ATA. ATC. CAG. CTA. TCC. CAG. TAG. GAG. AAA. T -3'

SK39 5'- TT. GGT. CCT. TGT. CTT. ATG. TCC. AGA. ATG. C -3'

GH26 5'- GTG. CTG. CAG. GTG. TAA. ACT. TGT. ACC. AG -3'

GH27 5'- CAC. GGA. TCC. GGT. AGC. AGC. GGT. AGA. GTT. G -3'

2.5.2. Oligonuclide 5' -^{32}P Gamma-ATP end-labeling

Oligonucleotide probes were 5'-end labeled with ^{32}P-gamma-ATP using a standard polynucleotide kinase reaction (Maniatis et al 1982). Probes were independently incubated in a 50ul solution consisting of 10ul oligonucleotide (100 ng/ul), 10ul ^{32}P-gamma-ATP (10μCi/ul; Bresatec, Pty. Ltd. Adelaide, South Australia), 4ul 10 x PNK buffer (0.5M Tris-HCl pH 7.6, 0.1M $MgCl_2$, 10mM DDT, 10mM β-mercaptoethanol), 4ul DTT, 4ul T4 polynucleotide kinase (5U/ul; Bresatec), and 8ul sterile distilled water. The reaction mixture was incubated for 45 minutes at 37° in a radiation hood, the nucleotides were purified by spun column chromatography and the radioactivity of the completed probe measured with an image counter (Packard 1900 TR Liquid Scintillation Analyzer).

2.5.2 Spun column chromatography

To remove unincorporated nucleotide the polynucleotide kinase reaction mixture was separated by spun column chromatography (Sambrook et al 1989). The spun column was manufactured with a one milliliter syringe (Terumo) plugged with a small amount of siliconized glass wool using a flame sterilized spatula. Sephadex G-25 medium (Pharmacia, North Ryde, Sydney, Australia) was added and spun at 2,500 rpm (350 gms) for three minutes (with the syringe resting in a 10ml tube). More sephadex was added and spun to make a compacted volume of 0.8-1 ml. The column was washed with 100ul STE (100mM NaCl, 10mM Tris pH 7.6, 1mM EDTA) and spun as above.

A screw cap eppendorf syringe was placed below the column, the labeling mix was pipetted onto the column and spun as above. The column was washed with 10ul STE and spun as above. The eluent collected in the eppendorf beneath the syringe was the labeled probe free of unincorporated nucleotides. The final volume was adjusted to 250ul with the addition of sterile distilled water.

To measure the radioactivity 1ul of purified oligonucleotide was placed on Whatman 3MM filter paper, air dried, placed into scintillation vials along with 1ml Optiphase 'HiSafe' 3 scintillation cocktail (LKB Scintillation Products) and counted in a Packard 1900 TR Liquid Scintillation Analyzer to obtain radiation counts/minute/microlitre (CPM/ul). Specific activity was greater than 10^9 CPM/ul. Labeled nucleotides were used immediately or stored overnight at-20°C in lead pigs.

2.5.3 Polymerase chain reaction conditions

The PCR method (Saiki et al 1985); (Saiki et al 1988) was adapted by having one oligonucleotide of each pair labeled with ^{32}P gamma-ATP. The ^{32}P-labeled PCR product was then obtained by amplification and visualized directly by acrylamide gel electrophoresis, autoradiography and computer densitometry (Pang et al 1990a).

Primers GH 26 and SK 38 were independently 5' labeled with ^{32}P gamma-ATP and purified (section 2.5.2 and 2.5.3). After the addition of radiolabeled primers all PCR work was done in a radiation hood or behind a Perspex radiation shield.

The PCR reaction mixture of 100ul contained 5 ul 10 x Taq buffer (670mM Tris-HCl pH 8.8, 166mM $(NH4)_2SO_4$, 2mg/ml gelatin, 4.5% Triton-X-100; Bresatec, Pty. Ltd. Adelaide, South Australia), 2.25 mMol $MgCl_2$, one mM of each of the four deoxynucleoside triphosphates (Perkin Elmer Cetus, Roche Molecular Systems, New Jersey, USA) and 0.04 units Thermus Aquaticus (Taq) polymerase (Bresatec), 5ul of each primer dilution, DNA and sterile distilled water.

The 'hot start PCR' technique (Bloch 1992) was used with the reaction mixture (less enzyme and DNA) overlaid with ampliwax PCR gem 100's (Perkin Elmer) and heated to 60°C for 5-10 minutes. The reaction tubes were cooled to room

temperature and an upper reaction mixture consisting of Taq polymerase, water and DNA was added using a dedicated positive displacement pipette (Bresatec) in a cleaned Bio-safety cabinet with UV light and fresh gloves.

The PCR reaction was carried out in a Perkin-Elmer thermocycler and each amplification cycle consisted of 1.5 minutes at 95°C, one minute at 56°C and two minutes at 72°C followed by ten minutes at 72°C. Twenty five cycles was used in all experiments and has been validated for semiquantitative analysis by Lee *et al* (1991).

2.5.4 Electrophoresis and image quantification

An eight percent polyacrylamide gel electrophoresis system was used to resolve radiolabeled PCR products (Sambrook et al 1989) followed by autoradiography and/or phosphorimage scanning.

Polyacrylamide gels were prepared and electrophoresed in a vertical electrophoresis system (Bio-Rad). An 8% acrylamide gel solution was prepared with 1.2 mls 33% acrylamide / 0.9% bis-acrylamide, 2.75mls H_2O, 1ml 5 x TBE (0.9M tromethamine (hydroxymethyl)-aminomethane (Tris) hydrochloride base, 0.9M boric acid, 0.2mM disodium ethylenediaminetetra-acetic acid pH 8.3), 50ul 10% ammonium per sulphate, and 3.5ul N,N,N,',N'-tetramethylethylene-diamine. Gels were allowed to polymerize for at least one hour prior to use.

A molecular weight marker (pUC 19 DNA restricted with Hpa II, Bresetec) was ^{32}P gamma-ATP radiolabeled (section 2.5.2). 10ul of each sample and the marker were added to 2ul of loading buffer (0.25% Bromophenol Blue, 0.25% Xylene Cyanol (Labchem), 15% Ficoll 400 (Sigma) in distilled water). The samples and marker were electrophoresed at 100 volts for 60-120 minutes in 1 x TBE.

After running, the gel was separated and covered on one side with 3MM Chr chromatography paper (Whatman) and dried at 80°C under vacuum for one hour in a gel dryer (Bio-Rad model 5833 connected to a Hetovaac VR-1 vacuum apparatus). The gels were exposed to autoradiographic film (XAR-5, Kodak, Rochester, New York, USA) without enhancing screen for 90-180 minutes at room temperature. Radiograph films were developed using an Ilfospeed 2240 automated developer, and selected gels were exposed to a phosphor screen imager exposure cassette.

A clean Phosphor screen (storage phosphor screen, Molecular Dynamics) was exposed to the radiolabeled gels for 18 hours at room temperature. The Phosphor screen was scanned with a PhosphorImager (ImageQuant, model #400B, Molecular Dynamics, Sunnyvale, CA 94086) using molecular dynamics image quant version 3.0 software (Molecular Dynamics). Net optical densities for the specific bands were determined by volume integration with manual background subtraction for each sample. Two independent readings were taken from each individual gel by the phosphor-imager, the average and variation was recorded (less than 10% variation was observed).

2.5.5 Semi-quantitative polymerase chain reaction

Simultaneous amplification and detection of a single copy human genome and an HIV sequence was used as a semi-quantitative assay of HIV infection. The simultaneous amplification and detection method used an internal control to validate the efficiency of the PCR reaction and determine the HIV copy number relative to input cell DNA copy number (Lee et al 1991); (Pang et al 1990a).

Controlled dilutions of H3B cells (which contain a single HIV gene segment (Li et al 1992a) were prepared from HUT-78 cells spiked with ten-fold serial

dilutions of 10^{-2} to 10^{-5} H3B cells. Chromosomal DNA was extracted from HUT-78 cells, H3B cells, and H3B cell dilutions (section 2.4.1). Five micrograms of DNA was coamplified for HIV-1-*gag* and HLA-DQ-α sequences by PCR (section 2.5.3). Autoradiographs from the serial dilutions were analyzed using an image analysis system (section 2.5.4) to obtain band intensities at each input cell level. The results of HIV/HLA band intensity ratios for each H3B/HUT-78 cell ratio were analyzed by linear regression analysis to construct a standard curve (Figure 5.4). The control curve obtained was used to determine the ratio of HIV infected cells from the radiolabeled PCR product of clinical samples.

Control curves were established in duplicate and new curves were constructed for each new reaction mixture or radiolabeled probe pair. Clinical samples were analyzed in triplicate. A negative control derived from HUT-78 cell chromosomal DNA was always included.

2.6 Statistics and mathematical illustrations

Linear regression analysis was used for the analysis of dependent variables (radiation dosimeters, semi-quantitative PCR, and irradiated virus titres). Microsoft Excel version 4.0 (Microsoft Corporation, Redmond, Washington, USA) was used to calculate the slope of the regression line, coefficient of determination of the data (r^2), and P-value. The regression line was obtained with the equation;

$$y = a_0 + a_1 \ x + \text{error}$$

and was illustrated with CA-cricket graph computer software (1990 Computer Associates International Inc, San Diego, USA).

3. BONE ALLOGRAFT BANKING IN AUSTRALIA AND THE RISK OF HIV TRANSMISSION

3.1 Introduction

To determine the frequency of usage and incidence of problems associated with bone allografting in Australia an audit of the South Australia musculoskeletal bank was undertaken. Of particular interest was the demographic data of the donor population and allograft bones that were discarded to determine the cause and incidence of bone being rejected. Bone harvested during a five year period including the years 1988 to 1992 was the subject of this review.

The risk of virus transmission to allograft recipients remains a concern regardless of allograft type. The estimated probability of not detecting an HIV infected blood donor in Australia is around one in 920 000 (Dax et al 1992). In the United States estimates range from one in 38, 000 to one in 300,000 per unit of blood (Ward et al 1988a); (Busch et al 1991); (Kaplan et al 1987).

In 1988 the risk of bone allograft HIV transmission in the United States was estimated to be less than one per million (Buck et al 1989) but the authors acknowledge the figures were early in the HIV epidemic and not exact.

Since the calculation of HIV incidence in bone allografts by Buck *et al* (1989) bone allograft banks have adopted more comprehensive protocols aimed at decreasing the likelihood of HIV transmission (La Prairie et al 1991); (Musclow 1992). The current risks of HIV transmission have not been calculated.

3.1.1 Aims

The aim of this review was to

1) obtain an overview of the pattern of bone allograft usage in an Australian bone allograft bank,

2) determine the nature and frequency of bone rejection from the bank, and

3) estimate the risk of HIV infection from a bone allograft in Australia using contemporary screening techniques.

3.2 Material And Methods

Bone donations are received from two sources. Specimens are obtained from live donors at the time of total hip replacement or hip hemiarthroplasty for fracture. Cadaver specimens are harvested from organ donors as part of the state wide general organ transplantation program.

Live donors are required to sign a declaration stating the absence of risk factors to potentially infectious disease transmission as outlined in a pre-donation proforma modelled on the American Association of Tissue Banks (American Association of Tissue Banks 1989). The donors are interviewed by the donating surgeon who completes the proforma listing exclusions directed at the possibility of transmissible virus diseases, bacterial infection, tumours and diseases of unknown aetiology including connective tissue diseases, diabetes and Pagets disease.

Pre-donation screening tests are performed at the time of completion of the risk declaration form. The screening tests may precede surgery and there is the potential for bone to be excluded before it is sent to the bone bank if there is an abnormal result. Screening for HIV and Hepatitis was introduced in 1985 and Hepatitis C antibody screening was introduced in May 1990. A 180 day quarantine and repeat serum test was introduced in 1993. The following screening tests are performed; HIV-1 and HIV-2 antibody (Recombinant HIV-1/HIV-2 third generation, Abbott, Chicago) , Hepatitis B antigen (Auszyme, Abbott, Chicago), Hepatitis C antibody (HCV EIA 3.0, Abbott, Chicago), Syphilis serology (Centocore EIA/G, Centocore, U.S.A.). ABO and Rhesus blood groups are recorded but donor and recipient matching was infrequent.

Cadaveric organ donors are screened by proxy via relatives and the treating physicians who are usually from an intensive care environment. Pre-donation screens are the same as for femoral head donors with the addition of blood cultures.

All specimens were removed under strictly aseptic conditions. Live donors and most cadaver donors were harvested in the operating room, 27 per cent of long bone donors were harvested in the autopsy room. Specimens are triple wrapped in sterile plastic bags and/or containers and stored at minus seventy degrees Celsius. Bacteriological culture swabs are taken at the time of harvesting and include the cut bone surface and synovial fluid. Swabs are incubated in culture medium and subcultured onto blood agar and culture broth. A biopsy of the specimen is examined by a histopathologist.

During the time of this review all specimens were irradiated with 25 kGy of gamma irradiation (Cobalt 60 source) while being maintained on dry ice.

All consecutive allograft specimens during this period where reviewed by analysis of donor and recipient demographic factors, donor questionnaires and pre-donation screening tests.

3.2.1 Estimate of HIV risk

The risk of HIV transmission was calculated by determination of the HIV prevalence in a bone allograft population and serially multiplying the risk of failure of each donor screening technique (chapter 1.4). Bone derived from live or cadaver donors are screened for HIV by separate protocols and are considered separately.

The Australian HIV incidence for age and sex was calculated from the cumulative number of new HIV infections to 30 September 1993 (National Centre in

HIV Epidemiology and Clinical Research. 1994) and the estimated resident population, analysed by age and sex (Australian Bureau of Statistics. 1994). The HIV incidence was multiplied by the fraction of bone allograft donors in each age/sex interval (Figure 2.1) to calculate the HIV prevalence in the donor population.

3.3 Results

3.3.1 Donations

2361 bone grafts were collected from 2176 patients during the period of review (Figure 3.1). 215 long bones were harvested from 30 donors.

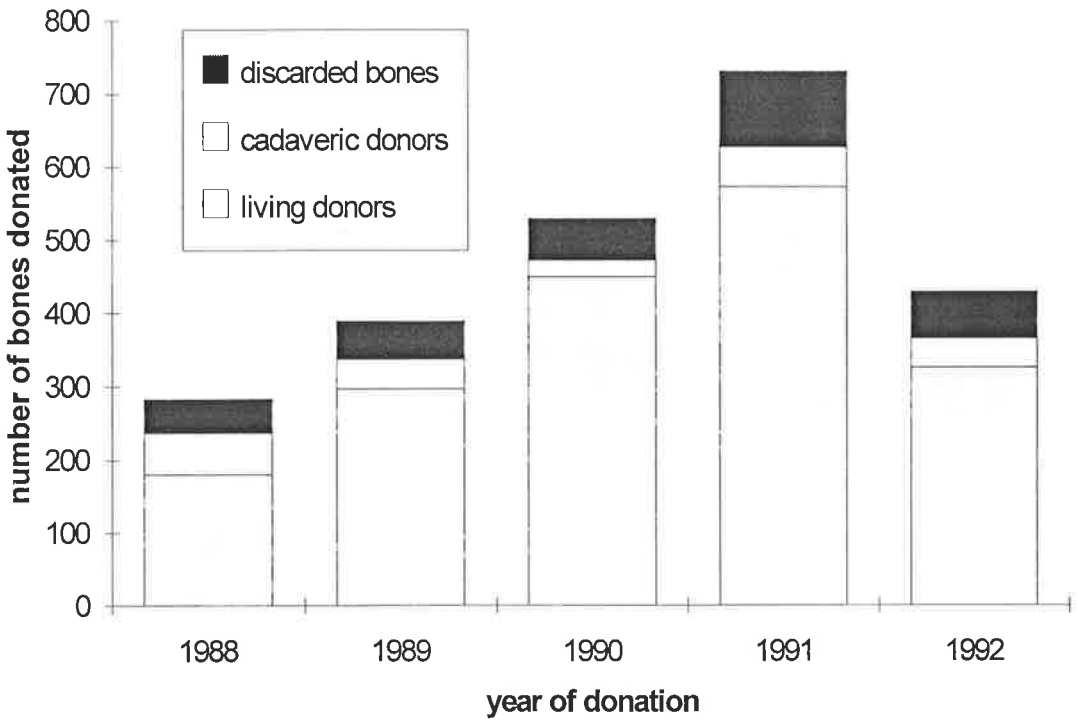


Figure 3.1 South Australian Bone Bank donations 1988 to 1992.

Donor demographical data is included in Table 3.1. There was an approximately equal sex distribution at each age range. The majority of living donors were aged greater than 60 years and most of the cadaveric donors were younger adults. 60% of cadaveric donors died from non violent medical illness (Table 3.2).

Table 3.1. Demographic details of 1824 living and 30 cadaveric bone allograft donors.

Age	Living donors		Cadaveric donors	
	males	females	males	females
3-19			2	1
20-29	3	2	5	2
30-39	3	8	2	2
40-49	42	27	3	3
50-59	136	92	3	4
60+	654	853	2	1
unknown	2	2		
total	840	984	17	13

Three hundred and twenty two donors were excluded and are not included in the table.

Table 3.2 Cause of death of cadaveric donors.

Cause of Death	Number of donors	Number of bones
intracranial hemorrhage	13	94
cardiogenic	7	49
vehicle accident	6	28
suicide	3	36
accidental shooting	1	8
total	30	215

3.3.2 Bone usage

The distribution of living and cadaveric allografts is included in Table 3.3. At the time of data collection 1740 of the 1824 live donor allografts had been used and 140 of the 215 cadaveric allografts had been used. The most frequent indication for bone allografting was arthroplasty surgery and spinal fusion. Femoral heads were used as structural grafts or milled bone chips and more than one femoral head was often used during the recipient procedure. Long bones used for arthroplasty surgery were most often utilized as proximal femoral allografts and one or two bones were required with the second being utilized as strut graft.

Table 3.3 **Allograft bone recipient procedures.**

recipient procedure	living donors	cadaveric donors
arthroplasty	768	80
spinal fusion	675	10
tumour	66	33
fracture/osteotomy	105	
maxillofacial	27	
other	47	17
unspecified	52	
total	1740	140

3.3.3 Discarded allografts

Three hundred and thirty six allografts were discarded. Of these 77 were discarded because of a positive donor history such as a previous tumour or Paget's disease. Seventy one bones were discarded because of incorrect handling procedures including incomplete donor history or screening tests, mishandling of the grafts (such as placing the graft in formalin), or an excessive delay in refrigeration after procurement. 103 bones were rejected after collection due to logistical errors mostly when the graft had been returned unused and allowed to thaw.

There were 85 exclusions because of positive patient or graft screens (Table 3.4). Biopsy exclusions were mostly non specific such as excess numbers of inflammatory cells but also included an undiagnosed Pagets disease and an undiagnosed lymphoma.

Culture positive results included 17 Staph. epidermidis, four Staph. aureus, two streptococci, two anaerobic diptheroids, one gram negative and one gram positive bacillus.

Three bone donations were rejected because of a positive HIV antibody test result. One test result was confirmed positive by Western Blot. This patient had an elective total hip replacement and donated his femoral head without knowledge of his antibody status which was determined as a result of bone bank screening for HIV antibody. The patient had unknowingly signed his consent form denying any risk factors; the proforma at that time did not include heterosexual sexual contact with sex workers from an area endemic with HIV (Thailand) and was the cause of infection in this case. Two other patients were rejected because of an initial HIV positive test result, one was an incorrect interim report from the laboratory, the

second was an elderly woman with no risk factors who returned repeat intermediate test results and did not seroconvert on repeat testing at six months.

Table 3.4 **Bone grafts excluded with positive screening test.**

positive result	total
biopsy	31
culture	27
VDRL	9
hepatitis C	8
Hepatitis B	7
HIV test positive	2
HIV true positive	1
total	85

3.3.1 Estimate of HIV risk

The calculated prevalence of HIV carriers in the unscreened donor population was 172 per million living donors and 1080 per million cadaveric donors. The estimated risk of missing an HIV infected donor after contemporary screening techniques was 0.025 per million living donors and 0.2 per million cadaver donors.

3.4 Discussion

During the five year period of this review 2361 allografts were collected from 2176 donors. During the same period the South Australian organ donation program harvested 510 organ and tissue allografts from 115 donors. During a six year period the Lions Eye Bank of South Australia collected corneas from 790 donors (Williams et al 1990). Nationally there were 3585 cornea transplants and 2787 kidney transplants from 1986 to 1991 (Chapman 1992). National figures for bone allografts are not recorded but the current study suggests the number of bone allograft procedures exceeds corneal grafting which was previously believed to be the most frequent tissue/organ allograft.

There have been well established reviews and guidelines describing the methods of harvesting and storage of bone in bone banks (Friedlaender 1987a); (American Association of Tissue Banks 1989); (La Prairie et al 1991); (Friedlaender 1982). A feature of bone allograft banks has been the priority of graft safety particularly since the majority of grafts were used for elective surgery such as arthroplasty and spinal fusion. Unlike organ recipients the majority of bone allograft procedures were not done for life threatening indications and few were done for tumour surgery (Table 3.3).

There was a high discard rate of 46% during the foundation year of the South Australian Bone Bank (Saies et al 1990) but this has been decreased to 14% in the current review. The majority of grafts continue to be rejected as a result of handling and logistical errors such as incomplete documentation, inadequate screening tests or poor graft handling at procurement or unused returns.

3.6% of grafts were rejected as a result of positive screening tests and the bacterial infection rate was 1.1%. The infection rate has fallen dramatically from the

initial year when 17% of grafts had positive cultures. This decline is partly explained by the inclusion of approximately 5% of grafts which had a light growth of staph. epidermidis in broth culture. Previous reports of positive cultures have varied from 2.2% to 22% (Hart et al 1987); (Kakaiya et al 1990); (Chapman et al 1992). The majority of banks perform microbiological screening of bone grafts but the significance of a positive culture varies with the philosophy of the tissue bank; some banks discard bone that is found to have bacterial contamination and others will accept known contamination and rely upon secondary sterilization (Chapman et al 1992).

All grafts were irradiated with 25 kGy gamma irradiation as recommended by the International Atomic Energy Agency (Van Winkle, Jr. et al 1967) but this may not be sufficient to inactivate viruses such as HIV (Campbell et al 1994); (Fideler et al 1994).

Infectious virus diseases continue to be a major concern to allograft banks particularly when the donor population has a significant prevalence of virus carriers. The American Red Cross Transplantation Services reported 0.46% Hepatitis B surface antigen positive and 0.3% HIV positive (Kakaiya et al 1990) which contrasts to the South Australian Bone Bank figures of 0.3% Hepatitis B positive and 0.04% HIV positive. A relative low prevalence of HIV infection amongst donors has been reported in other Australian blood and tissue banks. Forty six of 5.4 million Australian blood donations were HIV positive (Kaldor et al 1991) and the South Australian Lions Eye Bank identified two HIV antibody positive donors and three hepatitis B positive donors from 790 donors (Williams et al 1990).

Live donors tend to be of the age range least associated with HIV and hepatitis transmission. A more detailed medical history can be obtained from live donors. Cadaveric donor bone may be less safe than bone from living donors because of the

donors age and mode of death (Angermann et al 1992). Only 9% of bone allografts in this series were from cadaveric donors but it may be an important source of bone in some American tissue banks who receive donors from major trauma including social violence such as gunshot and knife wounds (Mankin et al 1983). Trauma patients presenting to trauma centers have a high incidence of HIV from 0.04% to 16% (Baker et al 1987); (Behrens et al 1992); 1.3% was reported in an Australian series (Garsia et al 1992). The majority of cadaveric donors in the current series died from non violent medical illness (Table 3.2) and the estimated HIV prevalence was 0.1%.

It is acknowledged that most HIV figures are imprecise however the estimated HIV antibody prevalence is consistent with findings from blood banks. Australian blood donations are subjected to similar social screening and have detected 46 HIV antibody-positive donors in 5.4 million donations (Kaldor et al 1991). The observed HIV antibody-positive prevalence in the South Australian Bone bank was one in 2176 donors. After social screening only, the calculated prevalence of HIV carriers is estimated to be 8.35 per million living donors and 54 per million cadaveric donors.

The rate of HIV carriers in Australian bone and blood donors remains very low and is further reduced by the screening methods. For live donors that are subjected to contemporary screening tests including a repeat HIV antibody test after quarantine the chance of HIV transmission is almost nil (0.025 per million donors). The risk of HIV transmission from cadaveric donors (0.2 per million donors) is similar to blood donors (one in 920 000 (Dax et al 1992).

An acceptable risk is poorly defined and inconsistent with over-representation of unusually visible or sensational hazards including acquiring HIV by transfusion or allograft transplantation (Whyte 1994). Starr and Browning (1980) estimate the involuntary workplace risk is 0.01 to 0.1 per million fatalities per exposure hour and

the background risk of death by disease is one per million (Whyte 1994). Using the estimates of Slovic *et al* (1979) and Starr and Browning (1980), a 'safe' allograft may be defined as a risk of 0.1 per million. Bone allografts from screened live donors could therefore be considered a 'safe' allograft. This degree of safety has not been approached by blood transfusions screened for hepatitis C but is closer for hepatitis B and HIV. The estimated risk of exposure to HIV in Australia is approximately one per million (Dax et al 1992). The risk of hepatitis B infection is about five per million in the United States (Dodd 1992) and the risk of post transfusion hepatitis C is one per 3000 (Donahue et al 1992); (Archer et al 1992). The risks from other potentially transmitted viruses that are not routinely screened are unknown. These risks should also be considered in the context of the recipient surgery which has a mortality of approximately one percent for allograft joint replacement surgery (Gross 1992) and 0.3% to 0.67% for non-allograft joint replacement surgery (Murray et al 1995); (Campbell et al 1995).

This review would suggest that HIV transmission is a topical but statistically small problem. In this series a greater number of bones were rejected because of other virus and bacteria contamination (Table 3.4). Transmissible diseases such as slow viruses and some hepatitis viruses which are not included in current serological screens may be of more concern.

4. STERILIZATION OF HIV BY GAMMA IRRADIATION IN A BONE ALLOGRAFT MODEL

4.1 Introduction

Gamma irradiation is used as means of secondary graft sterilization but excessive irradiation adversely affects the quality of the donor bone. In high doses it substantially compromises both mechanical and biological properties of the graft (Pellet et al 1983). Most viruses are inactivated by 20 to 40 kGy (Sullivan et al 1971) but it has been reported that perhaps as little as 0.25 to 2.5 kGy may be required to inactivate HIV (Bigee 1988); (Spire et al 1985).

With the increasing utilization of banked allograft bone there is an urgency to determine the minimum radiation requirement to inactivate HIV in infected bone without destroying its integrity. The aim of this work was to determine the minimum dose of gamma irradiation required to inactivate HIV utilizing a bone allograft model.

4.2 Method

Virus supernatant was obtained from H3B cells (section 2.1.4) and aliquots of 700ul of cell-culture fluid, containing $5x10^4$ tissue culture infectious dose 50 ($TCID_{50}$) of virus per milliliter, were stored at -70^0 Celsius. Control samples were thawed and assayed for reverse transcriptase activity (section 2.2.4) and $TCID_{50}$ (section 2.2.3).

Bovine femora and tibia were obtained in lengths of 10 to 15cm (the intra-medullary contents of which were removed). A vial of virus stock was placed within each bone in addition to a commercially available dosimeter (Nordion International, Kanata, Ontario, Canada) and cotton wool padding. The bone ends were sealed with lead wool to ensure the least radio-opaque window to the virus was at least one cortical width of bone (Figure 4.1).

The bone and its contents were irradiated at -70^0 C (section 2.3.1). The first five samples served as non-irradiated controls, the next five samples received 10 kGy of gamma irradiation and subsequent groups received a further five kGy to a maximum of 40 kGy.

Radiation exposure was measured (section 2.3.1) immediately on completion of irradiation of each package of five samples, extra-medullary radiation exposure to each package was established by an external dosimeter.

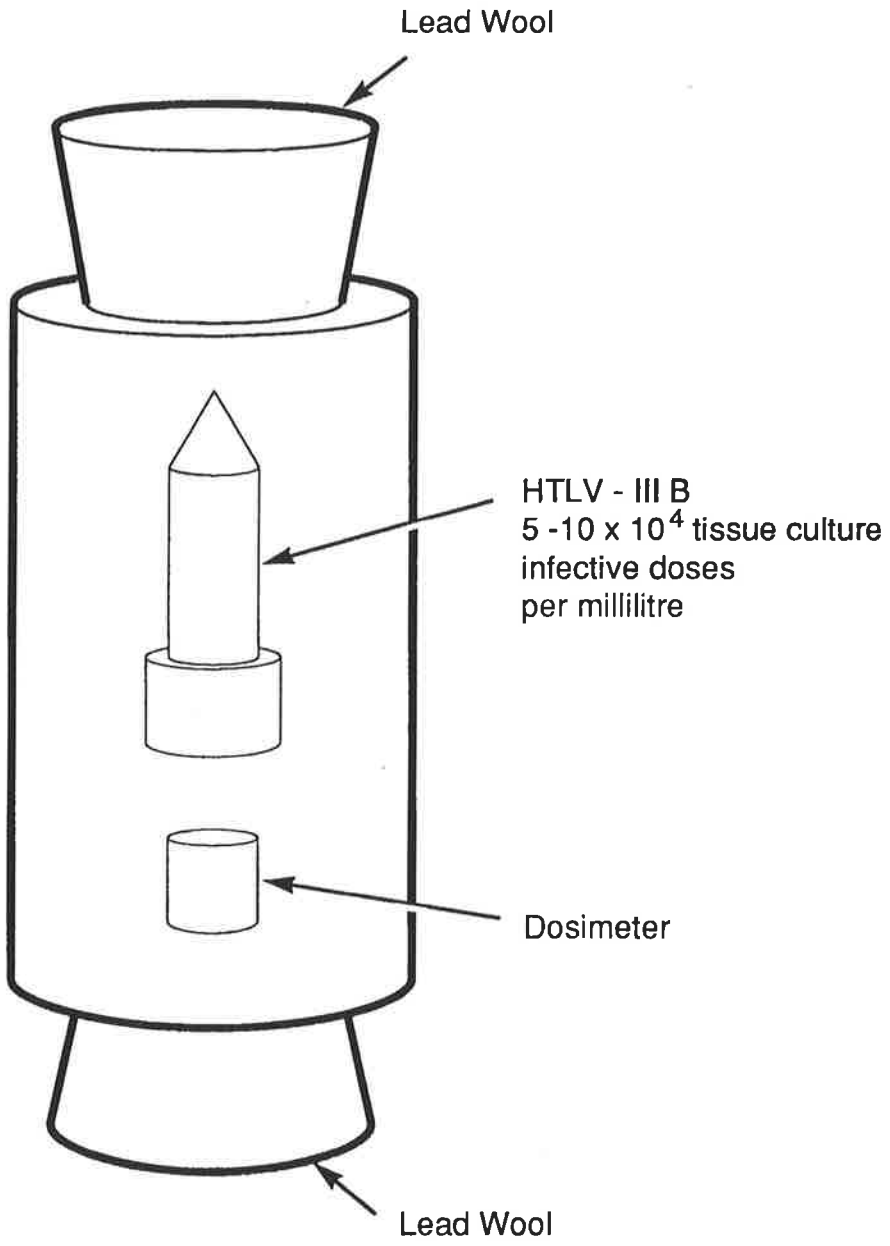


Figure 4.1 HIV infected bone allograft model with virus and dosimeter within the medullary canal of bovine femur or tibia.

4.3 Results

Virus inactivation results are illustrated in Figure 4.2. The five non-irradiated controls demonstrated the maximum response of thirty (CPE positive). Following 10 kGy of irradiation four samples showed no inactivation, one sample had live virus in four of the six wells suggesting some inactivation. At 15 kGy two samples had complete inactivation, at 20 kGy and 25 kGy four samples had complete inactivation consistent with considerable virus inactivation. Complete inactivation of virus occurred when samples were subjected to 30 kGy and 35 kGy. At 40 kGy there was no evidence of live virus in four samples, but from a fifth sample live virus was observed in two wells.

Using the intramedullary dosimeter the radiation absorption through a single cortex of the bovine bone was determined by comparison to the external dosimeter (Figure 4.3). There was little variation between samples, the coefficient of determination was $r^2 = 0.998$ with 4.1% of the radiation being absorbed ($p < 0.01$).

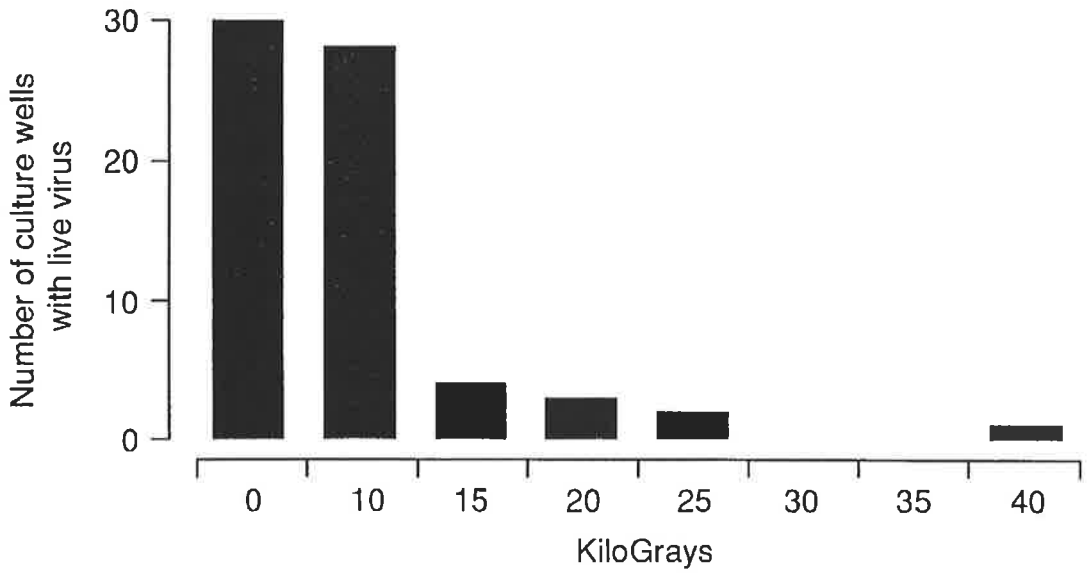


Figure 4.2 Lymphocyte culture response after inoculation with gamma irradiated HIV virus.

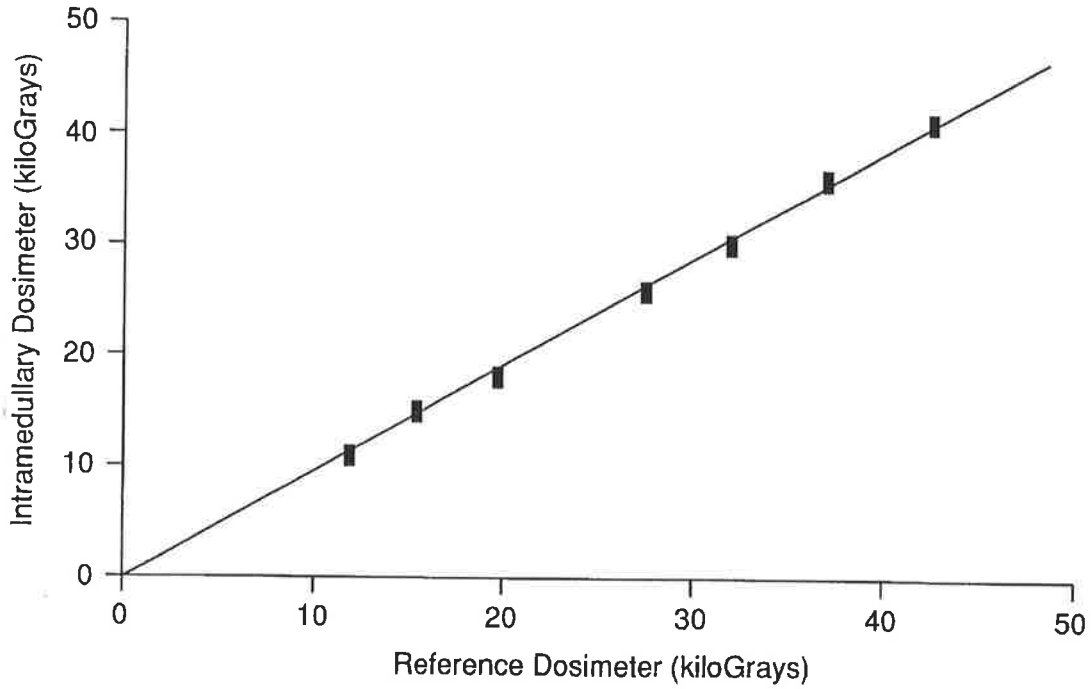


Figure 4.3 Radiation absorption from HIV infected bone model. Comparison of intramedullary dosimeters to extramedullary dosimeters examining radiation absorption from one cortex of bovine bone.

4.4 Discussion

In this study a model was developed to simulate a situation of HIV infected bone transplantation at a concentration in excess of the maximum known human dose. The natural history and antibody titres have recently been elucidated and HIV infection is associated with high titres of virus during an acute virus syndrome followed by a period of quiescence until clinically recognizable acquired immunodeficiency syndrome (AIDS) or AIDS related complex has supervened (Clark et al 1991); (Daar et al 1991). During this acute virus illness infected donors are not only most likely to go unnoticed because of their absence of symptoms and detectable antibody production but they have the greatest virus load quantified as $10\text{-}10^4$ tissue culture infective doses per milliliter of plasma (Clark et al 1991); (Daar et al 1991). For this infected bone allograft model a virus load of 5×10^4 tissue culture infective doses per milliliter was used approximating the expected maximal contamination.

Sterility is a dose dependent measure of probability which is determined by the unique radioresistance of a given organism, the initial concentration, and radiation dosage administered. Viruses are moderately or highly radioresistant and the sterilizing or inactivating dose varies within 20 to 40 kGy (Sullivan et al 1971). Two previous reports of irradiated HIV suggested the virus was perhaps uniquely radiosensitive requiring much lower doses for complete inactivation at 0.25 to 2.5 kGy (Bigee 1988); (Spire et al 1985). A more recent study using a variety of concentrations of HIV demonstrated that although delayed, infection developed following treatment with 5.6 kGy (Conway et al 1990).

It is known that temperature, state of hydration and oxygenation are important factors related to radiation sensitivity (Burt et al 1963); (Gardiner et al 1986); (Grecz et al 1967). The radiation sensitivity is reduced by suspension in media probably

because of free radical scavengers (Gardiner et al 1986); (Sullivan et al 1971). In this experiment the current practice of bone allograft banks was simulated by irradiating the bone allograft model. The model used frozen, non-lyophylised, aerated media. The bone was not in direct contact with the virus and its purpose was to simulate the radiation shielding effect that could occur in the medulla of an allograft bone. Samples were irradiated with dosages of gamma irradiation currently used in clinical practice. This was done in preference to quantifying the exact radiosensitivity of the virus for which lesser radiation dosages are thought to be required.

The results demonstrated a significant reduction in the cytopathic effect of the virus following increments in irradiation after 15 kGy. Between 25 kGy and 30 kGy a sterilizing dose was observed consistent with other RNA viruses (Sullivan et al 1971).

At 40 kGy live virus was detected in one virus/bone sample (from the five tested) . The significance of this finding is uncertain. The sterilizing dose is an arbitrarily determined probability of complete virus inactivation and the possibility of live virus remaining is always present (International Organization for Standardization. 1993). The detection of live virus may indicate the dose used was less than the sterilizing dose. Alternatively there may have been an error in the experiment with the virus aliquot migrating within the bone into the lead wool encapsulating the bone ends with subsequent shielding of the virus. Contamination whilst plating the virus into the culture plates could have occurred.

The effect of radiation absorption as gamma rays passed through a single Bovine cortex was minimal with intramedullary dosimeters recording 95.9% of the externally recorded dose. It is recommended the amount of irradiation absorbed by bone (4.1%) can be ignored for clinical applications.

From the results of this study the contention that effective sterilization of HIV infected bone allograft material by gamma irradiation doses as low as 0.25 or 2.5 kGy as previously reported (Bigee 1988); (Spire et al 1985) cannot be supported. The experiment suggests that in a bone allograft model utilizing an HIV dose approximating the maximum known bioburden of virus a significant kill was achieved with 15 kGy but sterility was not achieved until 30 kGy.

The finding of live virus following 40 kGy irradiation was a dilemma that required further examination. This experiment was done only once but the findings suggest an alternative method rather than repeat experiments were required. If repeat experiments had been performed and demonstrated virus surviving 40 kGy irradiation it would suggest irradiation may be unsuitable for bone allograft sterilization but the sterilizing dose would remain unknown. Alternatively if repeat experiments did not demonstrate live virus after 30 kGy or more irradiation it could be argued that there were insufficient samples (Lieber 1990).

It was suggested that further studies to accurately define the dose-response curve for HIV were required. It was suggested the virus bioburden in bone be examined and the radiosensitivity of HIV be determined.

5. SEMI-QUANTITATIVE POLYMERASE CHAIN REACTION ANALYSIS

5.1 Introduction

Quantitative determination of the HIV load (bioburden) in clinical specimens requires the measurement of very small quantities of virus. End point dilutional analysis of infected peripheral blood mononuclear cells has been used but this method is not applicable to solid tissue or frozen cells which form aggregates and preclude accurate counting.

Because of the low level of circulating free virus direct detection of HIV in patient samples is difficult without *in vitro* propagation. Even with co-cultivation the successful recovery of HIV varies from 10-75% (Kellogg et al 1990). Polymerase chain reaction (PCR) analysis involves *in vitro* amplification of specific DNA segments allowing the detection of a single specific DNA molecule against a background of 10^5 cells (Saiki et al 1988). PCR has been used to diagnosis infection of HIV and is at least as sensitive as virus isolation in clinical specimens (Jackson et al 1990); (Ou et al 1988).

Quantitative determination of HIV copy number in human cells has been reported. This was done by end point dilutional analysis of HIV PCR signal derived from infected fresh peripheral blood mononuclear cells that had been counted to determine cell input (Schnittman et al 1989); (Simmonds et al 1990). Simultaneous amplification and detection of a single copy human genome and an HIV sequence has been developed to allow a quantitative assay of HIV infection when the cell number or DNA input is unknown (Lee et al 1991); (Pang et al 1990a). The simultaneous amplification and detection method uses an internal control to validate the efficiency of the PCR reaction. The internal control may also be used to determine the HIV copy number relative to input cell DNA copy number.

In this chapter the standard PCR protocol was modified to allow quantitative analysis of samples. The modification of PCR employs synthetic oligonucleotide primers for amplification that were 5' end-labeled with ^{32}P gamma-ATP, followed by direct autoradiography of gel-resolved products (Arrigo et al 1989); (Lee et al 1991); (Pang et al 1990a). By using an end-labeled oligonuclide primer the transfer and hybridization steps used in other protocols were omitted to decrease the number of PCR cycles. Reducing the cycle number to 25 has maintained PCR reactions in a quantitative range when validated with other multiple primer reactions (Pang et al 1990b).

5.1.1 Hypothesis

That the modified quantitative PCR protocol will allow resolution of a 10-fold difference between samples over a clinically significant range.

Pang *et al* and Lee *et al* (1990a); 1991) reported resolution of twofold differences between samples over a 3-log range. The measure of HIV bioburden in clinical samples required sufficient accuracy to determine weather the virus bioburden in bone exceeded that of blood. For the purpose of this thesis a resolution of tenfold differences was estimated to be a sufficient measure of the HIV load in clinical samples.

The quantitative PCR method was developed to determine whether the virus load in bone and cartilage would exceed the known maximum in blood. The maximum HIV bioburden in blood is 4×10^3 copies per 1×10^6 peripheral blood mononuclear cells using a coamplified PCR method and end-point analysis (Lee et al 1991). The minimum number of HIV molecules that can be detected in clinical specimens may be as few as 1peripheral blood mononuclear cells (Kellogg et al

1990). The clinically significant range of the quantitative PCR method is defined as 1×10^{-5} minimum to a maximum of 4×10^{-3} HIV/ HLA copies.

5.2 Method

5.2.1 Primers and optimization

Several primer pairs were examined for coamplification of a region of the HIV-1-*gag* gene and coamplification of a single copy gene to be used as an internal control. A 147 base pair region of the HIV-1-*gag* gene was amplified using P3 and P4 primers (obtained as a gift from Dr Li Pengs laboratory, Institute of Medical and Veterinary Science, Adelaide) together with a 258 base pair region of the Beta-Globin gene using primers B1 and B2 (obtained as a gift from Dr. Hall's laboratory, Institute of Medical and Veterinary Science, Adelaide). Using an approximation for the preferred annealing temperature (Thein et al 1986) where;

$$T_M = 4(G+C) + 2(A+T)$$

(G, C, A, T are nucleotide sequences), the preferred annealing temperature was 37° for the HIV-1-*gag* gene segment and 42° for the Beta-Globin gene segment. Temperature difference plus the close proximity of both amplified fragments after gel electrophoresis produced an unsuitable product for coamplification.

A 115 base pair region of the HIV-1-*gag* gene was amplified using SK38 and SK39 primers (Kellogg et al 1990). A 242 base pair region of HLA-DQ-α gene was amplified using primers GH26 and GH27 (Saiki et al 1985). This primer pair was successfully coamplified in the same reaction with an annealing temperature of 56° (mean of preferred annealing temperatures) and bands were readily separated by electrophoresis.

5.2.2 Primer radiolabelling

The PCR method was adapted by having one oligonucleotide of each pair labeled with ^{32}P gamma-ATP. The ^{32}P-labeled PCR product was then obtained by amplification and visualized directly by acrylamide gel electrophoresis, autoradiography and computer densitometry. The probes GH26 and SK38 were

independently end-labeled on the 5' terminus using T4 polynucleotide kinase (PNK) and ^{32}P gamma-ATP (section 2.5.2).

5.2.3 Optimization of polymerase chain reaction conditions

Chromosomal DNA was extracted from H3B cells. H3B cells and HUT-78 cells spiked with 10^{-2} and 10^{-4} H3B cells, and chromosomal DNA from the blood of an anonymous donor were used to determine optimal PCR conditions.

Serial dilutions of chromosomal DNA from H3B cells were used to assess the optimum quantity of input DNA. 5ug, 4ug, 3ug, 2ug, 1ug, 0.5ug, 0.1ug and ten fold dilutions to 10^{-5}ug of DNA were used in a standard polymerase chain reaction mixture. 5 ug/chromosomal DNA equates to 2.5 x 10^6 cells and 10^{-5} ug equates to five cells (Sambrook et al 1989). Inhibition of the HIV or HLA bands was not observed on microradiographs when DNA concentrations up to 5ug were used. Further experiments used 5ug of DNA.

The coamplification of these probe pairs has been validated using 25 cycles of a reaction mixture containing 9mM $MgCl_2$ with an annealing temperature of 56°C (Lee et al 1991) and these conditions were maintained during the study. Coamplification of H3B cell dilutions and normal donor chromosomal DNA was performed with and without the hot start technique (Bloch 1992) with AmpliWax PCR gem 100 wax beads (Perkin-Elmer, Roche Molecular Systems, New Jersey, USA). The precision of diluted H3B DNA was improved with the hot start technique and less miss-priming was observed. The hot start method was used for all PCR reactions.

5.2.6 Standardization of HIV/HLA coamplification.

The feasibility and quantitative efficiency of simultaneous radiolabeled coamplification was evaluated with H3B cell DNA dilutions. DNA dilutions were independently subjected to PCR analysis with either one of the radiolabeled pairs or with both primer pairs simultaneously amplified in the same reaction mixture.

5.2.7 Quantitative polymerase chain reaction

Controlled dilutions of H3B cells were used to evaluate the reproducibility of coamplified PCR, and to identify the range of input cell ratios yielding quantitative results. HUT-78 cells were spiked with ten-fold serial dilutions of H3B cells and coamplified for HIV-1-*gag* and HLA-DQ-α sequences. Autoradiographs from the serial dilutions were analyzed using an image analysis system to obtain precise band intensities at each input cell level. The results of HIV and HLA band intensities (OD/mm2) for each H3B/HUT-78 cell ratio were analyzed.

5.3 Results

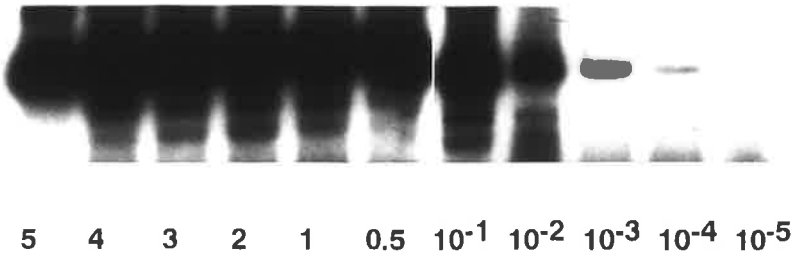
5.3.1 Discrete PCR amplifications

Dilutions of 10^{-5} to 5 ug of H3B cell derived chromosomal DNA were successfully amplified by the probe pairs GH 26 and GH 27 or SK 38 and SK 39 (Figure 5.1). Radiolabeled bands were separated at all input DNA concentrations and interfering bands were not observed in the molecular weight range of the PCR product.

The sensitivity of both primer pairs allowed detection of DNA segments at the minimal concentration of 10^{-5} ug which is approximately five cells per reaction. Image analysis of the radio-labeled gels demonstrated an approximately linear relation between input DNA and band intensity.

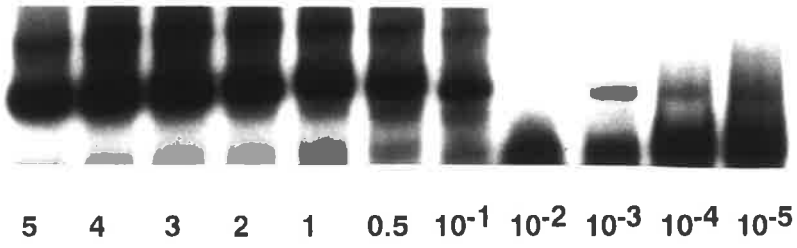
Figure 5.1 PCR amplification with dilutions of H3B cell chromosomal DNA. **(A)** SK39 and ^{32}P-labeled SK38 probe pair detect a 115 base pair region of the HIV-1-gag gene. **(B)** GH27 and ^{32}P-labeled GH26 probe pair detect a 242 base pair region of the HLA-DQ-α gene. **(C)** coamplification using both primer pairs simultaneously detects HIV-1-gag and HLA-DQ-α genes.

A



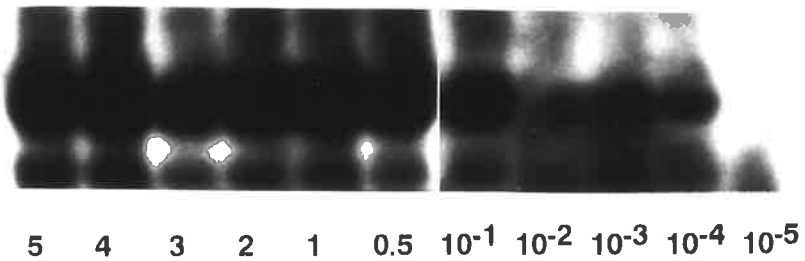
ug H3B cell chromosomal DNA

B



ug H3B cell chromosomal DNA

C



ug H3B cell chromosomal DNA

5.3.2 Coamplified PCR

Figure 5.1**(C)** includes the results of both primer pairs coamplified with serial dilutions of H3B chromosomal DNA. The two amplified products can be discriminated in the diagnostic region and no potentially interfering bands were seen.

End point sensitivities of coamplified PCR reactions were similar to discrete reactions using only one primer pair and signal was detected from the 10^{-5} ug DNA dilutions.

There was a logarithmic relation between coamplified PCR product and input DNA. Image analysis of the coamplified products (Figure 5.2) confirmed the relation between primer pairs over the input DNA range (coefficient of determination for GH26 was r^2=0.86, coefficient of determination for SK38 was r^2=0.91) There was some signal inhibition when five ug of DNA was used.

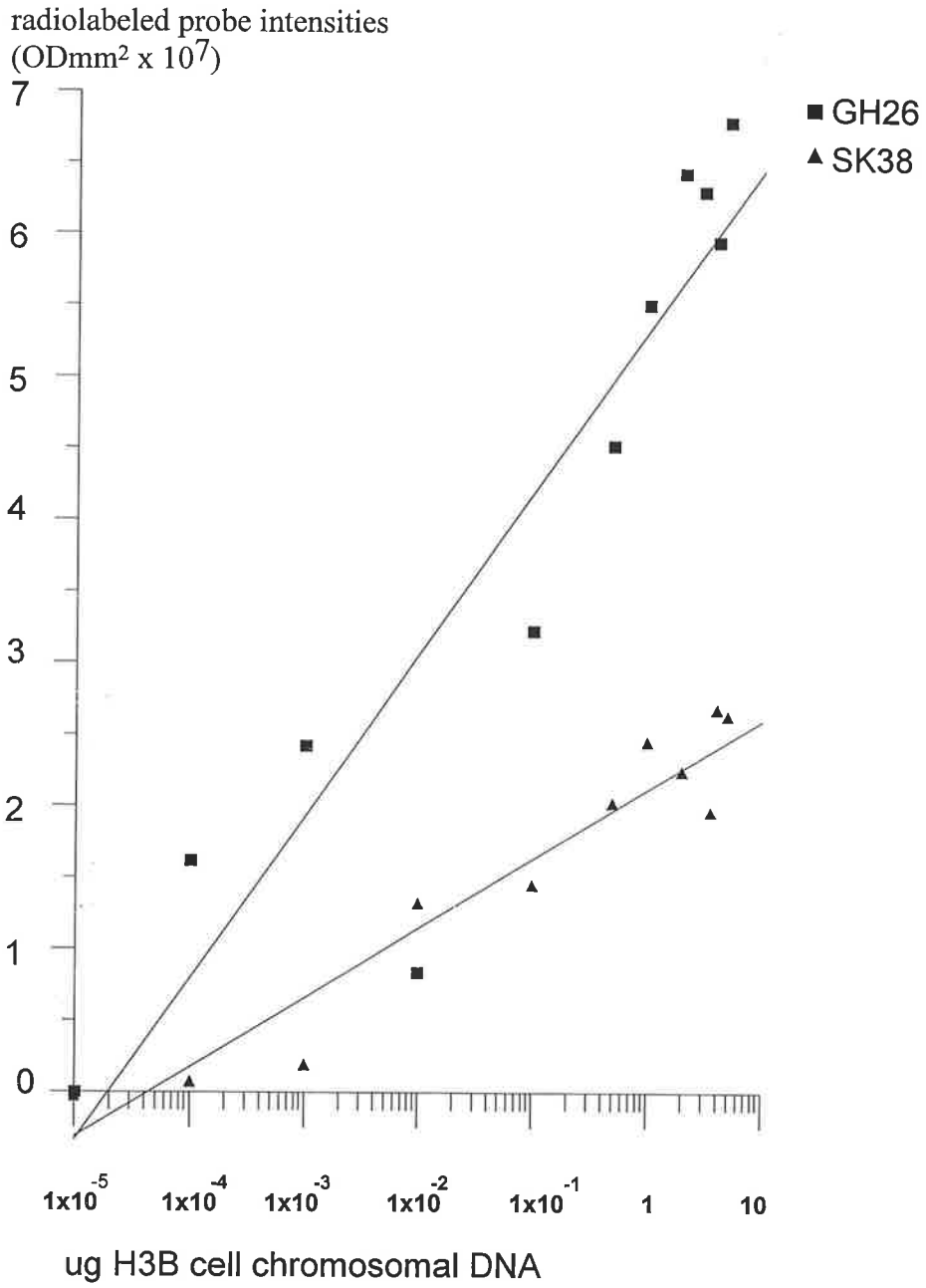


Figure 5.2 Image analysis of H3B cell DNA coamplified with probes to detect HIV-1-gag and HLA-DQ-α genes. The HIV-1-gag gene was amplified with a ^{32}P-labeled SK38 probe and the HLA-DQ-α gene was amplified with a ^{32}P-labeled GH26 probe.

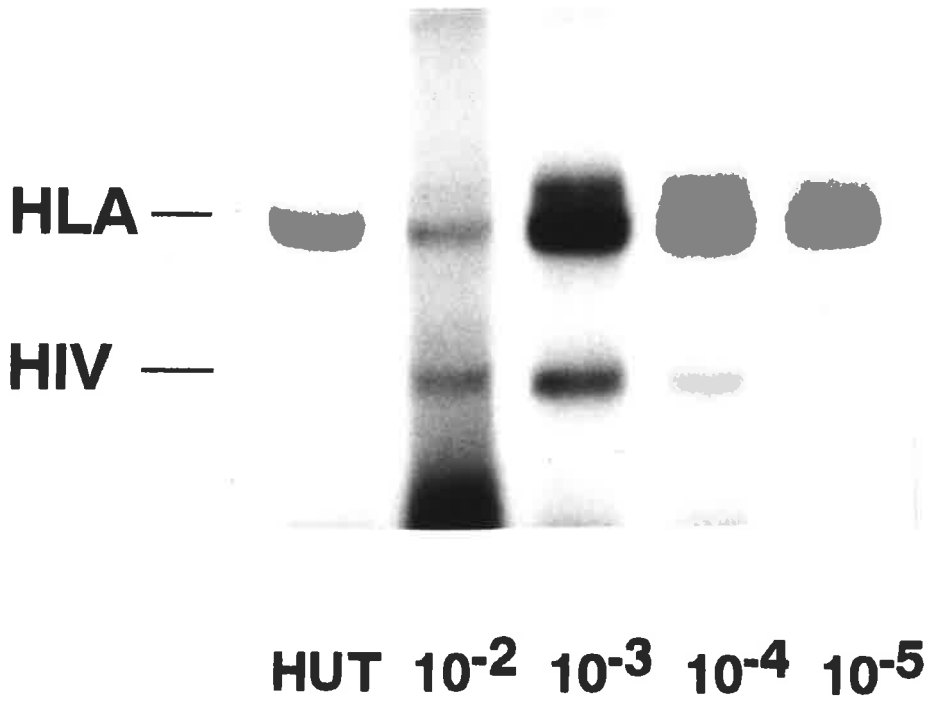
5.3.3 Quantification by coamplification

Autoradiographs from H3B/HUT-78 cell dilutions showed some variability of the HLA-DQ-α signal as seen previously with the discrete and coamplified H3B cell experiment. The end point sensitivity of HIV signal at the minimum H3B cell dilution was not adversely affected by the inclusion of a large volume (5 ug) of non infected chromosomal DNA.

The relation between HIV-gag signal and HLA-DQ-α approximated the cell dilutions (Figure 5.3). Using the image analysis system paired autoradiographs were analyzed for HIV and HLA band intensity for each input cell ratio (Figure 5.4). There was a linear relation between the observed HIV/HLA PCR signals and the input H3B cell dilutions ($r^2 = 0.97$, $p < 0.01$). The curve was linear over a 2-3 log range with H3B cell dilutions of 10^{-2} to 10^{-4} H3B/HUT-78 cells. Differentiating 10^{-4} and 10^{-5} dilutions was not accurate.

An individual curve was prepared for each newly labeled primer pair as primer labeling altered the probe intensity. Multiple curves were prepared prior to clinical experiments and the linear relationship was maintained (coefficient of determination $0.79 < r^2 < 0.97$, $p < 0.05$).

Figure 5.3 Coamplified PCR of HUT-78 cells spiked with H3B cell dilutions. The HIV and HLA band intensities were analyzed using an image analysis system for the quantitative PCR method.


HLA
HIV
HUT
10-2
10-3
10-4
10-5

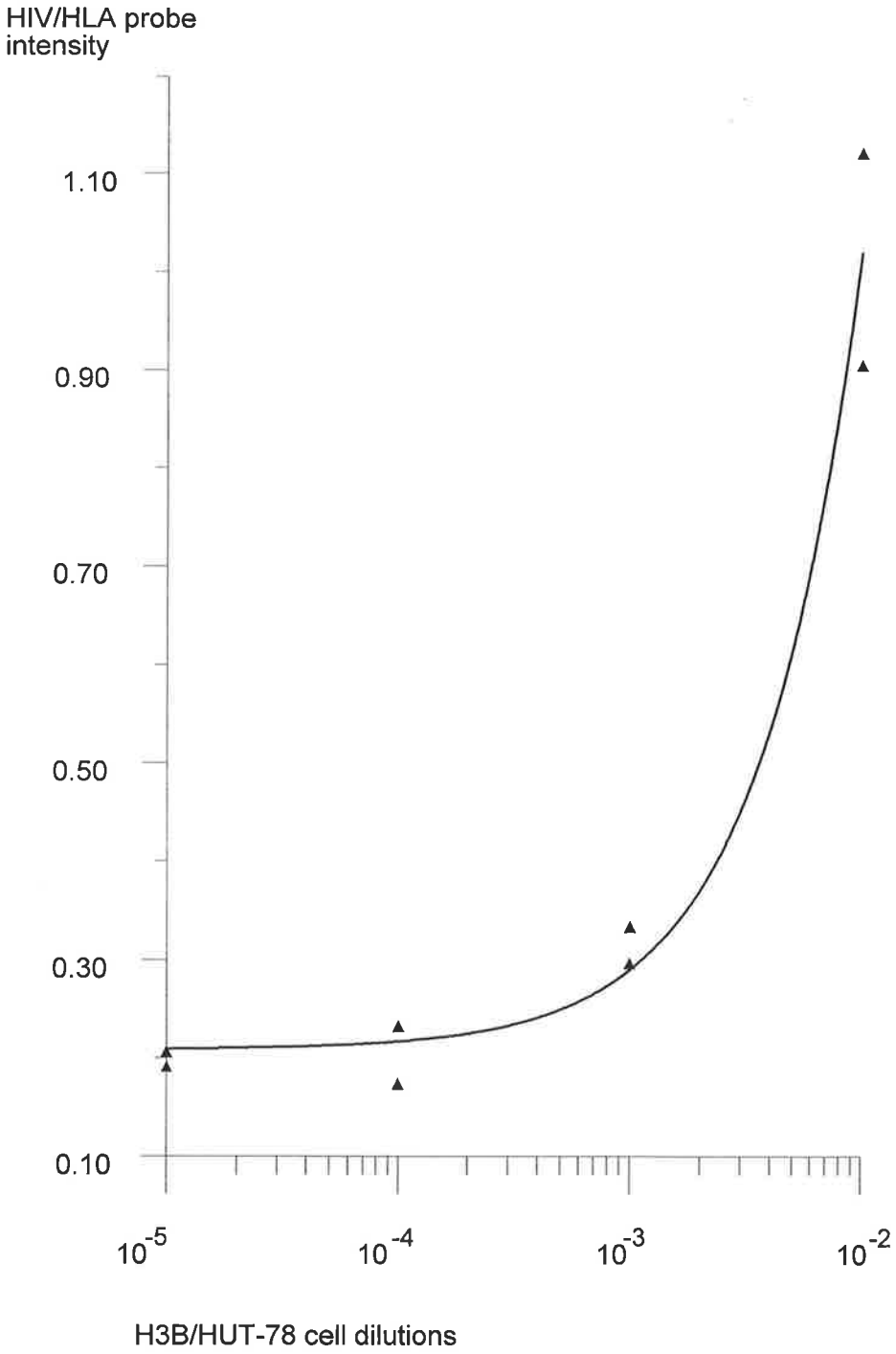


Figure 5.4 Quantitative PCR control curve from HUT-78 cells spiked with H3B cell dilutions. HIV/HLA intensity ratios derived from image analysis of coamplified radiolabeled PCR product.

5.4 Discussion

Development of a PCR coamplification methodology was achieved *in vitro* with simultaneous quantitative analysis of radiolabeled PCR product from two gene segments of HIV infected cells. Using the optimized PCR conditions described by Lee *et al* (1991) it was possible to have an internal control of DNA quantity, integrity and reaction efficiency. The signal sensitivity determined by end point dilutional analysis was not adversely affected by the inclusion of the heterologous HLA primer pair. The coamplification PCR method reflects the competency of each PCR aloquot and the method should be an accurate method to examine clinical samples where inhibitors such as iron from blood samples may adversely affect the reaction mixture.

Using end-labeled oligonucleotide primers fewer PCR cycles are required and the transfer and hybridization steps of conventional PCR method can be eliminated (Pang et al 1990a); (Arrigo et al 1989). End-labeling allows direct quantification of the reaction product by autoradiography and image analysis. The number of PCR cycles was empirically determined to maintain the linear part of the curve and the protocol of Lee *et al* (1991) using 25 cycles was successful.

The current method used the HLA-DQ-α PCR product as the measure of input cell number rather than measured DNA or cell counts. It was anticipated that *in vivo* application of this protocol would involve prepared DNA from clinical samples and validation with cell counts would not be possible. Lee *et al* (1991) found the amount of PCR product was proportional to input DNA over a 3 $\log_{10}$ range. These experiments demonstrated an approximately linear relation between PCR product and DNA dilutions (Figure 5.1 and 5.2).

Most PCR protocols use DNA equivalent to 1.5 - 2.5 x 10^5 cells (Saiki et al 1985); (Kellogg et al 1990). It was possible to increase the input DNA by 10 times and still detect as few as five cell equivalents per reaction mixture. Unlike Lee *et al* (1991) there was some inhibition of PCR product when the DNA content was increased to five ug per reaction but the end point sensitivities of both probe pairs was maintained.

The heterogeneity of HIV-1 has been extensively documented (Shaw et al 1984); (Saag et al 1988). The HIV primer pair SK 38 and SK 39 (Kellogg et al 1990); (Ou et al 1988) amplify a highly conserved region of the virus genome that expands the gag sequence area of HIV-1. This sequence is one of the last synthesized therefore complete or almost complete virus DNA rather than incomplete reverse transcriptase product is detected.

The DNA extraction method (Sambrook et al 1989) provides chromosomal DNA and minimal unintegrated (non chromosomal) DNA. Unintegrated DNA may be detected more frequently than chromosomal DNA in symptomatic patients (Pang et al 1990a) and over estimate the infected cell ratio when the coamplification method is used. The current method was validated using the H3B cell line which has a single HIV genome (Li et al 1992a). It is reported that in clinical samples each infected cell probably has at most four copies of HIV DNA (Schnittman et al 1989); (Simmonds et al 1990), hence this protocol may over estimate the virus load in clinical samples.

The quantitative PCR protocol was validated using H3B cells diluted in HUT-78 cells, the results demonstrated a quantitative relationship over the range 10^{-2} to 10^{-4} H3B/HUT-78 cells. The sensitivity allowed detection of 10^{-5} H3B cells. The hypothesis of this experimental series that *'that the modified quantitative PCR protocol will allow resolution of a 10-fold difference between samples over a*

clinically significant range' is correct. A standard curve of H3B/HUT-78 cell dilutions run in parallel with clinical samples allows quantitative analysis without cell counting or PCR dilution analysis.

6. SEMI-QUANTITATIVE ANALYSIS OF HIV INFECTED HUMAN BONE

6.1 Introduction

PCR analysis of clinical samples has been used successfully and a sensitivity of 99.0% for antibody positive, culture positive peripheral blood is reported (Sheppard et al 1991). Daar *et al* (1991) used a quantitative PCR method to demonstrate $10^{3.8-4.1}$ /million peripheral blood mononuclear cells (PBMC) were infected during the window period and $10^{2.3-3.2}$ /million PBMC's were infected during the asymptomatic infection period. Lee *et al* (1991) used a quantitative PCR method to demonstrate a rise in the HIV-PCR-units per million PBMC during stage two disease ($10^{0.3-3}$ copies) and stage three/four disease ($10^{2.3-3.6}$ copies).

There have been no reports of orthopaedic tissues examined by quantitative PCR analysis.

6.1.1 Aims

The aim of this experiment was to quantify the virus bioburden in bone from HIV infected patients.

6.1.2 Hypothesis

1. *That the virus bioburden in bone relates to the stage of disease and amount of virus in peripheral blood mononuclear cells.*

2. *That the virus bioburden in bone is not greater than peripheral blood mononuclear cells.*

The virus bioburden in bone is defined as the ratio of cells infected with HIV. HIV infection is defined as incorporation of the HIV genome into the host cells chromosome and includes cells that are actively infected and producing virus and those that are dormant. Dormant cells that have incorporated the virus genome may have the potential for reactivation and virus production (Zack et al 1992); (Hoxie et al 1985).

Disease stage is defined by the World Health Organization definition of clinical disease (Centers for Disease Control 1986). The bioburden of peripheral blood mononuclear cells is defined as incorporation of the HIV genome and is included to validate the clinical disease stage and anticipated bioburden of blood. It is anticipated that blood from patients with stage two disease (asymptomatic antibody positive) will have a low serum bioburden and patients with stage three and four disease (symptomatic antibody positive) will have a greater bioburden (Daar et al 1991); (Ho et al 1989); (Coombs et al 1989).

Peripheral blood mononuclear cells are defined as cells with chromosomal DNA and are detected by the single gene locus of HLA-DQ-α. The bioburden of PBMC's has been defined by semiquantitative techniques (chapter 1.2.4) and it was anticipated that bone specimens would be contaminated by blood containing PBMC's.

If the null hypothesis *'that the virus bioburden in bone is not greater than peripheral blood mononuclear cells'* is true, the cells in bone could be infected with HIV but their bioburden is the same or less than blood.

6.2 Material and Methods

6.2.1 Patient samples

Ethical approval for the use of cadaveric patient samples was obtained from the Royal Adelaide Hospital, Adelaide, South Australia (protocol no 921112). Samples were obtained from The NSW Institute of Forensic Medicine, Glebe, New South Wales by the state coroner (coroners act 1980, Section 48).

Specimens from ten anonymous autopsies were received. Limited clinical details were available (Table 6.1), all patients were HIV antibody positive, two patients had AIDS and eight non AIDS patients were believed to have stage two disease. 10 milliliters of whole blood was obtained and mixed with an even volume of 70% ethanol. One patella from each patient was obtained (the patella was chosen by the coroner as a convenient source of bone without active red marrow). The donors blood was washed from the sample during collection and samples were transported in 70% ethanol.

Table 6.1 Patient samples for Quantitative PCR.

patient	age/sex	disease stage	cause of death
A	unknown	unknown	unknown
B	31 M	AIDS	drug overdose
C	unknown	unknown	unknown
D	unknown	unknown	unknown
E	unknown	unknown	unknown
F	unknown	unknown	unknown
G	unknown	unknown	unknown
H	17 M	unknown	suicide
J	27 M	unknown	pulmonary embolism
K	32 M	AIDS	pulmonary sepsis

6.2.2 DNA extraction from bone

There is limited literature related to DNA extraction from bone, and four methods of DNA digestion were examined with bone, these were;

1) Hagelberg and Clegg's (1991) modification of Maniatis *et al*'s (1982) method of decalcification in 50 volumes of 0.5M ethylenediaminetetra-acetic acid (EDTA) for 72 hours with two changes of EDTA followed by DNA extraction with EDTA, proteinase-K, and N-lauroylsarcosin.

2) The standard method of Sambrook, Fritsch and Maniatis (1989) incubating bone in a lysis solution consisting of EDTA, proteinase-K, and sodium dodecylsulfate (SDS) for 18-24 hours was used.

3) An additional modification of Sambrooks method was to extend the digestion period to 72-78 hours without previous decalcification.

4) The alkaline DNA solubilization technique of West (1985) incubating for 18-24 hours in 10mM EDTA adjusted to pH 12.3 with NaOH.

One gram of mortar pulverized bone was digested by each method followed by DNA extraction and measured spectrophotometrically using OD=260. 0.8ul portions of the final volume were electrophoresed through 2% Agarose gels with DNA markers of known size followed by Ethidium Bromide staining to visualize the DNA under ultraviolet light by standard methods (Maniatis et al 1982).

The 72-78 hour digestion with proteinase-K and SDS gave the most consistently clear bands and highest yield of DNA spectrophotometrically. Preliminary decalcification yielded similar results but were not consistent when repeated in triplicate.

For all experiments with bone and cartilage clinical samples the 72-78 hour digestion with SDS and proteinase-K buffer was used.

6.3 Results

DNA samples were obtained from ten bone specimens and nine blood, a blood sample was not available from patient E.

Bone and blood derived DNA from an anonymous HIV negative donor was examined in series, in triplicate, with clinical samples. The HLA-DQ-α genome was amplified successfully in all samples and the HIV-1-*gag* band was not.

The HIV-1-*gag* genome was detected in all blood samples (Figure 6.1). In two of three experiments patient F had an interfering band in the region of the 115 base pair HIV-1-*gag* genome and no band in the HIV-1-*gag* region. The HIV-1-*gag* band was observed in one of the three experiments. The 242 base pair segment of the HLA-DQ-α genome was amplified successfully in all samples.

Quantitative PCR analysis results are summarized in Table 6.2. Samples from patient F were representative of only one successful PCR reaction due to the interfering HIV region band. For the remaining nine samples the calculated ratio of infected cells was 10^{-5} to 10^{-2} infected/uninfected cells (10^4 - 10^1 cells per 10^6 PBMC). Samples B and K from patients with stage four disease (AIDS) had 10^{-2} infected cells (10^4 cells per 10^6 PBMC). Samples from patients H and J with presumed stage two disease had 10^{-3} and 10^{-2} infected/uninfected cells (10^3 and 10^4 cells per 10^6 PBMC).

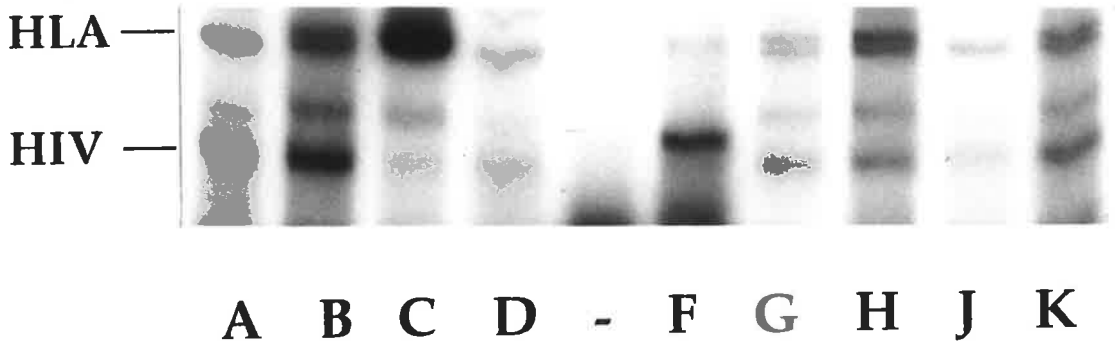
Autoradiographs from bone samples (Figure 6.1) demonstrated 115 base pair radiolabeled PCR product in all samples confirming detection of the HIV-1-*gag*

genome. There was a consistent finding in the triplicate analysis of clinical samples of the absence of the 242 base pair HLA-DQ-α band from patient C and a weak band from patients A, E, F and J.

Image analysis of the radiolabeled PCR bands from bone samples confirmed the observation of autoradiographs that the intensity of the HLA-DQ-α band was less than that of the HIV-1-*gag* band. The HIV/HLA band intensity ratio from seven samples exceeded the infected cell controls and from sample C the absence of a HLA-DQ-α band invalidates the intensity ratio method.

Figure 6.1 PCR coamplification of blood and bone from ten HIV infected patients. Detection of the 115 base pair HIV segment indicates HIV infection with incorporation of HIV into the host cells chromosomal DNA. A 242 base pair segment of the HLA-DQ-α genome is included as an internal control of PCR efficiency and to determine the HIV copy number relative to cell copy number.

blood



bone

Table 6.2 PCR coamplification of clinical samples. Combined results of three PCR experiments.

SAMPLE	BLOOD (log_{10} HIV infected cells)	BONE (log_{10} HIV infected cells)
A	-2	>-2
B	-2	>-2
C	-5	HIV
D	-2	>-2
E	CONTROL	>-2
F	>-2	>-2
G	-2	>-2
H	-3	-2
J	-2	-2
K	-2	>-2

6.4 Discussion

The HIV positive status of the ten clinical samples was confirmed by the PCR technique. Quantitative PCR results of blood samples suggest a virus load of 10^{1-4} HIV-PCR-units per 10^6 PBMC and six of the nine samples had 10^4 HIV-PCR-units per 10^6 PBMC. These results differ from the results of Lee *et al* (1991) who report maximal figures of 10^3 HIV-PCR-units per 10^6 PBMC during asymptomatic infection and $10^{3.6}$ HIV-PCR-units per 10^6 PBMC during stage three and four disease. Daar *et al* (1991) reported $10^{3.2}$ HIV-PCR-units per 10^6 PBMC during asymptomatic infection.

Two patients had stage four disease, two were presumed stage two as they were antibody positive but apparently asymptomatic and had no pathological evidence of stage three or four disease on the autopsy findings. Six patients were of unknown disease stage. Quantitative results from patients with stage four disease were not greater than results from the unknown patients as expected and one of the presumed stage two patients had 10^4 HIV-PCR-units per 10^6 PBMC and the other 10^3 HIV-PCR-units per 10^6 PBMC. It was anticipated that patients with stage two disease would have a lower virus bioburden than stage three and four patients but this finding was not observed.

HIV-1-*gag* was detected in all bone samples consistent with previous reports using non-quantitative PCR (Roder et al 1992) and co-cultivation methods. In one patient sample the HLA-DQ-α genome was not detected after three PCR experiments and the HIV/HLA ratio method of determining the virus bioburden is invalidated. Seven of the ten samples had an HIV signal intensity greater than the HLA internal control signal and the HIV/HLA signal intensity ratio was greater than that of infected cell controls for these patients and the quantitative PCR method was invalid.

The results suggest failure of the quantitative method from relative inhibition of HLA-DQ-α detection. HIV infected PBMCs include four or less copies of HIV DNA (Schnittman et al 1989); (Simmonds et al 1990) and it is unlikely that bone cells should include more. There is general correlation between the copy number of DNA and titres of infectious HIV in PBMC (Daar et al 1991). It is unlikely the quantitative PCR results in this experiment are valid as the results would suggest virus titres greater than previous observations of serum, PBMC and lymph nodes from AIDS patients (Daar et al 1991); (Ho et al 1989); (Coombs et al 1989); (Harper et al 1986).

It is likely that inhibition of HLA-DQ-α detection had occurred and this was demonstrated by the complete inhibition seen in one bone sample. Inhibition of HLA-DQ-α detection may also account for the greater virus bioburden than anticipated from the blood samples.

The hypotheses;

1) *that the virus bioburden in bone relates to the stage of disease and amount of virus in peripheral blood mononuclear cells,* and

2) *that the virus bioburden in bone is not greater than peripheral blood mononuclear cells.*

cannot be assessed by the quantitative PCR method as the method was invalid when applied to clinical samples of bone and possibly blood samples.

Inhibition of HLA-DQ-α detection was not seen with DNA derived from cell culture. It is suggested that the cause of failure resulted from DNA preparation of clinical samples, the inclusion of inhibitors relatively specific for HLA-DQ-α detection, or relative inefficiency of the probes to HLA-DQ-α when using less pure DNA from clinical specimens. Suggested methods to overcome these deficiencies include using an alternate DNA extraction technique, changing oligonucleotide

probes or alternative methods such as in-situ-PCR. Some of these methods may be available in other laboratories but this technology was not available in the Human Immunodeficiency Laboratory, Institute of Medical and Veterinary Science at the time of writing. It was elected to use an alternate method by study of the *in vitro* susceptibility of the major cells of bone to HIV infection.

7. HIV INFECTION OF HUMAN CARTILAGE

7.1 Introduction

Cartilage allografts are an important source of tissue for head and neck reconstruction surgery and have been used successfully in osteochondral allografts. Cartilage is also an important contaminant of many bone allografts. The success of cartilage allografts may be due to the relative isolation of the chondrocyte in its matrix (McGlynn et al 1981), cartilage is avascular and the mucopolysaccharide matrix forms a barrier to blood and immune cells. Viable allograft cartilage has been identified after seven years (Kandel et al 1985) and biopsies of osteochondral allografts have demonstrated live chondrocytes six years after transplantation (Czitroom et al 1990).

Human chondrocytes are considered not susceptible to HIV infection through a CD4-dependent mechanism as they lack this receptor (Bujia et al 1993b). Other mechanisms leading to possible HIV infection of chondrocytes have been examined. *In vitro* cell-free HIV infection of chondrocytes has been examined by two research centers with differing results (Ikeuchi et al 1990); (Bujia et al 1993a), the susceptibility of chondrocytes to HIV infection remains unclear.

In vivo infection of human cartilage with HIV has not been reported and is addressed in this chapter. The aim of this study was to examine cartilage from HIV infected patients for evidence of HIV infection.

7.1.1 Hypothesis

The in vivo susceptibility of human cartilage to HIV could be determined.

The method used to determine HIV infection of clinical samples must enable sufficient sampling to include an amount of tissue likely to be infected and have sufficient sensitivity of HIV detection.

The amount of tissue sampling required was estimated from the anticipated virus bioburden. Chondrocytes are mesenchymal cells and many of these cell types have been infected with HIV *in vitro* with varying expression of HIV. 20% of some cell lines express HIV proteins and increase to more than 80% in long term culture (Ikeuchi et al 1990); (Clapham et al 1983). Mellert *et al* (1990) found less HIV susceptibility with 0.1 - 0.5% of HIV infected fibroblast cell cultures from infected patients, and with *in vitro* studies they infected a maximum of 5% tumour cells and 10% of embryonic lung cells. Ikeuchi *et al* (1990) infected chondrocytes *in vitro* and found virus production only after co cultivation with lymphocytes which suggests a minimal bioburden.

The semi-quantitative PCR method developed in chapter five uses 5ug of chromosomal DNA which is equivalent to 2.5 million cells. If only 0.1% of chondrocytes are HIV infected a 5ug DNA sample of human cartilage should include 2.5×10^3 infected cells. The sensitivity of the quantitative PCR method allows detection of five cells per reaction mixture which is 500 times the anticipated minimum bioburden if human chondrocytes are infected *in vivo*. The quantitative PCR method should be sufficiently sensitive to conclude a negative result is a true negative.

7.2 Results

Autoradiographs (Figure 7.1) demonstrated delineation of the 115 base pair HIV-1-*gag* region and 242 base pair segment of the HLA-DQ-α genome with no interfering bands. HUT-78 cell and HIV negative donor cartilage DNA included as a negative control was amplified in all experiments and the 115 base pair HIV-1-*gag* region was not detected.

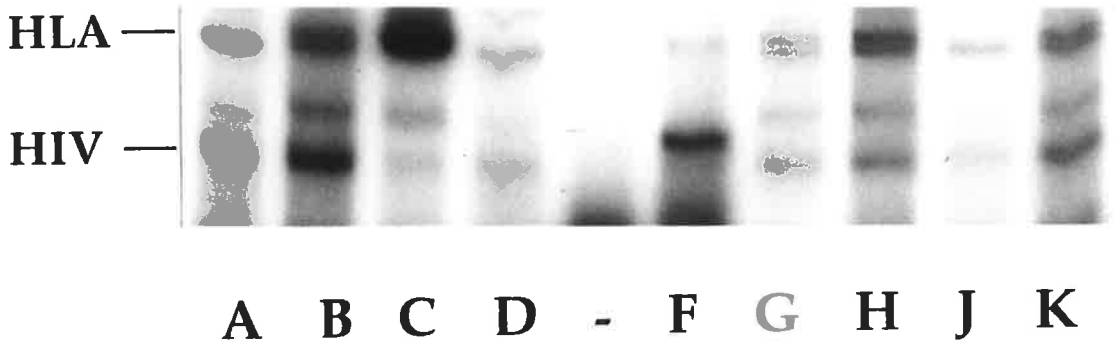
The two primer pairs detected from cartilage specimens and H3B cell dilutions had HIV and HLA specific bands that were successfully amplified. Detection of the HIV-1-*gag* region in chromosomal DNA from nine samples indicates *in vivo* infection of human cartilage with HIV.

PCR product was not detected from patient F after three PCR and repeated DNA digestion and extraction. Samples were examined in triplicate but PCR product was obtained from patients A, B, and D in only one experiment after repeat DNA extraction.

Semi-quantitative PCR analysis results are summarized in Table 7.1. The calculated ratio of infected cells was 10^{-2} to $<10^{-5}$ infected/uninfected cells.

Figure 7.1 PCR coamplification of blood and cartilage from ten HIV infected patients. Detection of the 115 base pair HIV segment indicates HIV infection with incorporation of HIV into the host cells chromosomal DNA. A 242 base pair segment of the HLA-DQ-α genome is included as an internal control of PCR efficiency and to determine the HIV copy number relative to cell copy number.

blood



cartilage

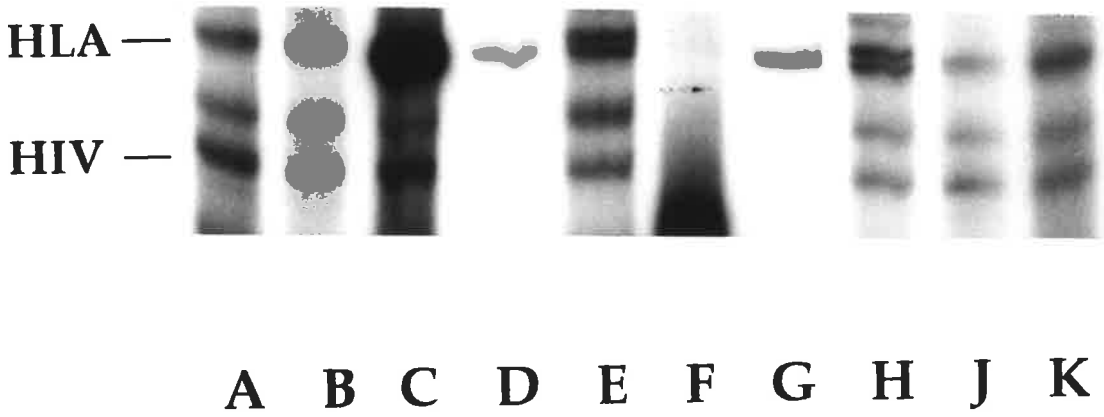


Table 7.1 PCR coamplification of human cartilage. Combined results of three PCR experiments.

SAMPLE	BLOOD (log_{10} HIV infected cells)	CARTILAGE (log_{10} HIV infected cells)
A	-2	-2
B	-2	-2
C	-5	-3
D	-2	<-5
E	CONTROL	-3
F	>-2	-
G	-2	-5
H	-3	-5
J	-2	-2
K	-2	-2

7.3 Discussion

In vivo infection of human cartilage has been determined in this study. HIV was detected in all samples where PCR analysis was effectively achieved. One of the ten samples did not react sufficiently to produce detectable radiolabeled PCR product despite repeat DNA digestion and extraction, the failure of this specimen to react may relate to the quality of the extracted DNA or the presence of inhibitors.

Human cartilage is avascular and the specimens examined were intact and macroscopically normal. In addition the specimens were thoroughly washed and not contaminated with blood or other body fluids, it therefore is assumed that the HIV DNA detected resided in infected chondrocytes. In nine of the ten samples where PCR product was obtained, HIV infection was identified in 10^{-2} - 10^{-5} cells. In the current study a finding of up to 1% HIV infection of chondrocytes *in vivo* is consistent with *in vitro* findings of HIV infection in cells of mesenchymal origin (Mellert et al 1990); (Ikeuchi et al 1990).

The mechanism of HIV infection of chondrocytes *in vivo* remains unknown. The mean 'pore' size of human cartilage is approximately the size of the serum albumin molecule (Maroudas 1979), therefore cell-to-cell HIV transmission is prevented but cell-free infection is possible. HIV infection independent of the CD4 receptor by phagocytosis and endocytosis has been reported (Tateno et al 1989); (Clapham et al 1989); (Harouse et al 1989) and it may be that this mechanism is effective *in vivo* with human cartilage. The differing results of *in vitro* infection of human chondrocytes (Bujia et al 1993a); (Ikeuchi et al 1990) cannot be explained by this study but the current results suggest chondrocytes can be infected under appropriate conditions.

The results are consistent with the finding of HIV in synovial fluid from HIV infected patients (Wirthington et al 1987). HIV arthropathy is described (Foster et al 1988) and it may be that chondrocyte infection is related to this clinical problem. In the present study the symptoms, function and microscopic appearance of the chondrocytes was not assessed so the clinical role of chondrocyte HIV infection remains unknown. In the context of systemic HIV infection cartilage may be a potential site for HIV infection. Although HIV infection from cartilage allografts has not been reported, the authors do not agree with Bujia *et al* (1993a) who suggest cartilage allografts may be less likely to transmit HIV than other allografts. Instead, cartilage allografts from donors of unknown HIV status should be vigilantly screened for the possible presence of HIV.

It may be possible to secondarily sterilize the allograft to further reduce the risk of HIV transmission. Irradiated cartilage allografts have been successful in short term animal models (Takahashi et al 1992) but the finding of live allograft chondrocytes in successful human cartilage allografts suggests viable chondrocytes may be obligatory to their success (Czitroom et al 1990) and irradiated allografts may not survive long term in humans.

8. HIV INFECTION OF HUMAN BONE DERIVED CELLS

8.1 Introduction

HIV has been cultured from the bones of HIV infected patients (Buck et al 1990); (Merz et al 1991); (Nyberg et al 1990) and it has been detected in DNA from the bones of infected patients by polymerase chain reaction analysis (Roder et al 1992); (Salzman et al 1993). It is not clear from studies of human bone that the bone specimens are infected or contaminated by HIV infected blood. There have been no reports documenting HIV infection of human bone at the cellular level.

Culture of human bone derived cells has been reported (Auf'mkolk et al 1985); (Beresford et al 1983); (Piche et al 1989); (Robey et al 1985b). These cells are osteoblastic and may be studied in a controlled environment with the potential to assess *in vitro* infection with HIV. They do not include haemopoitic or endothelial cells (Graves et al 1996).

8.1.1 Aims

The aim of this experiment was to determine if a human bone derived cell line could be infected with HIV using ideal *in vitro* conditions.

8.1.1 Hypothesis

That human bone derived cells can be infected with HIV.

8.2 Methods

Two systems of HIV infection were examined using an eight week incubation. Receptor based infection of the bone cells was examined using co-cultivation of human bone derived cells with virus obtained from a clone of productive lymphocytes from which the supernatant has been shown to include complete infectious virus. Infection via a cell-to-cell method was utilized via co-cultivation with chronically infected lymphocytes as described by Li *et al* (1992a); 1992b).

Infection of the human bone derived cells was defined as;

1) virus induced cytopathic changes or giant cell formation,

2) immunofluorescence of virus antigens,

3) reverse transcriptase activity (an indicator of virus production),

4) infection of co-cultivated human T-lymphoid cells receptive to HIV, and

5) incorporation of virus DNA into the bone cell genome.

8.2.2 HIV infection of human bone derived cells

The cell-free infection format was established with 10% volume virus supernatant added to the culture system. Human bone derived cells were chronically incubated with fresh virus supernatant added at day 0, 30 and 45.

Pre treatment with DEAE-Dextran incubation (section 2.2.3) to enhance virus absorption (Duc-Nguyen 1968); (Levy et al 1985) was examined. 1% DEAE-Dextran (100 micro liters/ml) was added to human bone derived cells, incubated at 37° for 30 minutes, then inoculated with 10% volume virus supernatant for two hours. Cells were washed three times with phosphate buffered saline, resuspended in culture medium and plated in 24 well plates.

When the cell-to-cell transmission method was used H3B cells were spun down, washed with serum free RPMI-1640, resuspended in growth medium and immediately co-cultured with human bone derived cells at a density of $5x10^3$ H3B cells/ml suspended in DMEM. The cell density was modified during regular medium changes which removes a proportion of the H3B cells (suspension cells) to maintain a simultaneous culture of both cell types.

8.2.3 Microscopy

Cells were examined daily for the first seven days, then before and after medium changes three times per week. Light microscopy observations were made for cytopathic changes and the formation of multinucleated giant cells, or syncytia. HUT-78 cells were incubated with virus or H3B cells as the positive controls and consistently demonstrated giant cell formation and cytopathic changes.

8.2.4 p24 antigen immunofluorescence

Cells were routinely removed from the 24-plate culture system on days one, three, five and seven, then weekly after inoculation and examined for the expression of p24 core antigen by immunofluorescence (section 2.2.2). Cells cultured in 25cm flasks were examined only at eight weeks post inoculation.

p24 antigen positive cells were evaluated using a Zeiss fluorescence microscope and graded negative, indeterminate or positive immunofluorescence. Non-infected HUT-78 cells were the negative controls and H3B cells were the positive controls which consistently immunofluoresced.

8.2.5 Reverse transcriptase activity

Half volume medium changes from human bone derived cells inoculated with virus supernatant were stored from days three, five, seven and then weekly for a seven week period. Supernatants were frozen at -20° until thawed for reverse transcriptase activity analysis (section 2.2.4).

The cell-to-cell infection system was not examined by reverse transcriptase analysis because H3B cells actively produce virus and have elevated reverse transcriptase activity which would mask virus production of the human bone derived cells.

H3B cell virus supernatant was the positive control and Human Bone Derived Cells cultured in non infective media was the negative control.

8.2.6 Lymphocyte co-cultivation

Subcultures of the cell-free infection system were co-cultivated with HUT-78 cells acting as indicator cells of HIV infection. On days 7, 14, 21, 28 and 35, after virus supernatant inoculation the cells were washed three times in phosphate buffered saline and HUT-78 cells were added to the cultures at a density of $1x10^5$ cells/ml and observed for syncitia formation daily for ten days. Half volume medium changes were continued three times per week. At ten days the medium was carefully removed leaving the majority of the HUT-78 cells in suspension. The slides were air dried, fixed and stained for p24 antigen immunofluorescence as previously.

The HUT-78 cells which are small round suspension cells were readily differentiated from the larger adherent bone derived cells. The co-cultivation method was not applied to the cell-to-cell infection system as it was not possible to remove all the H3B cells and avoid a false positive result.

HUT-78 cells incubated with virus were the positive control and consistently demonstrated giant cell formation, cytopathic changes and positive immunofluorescence.

8.2.7 Polymerase chain reaction:

Human bone derived cells incubated for eight weeks with virus supernatant or H3B cells were analyzed for the inclusion of virus DNA into their genome. Semi-quantitative assessment of HIV incorporation utilizing a modification of the co-amplification method of Lee *et al* (1991) was used (section 2.5.5).

Human bone derived cells were grown to subconfluence in 25 cm flasks and infected with either virus supernatant or H3B cells at weeks 0, 3 and 6. Complete medium changes were performed three times per week.

H3B cells diluted in HUT-78 cells were the positive control for PCR reactions. A standard curve of HIV/HLA probe intensity and H3B/HUT-78 cell dilution was constructed in duplicate. Non-infected human bone derived cells were used as the non-infected control group.

8.3 Results

8.3.1 Cell-free virus infection

There was no difference in the light microscopic appearance in the cell-free system compared to the negative controls during the period of incubation. No cytopathic changes were observed and giant cells were not seen.

Dilutions of virus supernatant (positive controls) were inoculated with HUT-78 cells and produced a $TCID_{50}$ greater than 10^5. The infected HUT-78 cells usually demonstrated giant cell formation within three days for virus that had been diluted up to 1/100 and usually within five to seven days for greater dilutions. These findings were not seen with the human bone derived cells which had been inoculated with identical virus supernatant.

8.3.1.1 Reverse transcriptase activity

Figure 8.1 is the result of reverse transcriptase activity of Human Immunodeficiency Virus infected human bone derived cells from the cell-free infection model including non-infected human bone derived cells as a negative control. Supernatants obtained from H3B cell cultures routinely had greater than 40,000 counts/millilitre. Elevations in reverse transcriptase activity from the human bone derived cell supernatants was seen only after the addition of fresh virus supernatant. The virus supernatant added to the culture system has reverse transcriptase activity which decreases at a rate determined by absorption and cell metabolism, the difference in reverse transcriptase activity between samples is a normal variation in these parameters. Virus supernatant was added on days 0, 30 and 45, and a subsequent rise in the reverse transcriptase activity was seen up to five days following this inoculation. Following half volume medium changes there was a rapid dilution of the reverse transcriptase activity suggesting no de novo production of reverse transcriptase activity from the culture system.

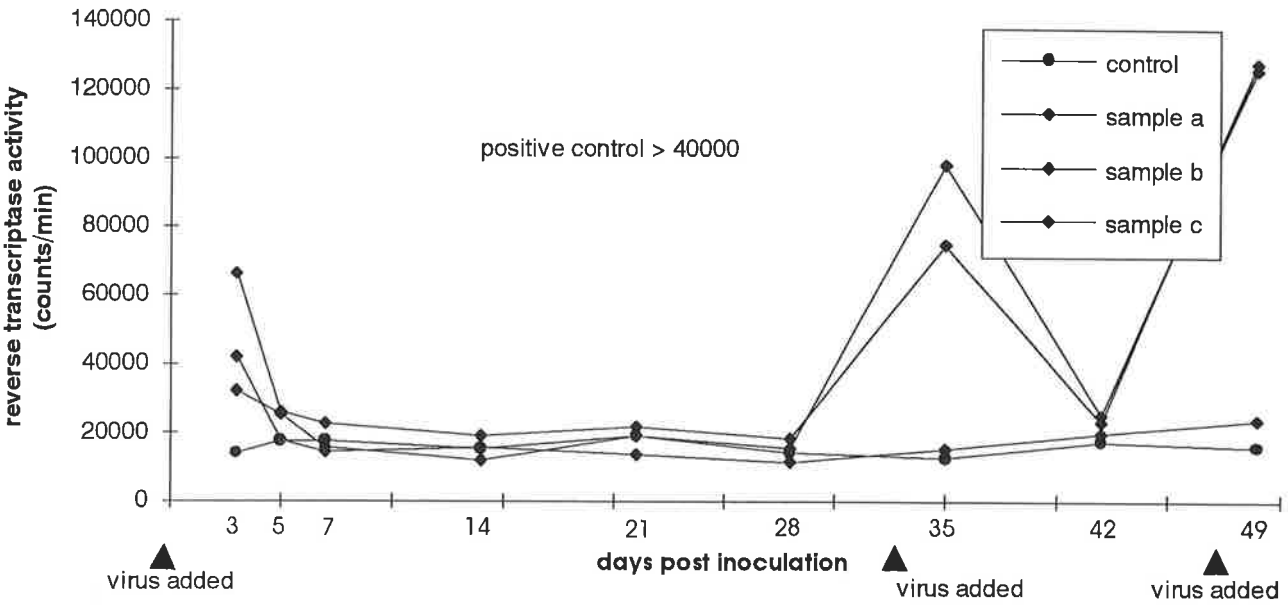
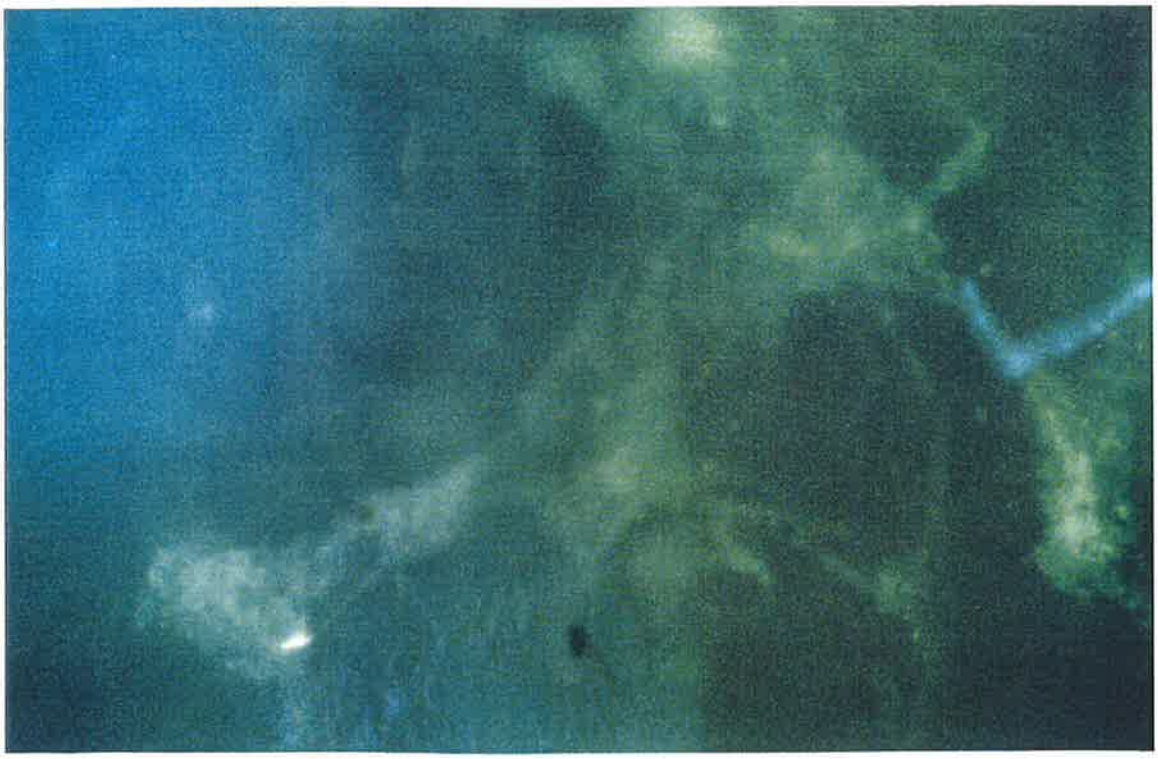


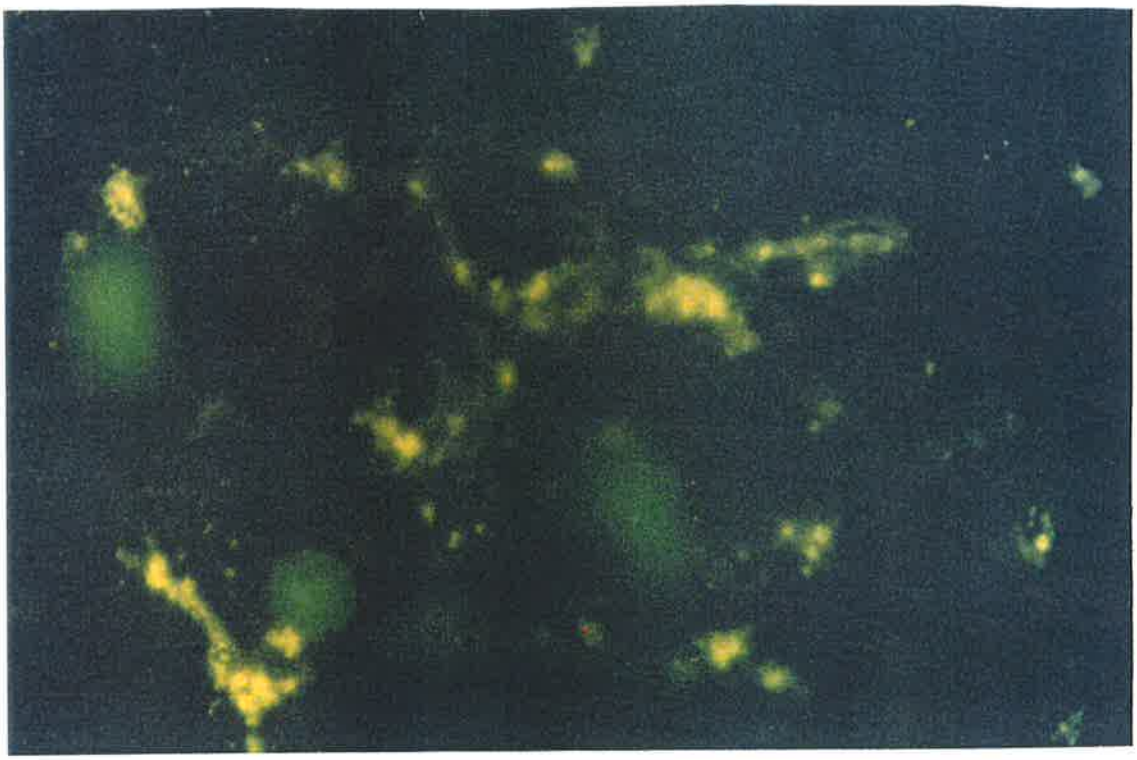
Figure 8.1 Reverse Transcriptase activity of Human Bone Derived Cells incubated for seven weeks with HIV. Reverse transcriptase activity is a measure of productive virus infection.

8.3.1.2 p24 antigen immunoflorescence

Immunofluorescence was graded as definite immunofluorescence, indeterminate immunofluorescence, and no immunofluorescence above background. Cells were generally readily differentiated to either definitely infected or not infected, and an indeterminate pattern was infrequently observed. There was no evidence of p24 antigen immunofluorescence from cells examined at intervals using a 24-well plate system. Human bone derived cells that had been cultured for eight weeks in 25cm flasks demonstrated an indeterminate result. There was some activity above background in a random distribution but corresponding to the cytoplasm of several of the human bone derived cells (Figure 8.2). Cells from this culture system were examined by PCR and found not to contain HIV DNA.

Figure 8.2 p24 antigen immunofluorescence of human bone derived cells chronically incubated with HIV for eight weeks. There was some activity above background in a seemingly random distribution but corresponding to the cytoplasm of several of the human bone derived cells. Magnification, x40.





8.3.1.3 Lymphocyte co-cultivation

When HUT-78 cells were added to the cultures as indicator cells they rapidly overran the more slow growing human bone derived cells. With half volume medium changes it was possible to continue the HUT-78 cell line until slides were made for immunofluorescence at 10 days. When infected with virus supernatant or H3B cells for a positive control, the HUT-78 cells rapidly coalesced to form giant cells and immunofluoresced within three to five days.

There were no cytopathic changes or giant cells observed when the HUT-78 cells were added to the cell-free infection system. Slides were removed for immunofluorescence at day 10. The human bone derived cells had been largely overrun and limited numbers remained upon the fixed slides, most of the HUT-78 cells which are smaller suspension cells were retained during fixation. The HUT-78 cells and the limited number of human bone derived cells did not immunofluoresce.

8.3.2 DEAE-Dextran treated cells

The DEAE-Dextran treated cells were inoculated with virus supernatant and maintained in culture for three weeks. There was no evidence of cytopathic changes of the human bone derived cells or giant cell formation.

The human bone derived cells did not immunofluoresce. HUT-78 cells which had been added as indicator cells showed no evidence of syncitia formation and did not immunofluoresce for p24 antigen at 10 days.

8.3.3 Cell-to-cell infection of human bone derived cells

When human bone derived cells of at least 90% confluence were infected with H3B cells they were overrun by this rapidly dividing cell population when inoculated by more than $1x10^5$ cells/ml. The culture system was successfully established using

$5x10^4$ H3B cells and medium changes adjusted to remove excess H3B cells. Usually 75-100% medium changes were required three times per week to maintain viable cultures of both the human bone derived cells and H3B cells.

The human bone derived cells established in culture with H3B cells showed no evidence of cytopathic changes. The H3B cells showed no evidence of fusion with the human bone derived cells or other H3B cells.

H3B cells do not have the CD4+ receptor and do not coalesce to form syncitia. When H3B cells are added to receptive cells such as HUT-78 cells they fuse to form giant cells (Li et al 1992a) and this was confirmed in a subculture of the H3B cells added to HUT-78 cells (positive control).

8.3.3.1 p24 antigen immunoflorescence

Cells examined by p24 antigen immunofluorescence for seven weeks showed no evidence of fluorescence. The majority of H3B cells had been washed away after three saline washes and were readily differentiated from the larger adherent human bone derived cells.

From a separate experiment of human bone derived cells co-cultivated with H3B cells in 25cm flasks, cells were removed and re-seeded onto glass microscope slides. Forty eight hours after adherence of the human bone derived cells, they were washed three times with phosphate buffered saline, fixed and examined for p24 antigen immunofluorescence. The result was indeterminate with some activity above background in occasional human bone derived cells (Figure 8.2).

8.3.4 Polymerase chain reaction

One flask of each of the cell-to-cell and cell-free infection methods was infected with bacteria and discarded. Two 25cm flasks inoculated with the cell-to-cell infection method and two flasks inoculated by the cell-free infection method and one non infected human bone derived cell negative controls were available for PCR analysis.

Discrete HLA-DQ-α bands were seen in all reactions confirming satisfactory PCR reaction conditions and DNA quality. The *HIV-1-gag* band was detected in all positive controls and not with negative controls. No HIV bands were seen with the cell-free infection model suggesting the absence of incorporation of HIV DNA into the host cell genome (fig. 8.5). Faint bands of the HIV DNA segments were present in all PCR reactions from the cell-to-cell infected model. Quantification of the band intensity ratios of HIV/HLA band regions demonstrated 0.1% infected cells in PCR reactions from one flask and less than 0.001% infected cells from the other flask. This result is consistent with some residual H3B cells remaining after saline washing or infection of less than one per thousand human bone derived cells.

Figure 8.3 Electrophoresis of radiolabeled PCR product from Human Bone Derived Cells incubated with HIV virus or H3B cells for eight weeks.

HLA
HIV
bone + H3B
bone + H3B
bone + virus
bone + virus
control

8.4 Discussion

In culture human bone derived cells synthesize predominantly Type I collagen, express high levels of alkaline phosphatase under appropriate culture conditions (Beresford et al 1986) and synthesize the bone/dentine specific protein osteocalcin establishing the validity of using human bone derived cell populations to study cells of the osteoblast lineage (Beresford et al 1984); (Auf'mkolk et al 1985); (Marie et al 1989); (Chavassieux et al 1990); (Wergedal et al 1984); (Crisp et al 1984); (Robey et al 1985a); (Ashton et al 1985).

For the cell-free infection system used in these experiments the minimum virus titre was 10^5 $TCID_{50}$/ml which is at least 10 times the maximal *in vivo* virus titre in human infection (Daar et al 1991); (Ho et al 1989). Virus absorption was further enhanced by DEAE-dextran pre treatment in some of the cell-free infected human bone derived cells.

The human bone derived cells did not demonstrate virus production when infected with virus supernatant as determined by microscopic changes, co cultivation and reverse transcriptase activity. Other mesenchymal cell lines (fibroblast and osteosarcoma cells) have been infected by the cell-free system and syncytia were seen at 14 days with nearly 100% of cells infected at three weeks (Mellert et al 1990). Reverse transcriptase levels rise in parallel with the formation of syncytia formation and when lymphocytes were added to this system as indicator cells syncytia were observed. Continuous observations of HIV inoculated human bone derived cells did not demonstrate these changes despite the duration of culture exceeding those reported in other series. Previous studies have used limited periods of HIV virus incubation (up to 48 hours) with and without DEAE-dextran pre-incubation, a longer incubation period with chronic virus inoculation for the duration of culture was used.

p24 antigen immunofluorescence of cell-free HIV inoculated human bone derived cells for seven weeks did not show evidence of infection of the human bone derived cells or co cultivated lymphocytes. At eight weeks an indeterminate result was observed but this does not imply infection as polymerase chain reaction analysis of the samples failed to demonstrate virus DNA segments. It is concluded that human bone derived cells are not susceptible to cell-free mediated infection with ideal *in vitro* conditions.

Some mesenchymal cells are susceptible to HIV infection with the cell-to-cell infection method but not with the cell-free format . Ikeuchi *et al* (1990) reported chondrocytes had become infected after 20 days of cell-to-cell HIV infection detected by co cultivation with indicator lymphocytes and polymerase chain reaction but not with reverse transcriptase activity, p24 antigen production or immunofluorescence. Human bone derived cells inoculated with the cell-to-cell infection format were not susceptible to HIV infection despite prolonged co cultivation when assessed by microscopy and immunofluorescence. It is concluded the cells are not susceptible to productive HIV infection.

Levy (1993) and Embretson *et al* (1993) have identified latent infection in lymphoid cells with polymerase chain reaction techniques. The HIV infected cells contained HIV DNA but 95% did not express sufficient viral RNA, and HIV infection in these cells could not be detected by microscopy, immunofluorescence or reverse transcriptase activity. PCR analysis of human bone derived cells inoculated with the cell-to-cell infection format demonstrated less than one HIV infected cell per 10^5 human bone derived cells in one experiment, and one HIV infected cell per 10^3 human bone derived cells in another experiment. It is possible that the infected cells detected by PCR were H3B lymphocytes used in the cell-to-cell infection format that had not been removed from the culture system prior to DNA extraction. Alternatively, latent infection may have been established in the bone derived cells

with no apparent expression of the provirus genome. Because of the sensitivity of the PCR technique it is not possible to differentiate between the detection of an HIV infected lymphocyte or the detection of latent infection of a human bone derived cell. This is discussed further in section 10.1 Directions for Future research.

Latent HIV infection of human bone derived cells is at most very infrequent despite the favorable *in vitro* experimental conditions. It was concluded that under ideal *in vitro* conditions human bone derived cells are resistant to HIV infection using the cell-to-cell method

This observation is in contrast to the work of Merz *et al* (1991) who successfully infected whole pieces of bone with virus supernatant or HIV infected lymphoid cells and observed antigen immunofluorescence and reverse transcriptase activity at six days. The cells in the infected bones were susceptible to both cell-free infection and cell-to-cell infection with a rapid production of virus that has not previously been reported in non lymphoid cells. It is suggested that these workers may have infected the non osteoblastic cells in the whole bone infection system as previously reported (Freedman et al 1991); (Folks et al 1988).

8.4.1 Conclusions

The hypothesis *'that human bone derived cells can be infected with HIV'* was not proven when examined by traditional methods to assess productive virus infection including syncytia formation, reverse transcriptase activity and antigen (p24) expression by cell-free infection. The hypothesis was not proven with a more comprehensive indicator cell co cultivation and polymerase chain reaction technique. When the hypothesis was assessed using the cell-to-cell infection model it was not proven with assessment of syncitia formation and antigen immunofluorescence. Latent cell-to-cell HIV infection of human bone derived cells may have been

established but was at most very infrequent. It is concluded the hypothesis *'that human bone derived cells can be infected with HIV'* is not true for productive HIV infection but further work is required to determine if infrequent latent infection may occur.

It is clearly established that bone from HIV infected patients may contain the virus and procedures to minimize infection from HIV infected bone cannot be relaxed. It is unlikely that bone cells will contribute significantly to an HIV reservoir in excess of the amount of virus from blood found in the donor bone. It would be prudent to assume bone to have a bioburden similar to serum which is 10^4 $TCID_{50}$/ml during the window period (Daar et al 1991), 30 $TCID_{50}$/ml during the asymptomatic period and 3500 $TCID_{50}$/ml for patients with stage four disease (Ho et al 1989).

HIV infection is of considerable interest to bone allograft banks because of the potential of not detecting an HIV infected donor during the antibody negative period of disease which is three to six weeks (Haseltine 1988); (Redfield et al 1988); (Gaines et al 1990). The *in vitro* inoculation of human bone derived cells with HIV was examined for eight weeks and it is concluded that bone is not productively infected during this time frame, but the potential for limited latent infection may exist. Methods to sterilize bone should be assessed by their efficacy to inactivate the virus in blood contaminating the graft and in a lesser number of potentially infected lymphoid and myeloid cells. It is suggested that methods to detect HIV directly from a bone graft (Merz et al 1992) may not be as sensitive as examining the donors blood which is likely to have a greater virus bioburden.

9. INACTIVATION OF HIV WITH GAMMA IRRADIATION

9.1 Aims

The aim of this chapter was to quantify the inactivation of human immunodeficiency virus by gamma irradiation to assess the application of this sterilization method to HIV infected bone allografts that have not been detected by routine bone bank screening.

9.1.2 Hypothesis

1. *Inactivation of HIV occurs as a first order reaction with doses of irradiation clinically relevant to bone allografts.*

2. *A sterility assurance level of 10^{-6} probability of infection is not achieved by irradiating potentially HIV infected bone allografts with 25 kGy of gamma irradiation.*

Previous experiments of virus inactivation with gamma irradiation have shown a first order reaction (Gardiner et al 1986) and it was anticipated that this would be demonstrated with HIV. If this first hypothesis was proven, the second hypothesis and primary aim of this work could be addressed. From previously studies it has been questioned whether HIV can be inactivated by the currently recommended dose of 25kGy. It may be that the recommended dose will inactivate most of the virus contaminating bone but the hypothesis requires additional virus inactivation to achieve the sterility assurance level. The sterility assurance level is arbitrarily determined and most standards accept a 10^{-6} probability of virus survival.

9.2 Results

Productive infection was observed in all HUT-78 cell cultures inoculated with controls or irradiated samples but varied in the virus titre. In most instances the presence of giant cell formation was evident by day five particularly when HUT-78 cells had been inoculated with large titres of virus (Figure 2.1) but with increased virus dilutions giant cells appeared later and were less frequent. A limited number of equivocal results were differentiated by day nine observations of cytopathic effect and giant cell formation.

There was universal agreement with p24 antigen immunofluorescence and microscopic observations of HUT-78 culture cells harvested for antigen immunofluorescence.

The virus titration results calculated by the $TCID_{50}$ method were subjected to linear regression analysis and values were derived from the regression curves. 23 samples were available for analysis from two independent irradiation episodes, a number of samples were lost due to events independent of the irradiation and titre calculation (bacterial contamination of the HUT-78 cell culture plates).

There was a linear relationship between the logarithm of the biological activity of the virus and the radiation dose delivered, the co-efficient of determination was 0.953, and $p < 0.001$. The linearity of the decrease in virus titre (log10 $TCID_{50}$/ml) as a function of the radiation dose indicates that the inactivation of HIV is a first-order process. From the radiosensitivity curve of HIV infected HUT-78 cells (Figure 9.1) the inactivation rate was -0.1134 $\log_{10}$ $TCID_{50}$/ml/kGy (95% confidence intervals, -0.1248 - -0.1020). The radiation dose that provides a 90% reduction of biological activity (D-10 value) was 8.82 kGy.

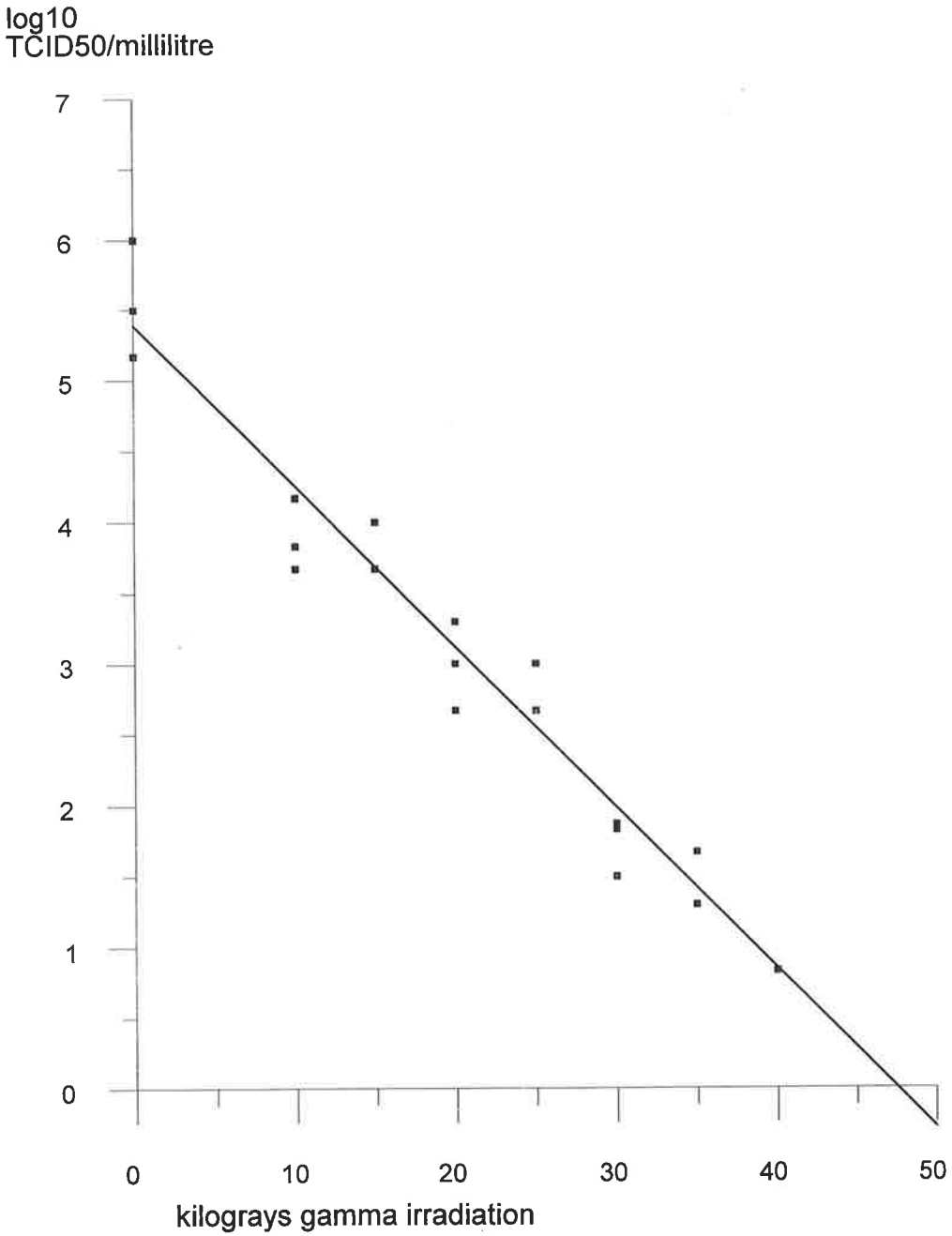


Figure 9.1 Virus titre of gamma irradiated HIV determined by HUT-78 cell culture inoculation.

9.3 Discussion

Irradiation of HIV in serum maintained on dry ice at approximately -70^{c} results in an inactivation rate of 0.113 $TCID_{50}$ dose per ml per kGy over a range of irradiation doses that are clinically relevant to bone allograft specimens. The results correspond to those of Hiemstra *et al* (1991) who reports the largest previous study of 11 HIV samples. The D-10 value of the current study was 8.82 kGy and is comparable to the D-10 value of eight kGy calculated by Hiemstra *et al*. These results correspond to reports of the irradiation inactivation of HIV where studies on smaller sample sizes have suggested the D-value to be 6.10 kGy (Kitchen et al 1989) and greater than four kGy (Conway et al 1991). The D-values for HIV approximate those of other viruses and retro-viruses (Bassin et al 1978); (Latarjet 1970); (Rainbow et al 1972) but HIV is one of the more radioresistant viruses.

The slight difference in susceptibility of HIV to gamma irradiation between the current and reported studies is not clear. The current study and that of Hiemstra *et al* (1991) and Kitchen *et al* (1989) used a variable irradiation dose and confirmed a first order inactivation curve. Conway *et al* (1991) examined six samples with a fixed irradiation dose of four kGy which should give an accurate representation of the inactivation curve but the small irradiation dose used may have been less sensitive to detect the decrease in virus activity than the higher doses used in the current study.

The $TCID_{50}$ assay is a direct measurement of viral infectivity and has several advantages over the alternative methods. Reverse transcriptase assay or antigen capture assays were used in early reports of HIV radiosensitivity and erroneously reported the virus to be unusually radiosensitive (Bigee 1988); (Spire et al 1985). The $TCID_{50}$ assay is several orders of magnitude more sensitive than either reverse transcriptase assay or antigen capture assays and is measured over a greater range (McDougal et al 1985a).

The virucidal effectiveness of gamma irradiation is directly related to damage to the virus genome (Ginoza 1968) and radiation inactivation has been used to determine the target size of the virus genome with virus in the frozen or lyophylized state. Hiemstra *et al* (1991) reported an estimated target size of HIV to be $3x10^3$ kDa which closely approximates the known size of the genome (Ratner et al 1985); (Wain Hobson et al 1985) suggesting the inactivation rate and D_{10}-value to be correct.

Sterilization of bone grafts infected with HIV is most important during the early stages of infection before antibodies can be detected and the donor eliminated by routine screening tests (window period). During this time the virus load in serum is at a maximum of 10^4 $TCID_{50}$/ml (Daar et al 1991) but the bioburden of HIV in bone is unknown. Human derived cells are not susceptible to productive HIV infection (chapter eight) and it is unlikely that the bioburden in bone will exceed serum, it would seem reasonable to consider the bioburden of bone to be not greater than serum. The most common bone allograft used is a femoral head (average 50mm diameter) and the maximum bioburden would be 5.8 $\log_{10}$ tissue culture infective doses if an equivalent blood volume is assumed.

An additional factor is the probability of one surviving organism in a million that causes an overt infection. Even if one or a few organisms are eluted from the bone the potential of causing an infection is minimal. Only very few of the most virulent micro organisms are capable of initiating infection when a single cell gains access to sub epithelial tissue, eg certain rickettsial, micobacterial and pasturella species, with other organisms, a 1000 or more may be required and most bacteria require a critical initial number of cells even to initiate growth *in vitro* in a nutritionally ideal culture medium (Gaughran 1985). The *in vivo* infectious dose of HIV remains unknown but the simian immunodeficiency virus (SIV) is an

approximate animal model for HIV and infection occurs in rhesus monkeys challenged with SIV at a dose of 100 $TCID_{50}$ (Murphey-Corb et al 1989). Two chimpanzees have been inoculated with a dose of 1120 $TCID_{50}$ and the other with 0.1 $TCID_{50}$ of HIV and infection occurred only at the higher dose (Francis et al 1984). If the simian immunodeficiency virus model is assumed the human-infection dose response curve for a gamma irradiated femoral head infected with HIV can be calculated (Figure 9.2).

The irradiation dose required for a sterility assurance level of 10^{-6} is 89 kGy and this dosage exceeds current recommendations for routine radiation sterilization of bone allografts. It is likely that a similar dose is required to inactivate other serious virus infections including Hepatitis B and Creutzfeldt-Jakob disease (Mikhailov et al 1987); (Gibbs et al 1978). The bioburden and D_{10} - value for Hepatitis C is unknown.

The HIV sterilization dose of 89 kGy exceeds current recommendations for sterilising medical products and the current practice of bone banks that use gamma irradiation for secondary sterilization. It is concluded that gamma irradiation must be disregarded as a significant virus inactivation method for bone allografts. If the irradiation dose was increased to the dose required to inactivate the HIV bioburden (35 kGy) or increased to the D-6 value (53 kGy) bone graft recipients could be infected and further increases of irradiation dose would result in a proportional decrease in the probability of infection. Achieving a sterility assurance level of 10^{-6} with 89 kGy would result in graft destruction.

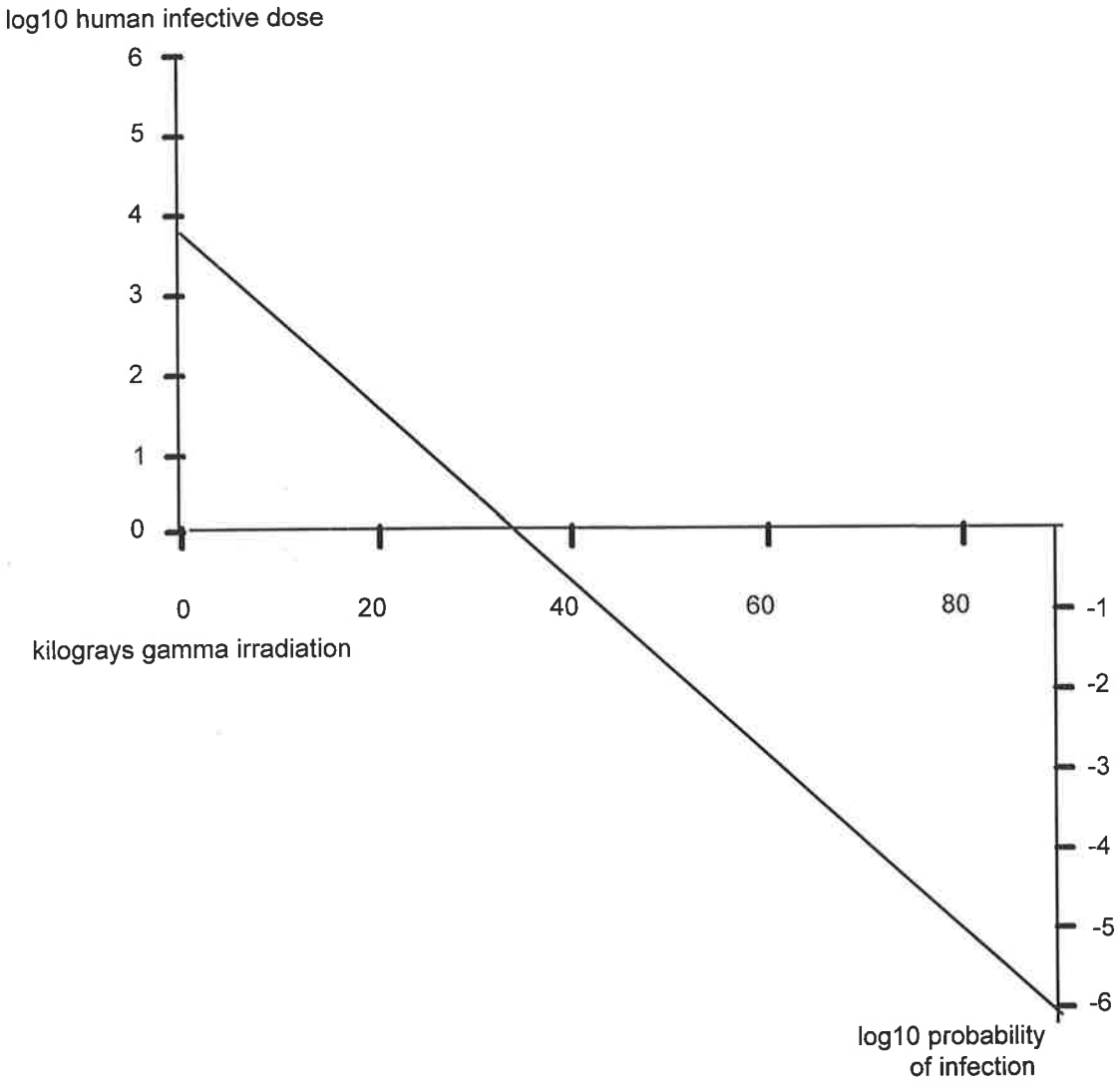


Figure 9.2 Dose response curve of an HIV infected femoral head treated with gamma irradiation. The bioburden is calculated as the human-infective dose that may occur in an acutely infected femoral head allograft donor during the window period (prior to HIV-antibody production).

9.3.1 Conclusions

The first hypothesis that *'inactivation of HIV occurs as a first order reaction with a linear reduction in log titre using a dose of irradiation clinically relevant to bone allografts'* was proven. Inactivation of HIV was examined using conditions simulating the current practice of many bone banks. The virus was suspended in media which has similar properties to a donors serum or bone and maintained at -70° C. With these conditions a first order virus inactivation curve was demonstrated with a high coefficient of determination ($r^2 = 0.953$).

The second hypothesis that *'a sterility assurance level of 10^{-6} probability of infection is not achieved by irradiating potentially HIV infected bone allografts with 25 kGy of gamma irradiation"* was proven and the radiation dose required to achieve a sterility assurance level of 10^{-6} exceeds current recommendations for routine radiation sterilization of bone allografts.

Previous reports suggesting the risk of virus transmission from allografts, especially HIV and Hepatitis non A, non B, and non C virus is nil due to radiation sterilization (Ferrante et al 1992) have not been supported by this study.

10. CONCLUSION AND SUMMARY

Patients requiring bone allograft surgery are being treated with increasing frequency. For many of these patients there are few alternatives, particularly following the massive defects that may result after limb salvage tumour surgery or multiple revision joint surgery. The steadily increasing demand for allograft bone has coincided with the HIV epidemic. In many countries the incidence of HIV continues to increase, with no potential for therapy in site other than preventative measures.

There have been concerns that if the safety of bone allograft surgery could not be adequately evaluated it may need to be discontinued. Already some allograft surgery has ceased eg. imported dura allografts have been prohibited since the occurrence of slow virus infection. The research outlined in this thesis was prompted by these concerns. Prior to the introduction of these studies one case of HIV infection from a bone allograft was reported (Centres for Disease Control 1988b). During the course of these studies the concern of HIV transmission was highlighted with the report of three bone allograft donors being infected with HIV despite strict adherence to contemporary bone banking standards (Simonds et al 1992).

One purpose of this study was to calculate the risks of HIV transmission from bone allografts. This has been addressed by epidemiological means and by an assessment of the effectiveness of secondary graft sterilization by gamma irradiation. Because there will always be a chance of HIV infection from bone allografts the degree of safety against HIV transmission is an arbitrarily determined probability termed the sterility assurance level. The sterility assurance level has several meanings; it can be applied to the effectiveness of the donor screening and also to the effectiveness of secondary graft sterilization.

The local pattern of bone allograft surgery was examined and the risks of HIV infection from a bone allograft donor was estimated. The prevalence of HIV in the donor population and the sensitivity of donor screening was used to calculate the HIV risk reported in chapter three of this thesis. The figures were calculated with an average estimate method and can be applied to most bone allograft banks using contemporary screening methods and modified to the donor population HIV prevalence. For the South Australian bone bank the estimated probability of receiving bone from an HIV infected donor after appropriate screening was 0.025 per million living donors and 0.2 per million cadaveric donors.

The chance of receiving bone from an HIV infected donor from the South Australian bone bank is very low and the need for further secondary sterilization against HIV may not be indicated from this allograft bank. The low probability of bone allograft HIV transmission is a comfort to Australians but there are concerns from other banks where the prevalence of HIV in the donor population is much greater.

At the current time the majority of bone banks use secondary sterilization as an additional method of reducing the chance of bacterial and perhaps viral infection. Different methods are used including chemical sterilization and physical treatments such as heating and irradiation. The majority of bone banks use gamma irradiation to sterilize the grafts against bacterial contamination and it is hoped that the irradiation confers some addition safety against the transmission of viral diseases.

Studies in this thesis have examined the efficacy of irradiation sterilization of HIV because it is both an important and topical potential contaminant of bone allografts, and because it is an accessible model of transmissible virus disease. The problem of HIV transmission has been significantly minimized by donor screening but other viruses which are more difficult to screen in the donor population remain

potential contaminants. The HIV virus was used as a model to examine radiation sterilization of viruses that may contaminate bone.

Previous studies from the United States and from the Pasteur Institute in France suggested that HIV was particularly radio-sensitive (Bigee 1988); (Spire et al 1985) and there may have been unjustified complacency regarding the risk of HIV transmission from irradiated bone and tissue allografts. A pilot study was performed using an HIV infected bone allograft model and is reported in chapter four. This study refuted the initial reports of unusually high radiation sensitivity of HIV and was consistent with more recently published work. The virus titre and volume of virus used in the initial studies may account for the differing observations of HIV inactivation when examined in a non quantitative manner.

The pilot study undertaken as part of this thesis was not able to determine the amount of irradiation required to achieve a satisfactory sterility assurance level of an acutely HIV infected bone. Two questions were then addressed;

1) What is the virus load in bone from acutely HIV infected donors (the most likely to be missed by contemporary screening techniques)?
2) What is the inactivation rate of HIV treated with gamma irradiation?

A novel approach to quantify the virus load in bone was developed using a quantitative polymerase chain reaction (PCR) technique (chapter five). The method was validated *in vitro* with HIV infected cells, but the adaptation of this method to bone samples was unsuccessful (chapter six).

Because the *in vivo* studies of HIV infection of human bone were inconclusive the susceptibility of human bone derived cells to HIV infection was examined. The *in vitro* studies with human bone derived cells did not demonstrate productive virus infection after an eight week inoculation under conditions that would be ideal for

virus infection. Previous *in vitro* studies using other cell types have used similar or shorter inoculation times and have demonstrated that cells susceptible to HIV infection should be identified within an eight-week period. The quantitative PCR method demonstrated no infection of the human bone derived cells inoculated with cell-free virus, but up to 0.1% cells had incorporated HIV DNA when incubated with the cell-to-cell infection format. It was concluded that the virus load in a given bone sample was not greater than the virus load due to the serum within that bone sample.

It was concluded that studies examining the sterilization of bone allografts could assume the virus bioburden in bone is not likely to exceed the bioburden of blood that contaminates the bone allograft.

The failure to detect significant levels of HIV infection of human bone derived cells may be an important finding relevant to the screening of bone allograft donors. Some bone allograft banks test the bone from allograft donors in addition to the donors blood and it is likely that the yield from this approach will be small. The current research suggests the virus bioburden in bone cells to be extremely low and screening efforts would be more beneficially directed to other areas of the donor likely to have a higher yield of HIV infection particularly during the early stages of HIV infection. This study suggests the target organs for HIV screening should not be bone but the early targets of HIV infection which are cells with the CD4+ receptor particularly nucleated blood cells and lymphoid cells. Examining these cells rather than bone would be a more logical selection.

The second question to be addressed by this thesis was the inactivation rate of HIV infected bone allografts treated with gamma irradiation. For this study the inactivation rate of HIV was examined using HIV infected serum maintained on dry ice. The results confirmed a first order inactivation curve and the inactivation rate (D_{10} - value) was determined. The results obtained were comparable to two previous

studies that examined smaller numbers of factor VIII preparations spiked with HIV (Hiemstra et al 1991); (Kitchen et al 1989).

The efficacy of irradiation sterilization was calculated to include blood that will contaminate the bone. The inactivation rate of HIV was used to calculate the dose of irradiation required to inactivate virus from an infected bone allograft. A sterility assurance level of less than one in a million infected bone allografts remaining non-sterile was not achieved with the dose of irradiation that is currently used. Increasing the dose of irradiation to achieve this sterility assurance level would be detrimental to the quality of the bone allograft.

It is likely that viruses with a genome size and blood concentration similar to HIV will require a similar dose. Viruses such as hepatitis B that have a greater blood bioburden and smaller viruses or virus like agents such as Jacob Creutzfeldt will require a greater irradiation dose and are not inactivated with the dose of irradiation that is currently used.

10.1 Direction For Future Research.

This study establishes the basic parameters for further research related to bone allograft surgery and HIV infection of bone and cartilage.

Irradiated bone allografts have been used successfully for many years. The principle purpose for irradiation has been to inactivate bacterial contamination. This study suggests that irradiation sterilization of bone infected with HIV or other viruses is unlikely to achieve sufficient virus inactivation. Gamma irradiation may confer some improved safety of the graft but the degree of sterility assurance cannot be achieved without irradiation levels that would be excessively detrimental to the graft. Therefore gamma irradiation should not be relied on to achieve viral sterilization. The quantitative radiosensitivity work in this paper has been confirmed by two independent laboratories (Hiemstra et al 1991); (Kitchen et al 1989) and it is recommended that further research is not likely to recommend a different bone allograft HIV inactivation dose.

Investigations using thermal treatment of bone allografts may establish a more dominant role in allograft sterilization if it can be supported by longer term clinical trials in humans.

In the longer term it is hoped that the use of bone allografts in orthopaedic surgery will be remembered as a satisfactory and safe method of skeletal reconstruction when no other viable alternatives existed. Future research examining alternatives to bone allografts is likely to be the direction of the future and at present progresses along two pathways. In tumour and revision arthroplasty surgery one direction is to use non biological methods with increasingly complex customized mechanical prosthesis which make no attempt to imitate the biology of the host bone. The second alternative is the use of biological bone alternatives. There has been

much research in the past using non organic tissue such as hydroxyapatite, glass, plastics and organic substitutes from other species treated to encourage incorporation into the host. With more understanding of the regulation and control of osseous tissues at the cellular and molecular level it may be possible to genetically engineer bone substitutes. At the present time it is possible to harvest bone from a potential recipient and grow colonies of bone forming tissue for later reimplantation, studies in humans with this technique are being investigated (Nolan 1995). Identification and development of factor delivery systems could lead to the long term goal of in situ bone formation.

The novel finding of *in vivo* infection of human chondrocytes with HIV has relevance to fresh osteochondral grafting. Using the quantitative PCR technique infection in human chondrocytes was demonstrated and this finding resolves the conflicting reports from two previous *in vitro* studies of human chondrocytes. The susceptibility of human cartilage to HIV infection does not directly relate to the main objectives of this thesis and has not been pursued further. The examination of human cartilage during HIV infection could prove useful to the understanding of the orthopaedic and rheumatological manifestations of HIV related disease particularly HIV related arthropathy. It is suggested this finding should be confirmed by alternative methods such as an in situ PCR technique to morphologically confirm the infected cell type.

In vitro infection of human derived bone cells with HIV has demonstrated that the bone cells are not productively infected but there remains the possibility that latent infection may have been established in the bone derived cells without expression of pro virus genome. If latent non productive infection has been established it should be possible to detect the estimated 0.1% to less than 0.001% HIV infected cells. It is suggested that two methods could be utilized; either *in vitro* by stimulating the infected cells to express virus with the use of mitogens, or *in vivo*

by examining bone specimens from HIV infected donors with an in situ PCR method. The in situ PCR technique could be applied to multiple bone samples and the results from the *in vitro* studies in this thesis could be used to calculate the number of specimens required.

This thesis has examined important and topical safety aspects of contemporary bone allograft use. Results arising from this work has provided direction for further work in defining bone and cartilage susceptibility to HIV infection. It is anticipated that future developments with the bone cell culture techniques utilized in this thesis will ultimately replace the need for major bone allograft surgery.

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