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VIRUCIDAL ACTIVITY AND BONE INCORPORATION EFFECTS
OF 98% GLYCEROL TREATMENT OR ETHYLENE OXIDE
STERILIZATION OF BONE ALLOGRAFTS:
IN VIVO AND IN VITRO STUDIES

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VIRUCIDAL ACTIVITY AND BONE INCORPORATION EFFECTS OF 98% GLYCEROL TREATMENT OR ETHYLENE OXIDE STERILIZATION OF BONE ALLOGRAFTS: *IN VIVO* AND *IN VITRO* STUDIES

By

George Steven Coronado, Jr.

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ABSTRACT

VIRUCIDAL ACTIVITY AND BONE INCORPORATION EFFECTS OF A 98% SOLUTION OF GLYCEROL OR ETHYLENE OXIDE STERILIZATION ON BONE ALLOGRAFTS: *IN VIVO* AND *IN VITRO* STUDIES

By

George Steven Coronado, Jr.

The feline leukemia virus (FeLV) was used to measure the affect of sterilization with ethylene oxide (ETO) or a 98% glycerol solution on antiviral activity and bone incorporation. FeLV-infected bone grafts were treated with ETO or glycerol and transplanted into 8-week-old specific pathogen-free (SPF) cats and introduced into cell cultures. Blood samples were obtained to monitor FeLV p27 antigen and antibody titers. Quantification of FeLV provirus was performed on blood and bone graft samples by the polymerase chain reaction (PCR). Cell culture and media samples were collected to monitor FeLV p27 antigen and FeLV provirus.

There was no evidence of transmission of the virus to cats or cell culture samples in the ETO groups. Transmission of virus to a cat in the glycerol group was evident, and glycerol-preserved bone samples contained a large amount of amplifiable provirus. Incorporation of bone grafts was similar among all groups.

This work is dedicated to

those cats that gave their lives for

the benefit of this research

...and to my cat and friend, Sid.

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KEYS TO SYMBOLS OR ABBREVIATIONS

AIDS ..... Acquired Immunodeficiency Syndrome

ANOVA ..... Analysis of Variance

AZT ..... 3'-Azido-3'-Deoxythymidine

BMP ..... Bone Morphogenic Protein

C ..... Celsius

cm ..... Centimeter

DMSO ..... Dimethyl Sulfoxide

DNA ..... Deoxyribonucleic Acid

ELISA ..... Enzyme-Linked Immunosorbent Assay

ETO ..... Ethylene Oxide

FEA ..... Feline Fibroblast Cell Line

FeLV ..... Feline Leukemia Virus

FIV ..... Feline Immunodeficiency Virus

FL74 ..... Feline Lymphoblastoid Cell Line

FOCMA .. Feline Oncornavirus Cell Membrane-Associated Antigen

gp70 ..... Envelope Glycoprotein (for Feline Leukemia Virus)

gsa ..... Group-Specific Antigen

H&E ..... Hematoxylin and Eosin

HIV ..... Human Immunodeficeincy Virus

HSV-1 ..... Herpes Simplex Type 1 Virus

IFA ..... Indirect Fluorescent Assay

IM ..... Intramuscular

IV ..... Intravenous

LSA ..... Lymphosarcoma

mrads ..... Megarads

µg/ml ..... Microgram per Milliliter

mg/kg ..... Milligram per Kilogram

mg/kg/h ..... Milligram per Kilogram per Hour

ml ..... Milliliter

n ..... Number (amount)

NaCl ..... Sodium Chloride

$NaHCO_3$ ..... Sodium Bicarbonate

OD ..... Optical Density

p15E ..... Envelope Glycoprotein (for Feline Leukemia Virus)

p27 ..... Major Core Protein (for Feline Leukemia Virus)

PBMC ..... Peripheral Blood Mononuclear Cell

PCR ..... Polymerase Chain Reaction

QPCR ..... Quantitative Polymerase Chain Reaction

S/P ..... Sample to Positive Ratio

SE ..... Standard Error

SIV ..... Simian Immunodeficiency Virus

SPF ..... Specific Pathogen Free

VN ..... Virus Neutralizing

INTRODUCTION

The most common indication for cortical bone allografts in veterinary and human orthopedics is for repair of large diaphyseal long bone defects. The inconvenience of harvesting, processing, storing, and assuring quality has restricted use of allografts in most veterinary practices. Preservation of bone allografts has been limited to ultra-low freezing or treatment with ethylene oxide (ETO). Gamma irradiation also has been used to sterilize and reduce immunogenicity of allografts.[33] However, problems have been encountered with use of these sterilization procedures. One major disadvantage is that ETO may reduce bone induction and incorporation of host bone at the graft site.[3,142] Ethylene oxide also may negatively affect mechanical strength of the bone graft and have a toxic affect on fibroblast activity.[73,119,142,152] As an alternative to sterilization with ETO, canine cortical bone allografts stored in a 98% solution of glycerol appeared to have good incorporation into host bone, although quantification of this assessment was lacking.[27]

Viral and bacterial transmission are possible adverse outcomes when stored tissues are used for transplantation. Concerns that current freezing and storage practices may not be adequate to inactivate retroviruses have been

substantiated by recent studies in animals that investigated viral transmission from retrovirus-infected transplanted allogenic cortical and cancellous bone and connective tissue grafts to recipient animals.[104,105] Specific pathogen-free (SPF) cats had evidence of exposure to (positive results for antibody), or infection with (positive results for antigen), the retrovirus feline leukemia virus (FeLV) by 2 to 6 weeks after implantation of infected allogenic donor tissues that had undergone 1 or 2 freeze-thaw cycles before implantation. These studies were important, because they documented that retroviruses may be transmitted through transplantation of infected bone and connective tissues and that freeze-thaw cycles were inadequate to prevent transmission.[104,105]

Although some biological effects have been investigated,[3,73,142] we are not aware of studies that have examined virucidal properties of ETO on cortical bone allografts. Reports of studies from Europe[27] and South America[27] advocated use of a 98% solution of glycerol for storage of bone and skin allografts. It is stated in other reports[57,97] that a 98% solution of glycerol is bactericidal and virucidal against enveloped and non-enveloped viruses, suggesting that efficacy against human immunodeficiency virus also might be possible. This latter claim was extrapolated from the observation that a solution of 98%

glycerol appears to have in vitro virucidal effects on herpes simplex virus, type 1 (HSV-1) and poliovirus. If proven effective against pathogens, a solution of 98% glycerol would be a simpler, less toxic, and more cost effective alternative to sterilization with ETO.

The purpose of the studies reported here was to compare the antiviral properties and effects on bone incorporation of allografts obtained from FeLV-infected cats, then treated with a 98% solution of glycerol or ETO sterilization. We hypothesized that use of the 98% solution of glycerol would have similar virucidal effects as ETO, but would not be detrimental to incorporation of bone allografts in SPF cats.

LITERATURE REVIEW

Use of cortical bone allografts in human and veterinary medicine

It is estimated that tissues from approximately 5000 cadaver donors are transplanted into more than 220,000 bone or soft-tissue human recipient patients annually in the United States.[11] Many advantages have been found with the use of bone allografts in both human and veterinary medicine. The use of allografts eliminates donor site morbidity, operative time, and length of hospital stay; there is no limit on the size, shape, or quantity of the graft; and most grafts are amenable to long-term storage.[76,95] Cortical bone allografts have been used to replace bone segments affected by tumors, to replace bone lost to trauma or autolysis of bone from cemented prostheses, and for oral reconstruction.[42,91,92,99,114] In addition, cortical bone allografts have been used in spinal surgeries either to assist in fusions or to act as a structural element.[17]

Twenty-five to 35% of patients who receive allograft transplants for limb salvage have a complication within the

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first 3 years after surgery.[131,136] Problems associated with allografts include nonunion of the graft-recipient bone interface, resorption and/or fracture of the graft, and infection. Nonunion is a relatively common event, and it has been proposed that nonunion may represent a subtle form of rejection.[20]

Cortical bone allograft incorporation

Incorporation of cortical bone allografts following implantation is a long process that often is not ever completed. The term "creeping substitution" has been used to describe the process whereby transplanted bone graft is invaded by osteoclasts. Initially, the graft is the focus of an inflammatory response characterized by vascular buds infiltrating the grafted bed. By the second week, fibrous granulation tissue becomes increasingly dominant in the graft bed, the number of inflammatory cells decreases, and osteoclastic activity increases. These osteoclasts channel into the bone graft and create a tunnel known as Howships lacunae. These tunnels become vascularized by capillaries that bring osteoblasts to the area. Osteoblasts are the cells responsible for laying down new bone in concentric layers until the tunnel is filled with new bone. The final

structure formed, containing a central arteriole canal, is known as a Haversian system or osteon.[15,44]

This process of incorporation continues until the bone graft has been replaced with Haversian systems belonging to the recipient. After a full year of bone incorporation, the graft may appear to approach its preoperative mechanical strength. Even then, only approximately 60% of the structure is composed of new bone.[4] Cortical grafts tend to remain a combination of necrotic and viable bone. Incomplete incorporation of bone grafts has been demonstrated by scintigraphy with a decline in scintigraphic activity with increasing distance from the host-graft interface 9 to 367 weeks after cortical bone allograft transplantation.[132]

Until the transplanted graft becomes vascularized, it does not have the potential to respond to loads physiologically by remodeling or by repairing subfailure damage.[110] Initially, as there is an increase in osteoclastic activity and a decrease in osteoblastic activity, the porosity of the bone increases, and the mechanical properties are impaired.[15] Microfractures may occur which are thought to be associated with rapid

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revascularization of the graft with resultant resorption leading to weakness. These microfractures do not necessarily cause harm as they can be filled with mineralized non-lamellar woven bone, and thus may be a possible route for new bone apposition.

Osteoinductive factors, such as bone morphogenic protein (BMP) contained in the cortical bone matrix, also are thought to be released by microfractures and may assist with healing.[41] The released BMP is believed to induce mesenchymal cells to differentiate into cartilage and bone and to stimulate DNA synthesis and cell replication.[72]

Osteoinductive and osteoconductive properties

In general, bone grafts may provide several different functions: osteogenesis, osteoinduction, and osteoconduction. Although cortical bone allografts cannot contribute living cells for osteogenesis, they are capable of osteoinduction in the recipient and of providing structural support for osteoconduction.

Osteogenesis is the process whereby cellular elements within a graft survive the transplantation process and produce new bone in the recipient site. In cortical bone

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allografts, few, if any, cells will survive the transplantation process, and it is generally accepted that cortical bone allografts do not contain osteogenic capabilities.

Osteoinduction is the mechanism whereby nonosseous tissue is influenced to change its cellular function and become osteogenic. For this to occur, there must be an inducing stimulus, a potentially osteogenic cell, and a favorable tissue environment. Although there is some controversy as to how this mechanism is initiated, it is generally thought that the cell responsible for bone formation is the osteoblast. It remains to be established where the progenitors of osteoblasts originate. It has been suggested that the endothelial cells in blood vessels may become osteogenic, however, there also is evidence that cells resembling fibroblasts in the limiting membrane of bone and in surrounding soft tissues may be induced to differentiate into osteoblasts. Another theory is that all somatic cells have the genetic potential for osteoblastic transformation.[4]

The collagen matrix of type I collagen in the bone matrix also may play a significant role in bone

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induction.[117] The geometry of the bone matrix may play a crucial role in determining its suitability as a foundation for osteoinduction. The implantation of a bone matrix powder with particle size 74 to 420 μm resulted in bone formation in one study, whereas matrix with particle size 44 to 74 μm did not induce bone formation. The inability of a fine matrix to induce bone formation was thought to be due to the role of the matrix geometry in triggering the biochemical cascade of endochondral bone differentiation *in vivo*.[117]

Osteoconduction, or "trellis" function, is the process of ingrowth of capillaries, perivascular tissue, and osteoprogenitor cells from the recipient bed into the graft. The bone graft in this case serves as a passive support for ingrowth of blood vessels and subsequent deposition of new bone from the recipient bed.[15]

Immunologic response to bone allografts

Acceptance or rejection of a graft is determined by the presence or absence of alien, genetically determined antigens in the grafted cells, known as transplantation antigens. The genes responsible for formation of these cellular antigens are known as histocompatibility genes,

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and the chromosomal locations of these genes are histocompatibility loci. Class I major histocompatibility antigens are found on virtually all nucleated cells, while Class II antigens are present on the surface of B lymphocytes and other antigen presenting cells. Class I and II antigens have been identified on osteocytes, although it is difficult to correlate the presence of circulating antibodies to these antigens with an adverse clinical or functional outcome. In one study, the highest titers of antibody were found in major histocompatibility complex Class I mismatched animals, and antidonor antibodies were identified 3 weeks after transplantation of a bone allograft. The strongest response was when both Class I and Class II antigens were present.[136]

Recipients of bone autografts have a minimal immunologic response to the graft compared with transplanted bone allografts. This immunologic response to an allograft may result in a reduced osteoinductive process and a slower increase of union strength.[151] The resulting inflammatory environment could potentially interfere with osteogenic cells that eventually will bind the graft in place.

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There are primarily two possible sources of antigen in a cortical bone graft - its cells and its intercellular substance. Since the mineral content of intercellular substances should not differ quantitatively from one animal to another, it has been suggested that the matrix of the transplant, which is composed of glycoprotein, collagen, and mucopolysaccharide, would not be sufficiently antigenic to incite a response.[15]

The most immunogenic element of bone is thought to be the bone marrow. The bone marrow is contained within a rigid cancellous meshwork surrounded by thick dense cortical bone and therefore is effectively sequestrated. The bone marrow of a bone graft is resorbed very slowly by the host, so the exposure of marrow (donor antigen) to host immunocompetent cells occurs over an extended period. This may allow a balance to be established between release of antigen and formation of antibody.[85] It also has been found that marrow-free bone can produce an antigenic response. This would suggest that a major histocompatibility antigen might be present in the bone itself.[4]

One study concluded that since no immunologic response was detected from implanted frozen allografts, the

technique of freezing bone grafts was assumed to kill all live cells. The immunogenic cell in bone was, therefore, thought to be alive to cause an optimal immunologic response. In addition, frozen grafts in a rat model elicited a weaker response and a response in fewer animals than fresh grafts.[136] It also has been found that even with reaming or further irrigation, bone marrow remnants were never completely removed. Therefore, these residual bone marrow cells may incite the immune response.[43]

In a study that measured the immunologic response to bone allografts, humoral cytotoxic antibodies in recipients of fresh allografts could be detected beginning the first week after transplantation. The maximum levels detected were at one and two weeks, the same times when the maximum degree of cellular immunity was observed. Humoral cytotoxic antibodies where shown to persist up to four weeks after transplantation. During this period of observation, an inflammatory reaction was found to be confined first to the periosteum, and later the outer cortex and metaphyseal portion of the medulla also were affected.[85]

One report suggested that cellular immunity is more important than humoral immunity in the destruction of

allografts. Humoral immune responses were judged to be generally insignificant in primary allograft rejection. A possible explanation for this is that although antibodies are formed to bone allografts, they may not be directly involved in the rejection episode. Other rejection factors may include chemotactic and osteoclastic activating factors, release of anaphylactic toxins, and features associated with inflammation, such as vasoconstriction, vasodilation, platelet activation, and thrombus formation.[15]

The incidence, strength, and duration of the recipient antidonor antibody response have been shown to be affected by both graft treatment and size. Massive bone allografts have been shown to elicit a sustained response compared with small cortical grafts.

Concerns of retroviral transmission via allografts in people

In 1989, the reported risk of obtaining an allograft from an unrecognized human immunodeficiency virus (HIV) infected donor was approximately one in 1.6 million. Since then, the epidemic has grown, but intense screening of donors and serologic testing for HIV antibodies, HIV antigen, and the polymerase chain reaction (PCR) for HIV

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have prevented this risk from increasing. Currently, the risk is not believed to be greater than it was in 1989.[11]

Human immunodeficiency virus antibody testing was first implemented for organ and tissue transplantation in 1985.[4] The rapid enzyme-linked immunosorbent assay (ELISA) is the currently used screening test. It is believed that persons who have HIV antibody in their serum are infected with HIV. Following exposure to and infection with HIV, 95% of individuals will have HIV antibody at detectable levels by 6 months.

The HIV antigen is thought to be circulating at detectable levels within 1 to 2 weeks of exposure. This viral antigen is seen to decline with the production of HIV antibodies.[4] The period of time associated with increased antigen levels is believed to correlate with early HIV infection, before antibody production, and may be the most infectious period.[28,137] Various studies have been performed screening blood donors for HIV p24 antigen, but the test failed to significantly demonstrate improved detection of HIV infected persons over the antibody test.[2,4,16,18,130]

The PCR on blood can detect HIV infection before seroconversion, but false negative results were obtained by PCR in about 50% of blood samples in one study.[36] Plasma PCR also has been shown to be less sensitive than leukocyte PCR.[127] Several factors including PCR inhibitors, variability relating to the degree of cellular lysis and site of blood collection might interfere with PCR results. Nested PCR on postmortem skin samples has been shown to detect HIV more reliably than PCR performed on blood. The rationale for performing PCR on skin is the presence of HIV proviral DNA and RNA in epidermal Langerhans' cells isolated from HIV infected individuals.[36] In general, the technical difficulty of performing a PCR has somewhat limited its use.

Coculture of patients' peripheral blood mononuclear cells (PBMCs) and detection of amplified provirus are sensitive methods that identify more than 90% of seropositive subjects. The limitation is that they do not distinguish between integrated nonreplicating provirus (latent infection) and actively replicating virus (persistent infection). In contrast, the presence of circulating virions capable of infecting normal PBMCs, and HIV antigen, does correspond to active (persistent) viral

replication. However, detection of HIV antigen is not as sensitive as HIV culture.[137]

Reports of retroviral transmission through soft tissue and bone allografts

Since 1985, there have been many reports of transplantation associated HIV transmission from seropositive donors to organ recipients.[4] Other reported sources of HIV transmission are from artificial insemination[22] and skin graft transplantation[25,77].

The first reported transplantation-associated transmission of HIV occurred following transplantation of an HIV infected kidney and liver.[21] Since then there have been multiple reports of transmission of HIV through kidney transplantation, some with subsequent development of AIDS.[79,88,113,116,128,155] Two possible mechanisms for transmission of the virus were proposed. Either infected blood in the donor kidney transmitted the virus, or the donor kidney itself was infected with HIV.[88] Infection with HIV also may negatively influence survival of renal allograft recipients. Moreover, these individuals are at risk for HIV-associated nephropathy, with an incidence of 6-10%.[115]

The first reported transmission of HIV following transplantation of bone was in 1988. The recipient was transplanted with an HIV-infected femoral head and was the first person reported to the Center for Disease Control as developing transplantation-associated AIDS.[23] Another report documented two bone recipients and one bone to patellar tendon allograft recipient developing HIV infection after receiving a transplant from an HIV infected donor.[11] Three recipients of unprocessed fresh-frozen bone from an HIV-infected cadaver were infected with HIV. However, another recipient of a femoral allograft from this same cadaver tested negative for HIV-1 antibody. Unlike the other bone grafts, the shaft of the femoral allograft was extensively excavated to permit introduction of a metal rod. It was theorized that the excavation removed most marrow elements and decreased the viral load of this graft. Four other recipients of organs from this cadaver also were infected with HIV-1.[130]

The HIV has been shown to reside in bone itself as well as in blood or marrow elements.[45,125] Fresh-frozen connective-tissue allografts also have been shown to transmit a retrovirus in an *in vivo* study.[104] This is in

contrast to one *in vitro* study which concluded that human bone derived cells are resistant to HIV infection using a cell to cell method. The authors of this study believed that infection arises from nonosteoblastic cells present in whole bone and suggested that methods to sterilize bones should concentrate on inactivation of the virus in blood contaminating the graft.[19]

One study used the PCR to compare the amount of HIV proviral DNA in bones that were processed and unprocessed. Since the amount of proviral DNA was not significantly different between groups, they concluded that the DNA present in processed bone was inactivated and not infectious. This study also concluded that the virus could be inactivated following multiple freeze-thaw cycles. This, theoretically, would cause cell disruption and lysis, and proviral forms of HIV would not survive this cell death.[125]

Another *in vivo* study disproved the efficacy of freeze/thaw cycles by showing that multiple freeze/thaw cycles with a water flush was not sufficient to prevent transmission of a retrovirus through bone allografts.[105] Although freezing allografts during processing was thought

to reduce HIV infectivity, it also is possible that freezing may have a preserving effect on the virus in bone.[142]

There have been no known cases of HIV or other viruses transmitted through freeze-dried tissue grafts. It is unknown if the processing itself or the nature of the tissue may decrease the viral load to zero or a level that is subinfectious.[4] Laboratory studies have demonstrated, however, that in some circumstances neither washing nor freeze-drying inactivate HIV in bone.[11] The ability of heat, sterilization chemicals, and gamma irradiation to inactivate HIV-1 in plasma has been demonstrated, but whether these processes can inactivate HIV in bone without affecting the functional integrity of the allograft is questioned.[130]

Most of these cases of HIV transmission following allograft transplantation occurred before 1985 when current donor screening protocols were implemented. At this time, the theoretical risk of processing bone from an unrecognized carrier of HIV is one in more than one million. However, if adequate precautions are not taken, the risk is as high as one in 161.[10]

Bone allograft harvesting and sterilization procedures

Various techniques have been reported for preservation and sterilization of canine cortical bone allografts.[27,33,73,119] However, their use in most veterinary practices has been restricted by the cost and inconvenience of allograft collection, processing, and storage.

Typical methods used for processing bone allografts include sterile harvesting of the graft followed by low-temperature storage (-20^0 to -40^0 C) or lyophilization (freeze-drying). Frozen bone grafts (at -20^0 to -40^0 C) have a shelf-life of only 6 to 12 months. Freeze-drying grafts has the advantage of allowing storage at ambient temperature, but this technique reportedly decreases mechanical strength of bone.[33]

Secondary sterilization methods can be used to eliminate the need for aseptic harvesting of bone grafts, thereby reducing cost and increasing convenience. Gamma irradiation is an effective sterilization technique for deep penetration of thick tissues, but it requires instruments that may not be available in most veterinary hospitals.

Bacterial infection rates in cortical allografts has been reported to range from 5 to 13% and as high as 12.5 to 31%, in human and veterinary allografts, respectively.[24,95,152] To attempt to eliminate transplantation of infected grafts, an intense protocol of multiple bacterial cultures are performed on each bone graft.[11,92]

Preservation techniques also can affect incorporation of the graft. This may occur by a direct effect of the preservation process on the biologic events of graft union, or an indirect effect on the immune responses against the preserved graft.[14]

Ethylene oxide

Ethylene oxide (ETO) sterilization of cortical bone allografts has become one of the most commonly used techniques. Advantages of ETO sterilization of bone allografts include room temperature storage after sterilization, elimination of aseptic harvesting, bacteriacidal efficacy, and reduced immune rejection.[74,75] Ethylene oxide-sterilized bone allografts can be stored up to one year at -20^0 C without risks of significantly

reducing their resistance to compressive, bending and torsional loads.[146] Although ETO reportedly is an effective bacteriacidal agent, its virucidal properties in tissues have not been documented.

Ethylene oxide is believed to kill bacteria by replacing an available hydrogen atom with a hydroxyl-ethyl radical within a chemical group such as sulphhydral, amino, carboxyl, or hydroxyl in the protein molecule. Proper concentrations of ETO, humidity, and time of exposure are thought to ensure sterility.[73] Alkylation by ETO also is thought to affect microorganisms. Alkylation of bacterial enzymes and viral RNA and DNA purine and pyrimidine bases results in inactivation and possible partial destruction of some etiologic agents.[142]

The concentration of ETO used is thought to affect biomechanical aspects of the bone graft. Use of 84% ETO was found to be superior to 12% ETO. Sterilization with 12% ETO resulted in greater dehydration which can cause the bone to become brittle and susceptible to formation of cracks on the cortical surface.[73] After sterilization with 12% ETO and 1 week storage at room temperature, pullout load or screw-stripping did not change. However, bones

stored for 16 and 32 weeks withstood significantly less compressive and pullout loads than bones stored for 1 week. Whether this decrease in load resistance is attributable to the treatment or the storage temperature was not determined.[119,146] Another study suggests that ETO-sterilization does not have an adverse affect on pullout strength, but this report failed to state the concentration of ETO or storage time used.[131]

Toxicity of ETO and its byproducts, ethylene glycol and ethylene chlorhydrin, and their affects on tissues have been documented, and chemical sterilization with ETO has been associated with recipient morbidity.[74,142] Persistent intraarticular reactions have been reported with ethylene oxide-sterilized allografts. In one report, detectable levels of the residue, ethylene chlorhydrin, was measured by gas chromatography in a bone-patellar tendon-bone allograft as well as in the synovium.[4,64] Ethylene chlorhydrin itself has been demonstrated to cause toxic reactions in biologic tissues. Implanted ETO sterilized bone-patellar tendon-bone allografts also were shown to have a high rate of graft dissolution (22%) which was thought to be due to ETO byproducts.[118] Sensitization with resulting anaphylaxis from ETO byproducts also has been

described.[64] Because of these complications, use of ETO-sterilized bone-patellar tendon-bone allografts has not been highly recommended.[135]

Decreased incorporation associated with ethylene oxide sterilized bone has been reported and is thought to possibly be due to either alkylation of amino acids by ETO in the graft-recipient environments or to its damaging affects on bone morphogenic protein.[142,152] An effect of ETO on collagen cross-linking and ground substance has been suggested.[140] Reduction in bone inductive properties by ethylene oxide has been shown to be dependent upon exposure time. When a short sterilization procedure (5 minutes) was used, ETO did not destroy bone induction properties, but viable bacterial spores were still present within the bone. When exposure time was increased to 240 minutes, no viable bacterial spores were present, but bone induction properties of the implant were absent.[3] Ethylene oxide treated allografts are believed to yield unacceptable results when used for spinal fusion techniques due to decreased incorporation.[17,76]

It also has been suggested that ETO may be carcinogenic, especially with chronic exposure. However,

there is no evidence that ethylene oxide-sterilized allografts have induced cancer.[64,148]

Glycerol solutions

The Swedish chemist, K.W. Scheele, discovered glycerol in 1779. It is syrupy, colorless, odorless, hygroscopic, miscible with water, and has become one of the world's most widely used chemicals. It is used extensively in pharmaceuticals as a solvent, in creams, and as a lubricant in many products such as gelatin capsules and elixirs. The use of glycerol as a preservative for soft tissue grafts was first implemented in 1983 and quickly became the technique of choice for the Dutch National Skin Bank.[57] Burn centers have successfully used glycerol preserved cadaver skin as a short-term biological dressing on wounds with a questionable bed.[7,109,134,153] Preservation with glycerol has been shown to be an inexpensive technique which does not require complicated equipment. Grafts may be stored in a refrigerator at 4^0 C or at room temperature (20^0 C). No significant ultrastructural changes have been observed in skin grafts preserved in glycerol. Glycerol-preserved skin maintains many of the characteristics of

fresh skin, including the collagenous and elastic architecture.[29,98]

One clinical study reported that the inflammatory response seen in glycerol-preserved skin allografts was less than that seen with fresh donor skin. This may be due to the fact that glycerol has been shown to decrease antigenicity of tissues used as transplantation.[56] The antigenic features of glycerol are believed to be associated with its effective lyophilization properties by hygroscopic action. Lyophilization, as in freeze-dried grafts, has been shown to decrease immunologic reactions to allografts. Glycerol, therefore, has been proposed as an effective lyophilization agent that could be used more simply and less expensively than by freeze-drying.[82] Greater growth of capillaries, fibroblasts and autologous epithelium after application of a glycerol-preserved allograft also has been observed. The response shown was comparable to that seen using meshed autografts.[62]

Other advantages of the use of glycerol as a preservative include its effective antibacterial and antifungal properties.[29,67,97] One study investigating preservation of heart valves in a glycerol solution

reported contaminating fungi within a valve and, therefore, questioned the effectiveness of glycerol as a sterilizing agent. However, the concentration of glycerol used was not stated.[107]

Glycerol was reported to have antiviral effects against herpes simplex type 1 virus (HSV-1) and poliovirus type 1. This has been shown to be temperature, time, and concentration dependent. One *in vitro* study showed that these viruses can be inactivated within 24 hours when preserved in 85% glycerol at 37^0 C.[29,147] In another *in vitro* study, an 85% glycerol solution did not fully inactivate poliovirus when stored at 4^0 or 20^0 C for 4 weeks. Conversely, a 98% glycerol solution did not fully inactivate poliovirus at 4^0 C, but did inactivate HSV-1 and poliovirus at 20^0 C after 2 weeks. Because of these results, it was suggested that a standard protocol for the use of glycerol should follow preservation with a 98% glycerol solution at a minimum of 20^0 C for 4 weeks. Although the exact mechanism of inactivation of viruses by glycerol remains unclear, it is possible that it exerts its affect through lyophilization.[38,97] Glycerol's antiviral activity was questioned in a study in which positive viral cultures for HIV were found in skin grafts harvested from

HIV-1 infected human cadavers that were preserved in 30% glycerol.[36] It is possible that this concentration was too low to allow for effective antiviral activity.

Investigations of the virucidal properties of glycerol on transplanted bone grafts have not been described. However, treatment of canine femoral cortical bone allografts with a 98% solution of glycerol at ambient temperature was adequate for storage and resulted in good healing when a bone plate was used for stabilization. Complete periosteal bridging was seen at the graft sites with a continuity of cortices at the host-graft interfaces 90 days after graft implantation.[27]

A high percentage of chondrocytes have been shown to survive when frozen in a 10% glycerol solution compared with cells frozen without glycerol. The mechanism by which this occurs is presumably related to an alteration in nuclear and cytoplasmic membranes as a result of a decrease in the size of ice crystals that may be formed during the freezing and thawing procedure.[95] Because of this effect, osteochondral allografts are commonly preserved by slow freezing in a glycerol solution (usually a 15% solution) as a cryoprotective agent.[11,92,93,94,102] Chondrocytes were found

to survive at a maximal viability of 65% when preserved in glycerol concentrations of 8-12%. Above and below these concentrations, the maximum viability of chondrocytes was never greater than 60%.[143] There also is a debate whether glycerol is a superior cryoprotective agent compared with dimethyl sulfoxide (DMSO).[63,93,94,96]

Other tissues reported to have been successfully preserved in glycerol solutions include vein grafts[139], heart valves[107], and dura mater[6].

Gamma irradiation

Low-level gamma irradiation is routinely used to eliminate surface contamination bacteria. One survey reported that 80% of tissue banks use 1.0 to 3.5 megarads (mrads) of gamma irradiation as a pretreatment before aseptic processing.[148] The International Atomic Energy Agency has adopted a dose of 2.5 mrads of gamma radiation as the standard dose for the sterilization of medical products.[32]

Passage of irradiation through tissue is a two-stage process. Energy is first transferred from photons into high-speed electrons known as Compton electrons. These

electrons release energy as they slow down in the tissue. The dose received by the tissue is related to the damage done by the Compton electrons. These electrons also have a definite 'range' within the tissue, and at depths less than this range the full dose will not be received. This depth is known as the 'build-up' region, which in bone is approximately 2 mm. Bone less than 2 mm thick exposed to gamma irradiation may not receive the full effect.[45]

In a liquid medium, gamma irradiation may affect infectivity of HIV. A low dose of 1.2 to 1.8 mrads was shown to decrease HIV concentration a thousand-fold.[148] In doses greater than 2.5 mrads, viral particles became noninfectious. Viral nucleic acids seem to be radiosensitive in this setting, but conclusive studies on bone and soft tissue models are lacking.[4]

The dose required to completely inactivate HIV in bone allografts is not yet clear. Irradiation with 1.5 megarads may inactivate the virus, although this is disputed. In bone-patellar ligament-bone grafts, 3.0 mrads has been shown to be effective against HIV.[32,45] A dose of 4 mrads has been suggested to be necessary to inactivate the HIV

genome, but biomechanical weakening and collagen alteration becomes the limiting factor at this level.[148]

High-dose gamma irradiation (2.5 to 3.5 mrads) has been shown to cause significant decreases in the breaking strength of irradiated bone. Irradiation below 3.0 megarads has been shown to have few effects on the grafts, but above this level a significant drop in the breaking strength of bone has been observed.[4,110,131] This is thought to occur by irradiation affecting the collagen intermolecular crosslinks in the bone graft and may be mediated by free radicals generated from water molecules, therefore affecting its mechanical stability.[4,44,131] Radiation produces reactive free radicals by the radiolysis of water. These cleave peptide bonds and thereby damage the collagen.[44] This effect has been seen in collagen in skin or tendon as breakdown of molecules into smaller subunits or by disorganization of the secondary structure of the triple helix. Histologically, gamma irradiation induced crimping and separation of collagen fascicles.[145] At standard doses of irradiation, elasticity of bone is unaffected, but the capacity to absorb work and strength are decreased.[45]

Bone grafts are thought to be further compromised if tissues are freeze-dried in addition to being irradiated. Effects of the combination of gamma irradiation and freeze-drying are dependent upon the order of procedures. Initially irradiated, then freeze-dried bone-patellar tendon-bone grafts had a 35% decrease in strength, whereas a freeze-dried then irradiated graft had a 75% decrease in strength.[4,110] Although the combination of freezing and irradiation is detrimental to bone, the effect of freezing is thought to give partial protection against embrittlement in comparison with irradiation at room temperature. It is possible that the affect of highly-reactive oxygen free radicals, produced by irradiation from the radiolysis of water, is decreased by irradiating when frozen.[44]

Treatment with gamma irradiation also is believed to reduce immunogenecity of the allograft.[69,81,86] Lymphocytes are very radiosensitive cells, and exposure to low-dose radiation produces almost complete destruction of lymphoid tissue, with suppression of immunological capacities.[34,78] It is thought that the DNA of the cell is the principal target for radiation-induced cell lethality. Therefore, proliferative cells would seem more sensitive to the effects of irradiation than resting cells.[40]

FeLV as a model for other retroviruses

The human immunodeficiency virus (HIV) is a retrovirus belonging to the lentivirus subfamily. It is designated as human immunodeficiency virus type 1 (HIV-1) or human immunodeficiency virus type 2 (HIV-2). The only animals susceptible to experimental HIV-1 infection are the chimpanzee, gibbon ape, and rabbit, but AIDS-like disease has not yet been reported in these species. The limitations of using simians to study HIV are the practicality, cost, safety, animal welfare, availability of the animals, and appropriate animal facilities.[37]

During the replication of a retrovirus such as the human immunodeficiency virus, the single-stranded RNA genome is copied by a preformed viral enzyme, reverse transcriptase, into a complementary DNA form, which then is converted to double-stranded DNA, called provirus. The proviral DNA then integrates within the chromosomal DNA of the infected cells of the host, resulting in viral replication whenever the host cell divides. The provirus can be detected using several molecular biological techniques.[32]

W.F.H. Jarrett first discovered FeLV in 1969 as a naturally occurring retrovirus. Since then it has become one of the most studied retroviruses affecting outbred species. Over 2% of the cat population in the United States is thought to be infected with FeLV, and resulting disease processes are responsible for most deaths in this species.[123,141]

FeLV is a retrovirus that belongs to the type C Oncornavirinae subfamily. The FeLV-associated immunodeficiency results in an acquired immunodeficiency disease (AIDS)-like syndrome similar to human AIDS and makes the feline model attractive for retroviral studies.[129] The effect of 3'-azido-3'-deoxythymidine (AZT) on the activity of retroviruses has been studied using FeLV as a model.[141] The efficacy of the drug Zidovudine, the first antiviral drug to be approved for AIDS therapy, was studied using FeLV to test its antiretroviral activity.[35]

Although FeLV differs from HIV in terms of its host, the mechanism of tumorogenesis, and the primary route of natural infection, it serves as a well-documented comparative model for the study of HIV.[48] Both viruses share a similar structure and replication cycle. Both lead

to an infection in which incorporated retroviral DNA results in the production of infectious virus particles by the host cell. The basic similarities and available reliable tests make FeLV an effective model to study retroviral transmission through transplantation.[104] Although HIV and FeLV have different genomic structure and resultant infections represent distinct retrovirus/host relationships, there are many similarities in their infectious activity. Infections with both retroviruses require close contact and transfer of secretions or blood for contagious transmission. Both infections are characterized by sequential stages in the progression of infection and disease and produce T-cell depletion *in vivo*. Both retroviruses manifest viral envelope glycoprotein heterogenicity, contain virus strain-related variations in tissue tropism and pathogenicity, and exhibit viral latency and activation. The pathogenic potential of the virus within an FeLV-induced AIDS strain suggests the possibility that a similar diversity may exist in other retroviruses such as within HIV.[103] Subtle changes in the envelope glycoprotein (gp70) of FeLV can convert a minimally pathogenic virus into one that induces an acute form of immunodeficiency.[58] It is thought that these changes may similarly occur in HIV-infected people.[37]

The feline immunodeficiency virus (FIV) is also a retrovirus, but belongs to the subfamily Lentivirinae. It is structurally and biologically similar to HIV and is associated with immunosuppression in domestic cats. Besides the simian immunodeficiency virus (SIV), FIV is one of the more closely related viruses to HIV.[108] Although FIV belongs to the same lentivirus subfamily as HIV, it is not antigenically related.[37] The prevalence of anemia, lymphopenia, neutropenia, and thrombocytopenia associated with FIV infection is similar to that seen in HIV-seropositive patients with AIDS. Use of this feline virus also could provide an understanding of marrow suppression from lentivirus infections and/or the hematologic effects of new therapies.[129]

Feline infectious viruses are good models for HIV infection in man. Cats are well-established research animal and almost all vivaria are equipped to handle them. They are relatively inexpensive to obtain, easy to handle, and naturally infected cats with AIDS-like disease could be recruited for therapeutic trials.[103,108]

Infectivity of FeLV

The pathogenesis of FeLV in experimentally and naturally infected cats has been desribed.[122,141] The major vehicle for transmitting the virus in nature is probably blood and possibly saliva. Biting enables direct injection of the virus, while licking may permit infection of cats via the ocular, oral, and nasal membranes.[53] Infection of local lymphoid tissue associated with the inoculation site occurs first where replication is amplified. Infected circulating mononuclear cells spread to various systemic lymphoid tissues. Virus replication can be detected in the bone marrow by 14-21 days after infection. After this point, infection is amplified by spreading circulating leukocytes and platelets and persistent (chronic) viremia becomes established. It is thought that the presence of antigen in circulating leukocytes and platelets reflects predominantly infection of hematopoietic precursors in bone marrow rather than phagocytosis of circulating virus or viral antigens.[60] Infection then can spread to epithelial tissues (28-56 days after infection). The virus spreads to and replicates in salivary glands and respiratory epithelium from where it is shed and may be transmitted to other cats.[53,58]

Continued FeLV replication in hemolymphatic tissues over a period of months to years leads to multiple proviral integrations within target cell genomic DNA and to progressive immunosuppression and lymphoid and myeloid cell depletion. Infection with FeLV may lead to death or illness shortly after the onset of viremia (4 to 8 weeks), as a result of leukopenia and acute immunosuppression.[58]

Most of the time (98%), the ability to recover infectious FeLV from the blood coincides with the presence of FeLV group-specific antigens (gsa), mostly corresponding to the FeLV major core *gag* gene-encoded protein, p27, in circulating neutrophils and platelets.[122] A *pol* gene also is present which codes for the viral RNA-dependent DNA polymerase (reverse transcriptase), the enzyme responsible for copying the viral RNA into DNA and permitting virus replication. The *env* gene encodes the viral envelope components gp70 and p15E. In contrast with envelope antigens, the FeLV internal core antigens are identical for all subgroups of FeLV and are, therefore, termed group-specific antigens.[50]

The property of the virus envelope glycoprotein is the basis for assignment of FeLV into subgroups A, B, and C.[58] Isolates of FeLV can be assigned to one of these three subgroups based on their susceptibility to neutralizing antibodies. The virus subtype also may play a role in the extent of virus replication. The subtype FeLV-A is believed to be most important in induction of viremia and in induction of latent infection, and it is present in 100% of infected cats.[53] The subtype FeLV-B is thought to enhance the ability of FeLV-A to cause a persistent viremia. The FeLV-C subtype is generally inefficient in establishing viremia except in newborn cats and is only found in association with FeLV-A in nature. The FeLV-C subtype is rare, in as few as 1% of viremic cats, and is associated with aplastic anemia.[103,122] The connection of FeLV-C with severe erythroid aplasia and nonregenerative anemia is the most consistent link of a specific FeLV subgroup with a specific disease.[58]

The crucial point for reversibility of FeLV-induced disease is infection of the bone marrow. Once this stage is reached, the viral genome integrates into progenitor cell DNA, and all cells originating from these precursors will be FeLV-infected. The actual time course depends upon

the virulence, dose, and speed of the emerging antiviral immune response. Cats which have an adequate antiviral immunity may develop an immune or latent infection. FeLV replication and virus expression is restricted in these cases. These cats usually have not fully eliminated the virus, but have a persistent, low-grade, latent, nonexpressed infection. Although onset of viremia of marrow origin usually signals the establishment of persistent infection, in rare cases some cats are able to reverse this state by clearing FeLV-infected cells and producing virus neutralizing (VN) and, frequently, feline oncornavirus-associated cell membrane associated antigen (FOCMA) antibodies. Viral antigens released during early stages by infected lymphocytes (1-14 days after infection) appear to stimulate the immune system to produce neutralizing antibodies to FeLV envelope glycoproteins.[122,141] In cats that fail to develop VN antibody, FeLV infection extends to multiple epithelial tissues.[47,52,121] If the immune system of the infected cat responds to the viral envelope antigens, it will produce antibody that can neutralize the infecting strain of virus. If high titers of this antibody are produced soon after FeLV infection, the cat may be able to reject the infecting virus and become immune to further infection.[53]

A majority of cats exposed to FeLV resist and recover from infection and establish a strong immunity to FeLV. This transient infection often results in latent nonviremic infections.[84] Transition to the fully viremic state is accompanied by a decrease in virus-neutralizing antibody titer to undetectable values, suggesting that immunologic control of latent infection has failed.[71]

After infection with FeLV, two events usually occur. Either an antigen-positive state develops with no development of virus-neutralizing antibody and variable development of titers against FOCMA, or an antigen-negative state is maintained and usually accompanied by both virus-neutralizing and FOCMA antibodies. Transiently infected cats usually remain negative for FeLV antigen and develop both FOCMA and VN antibodies.[121] Once the antigen-positive state is achieved, development of FeLV-related diseases and capability for contagious transmission results.[60]

Resistance to viremia has been shown to be acquired with increasing age and maturity of the cat's immune system. One study showed that 100% of neonatal cats developed persistent viremia after parenteral exposure.

Seventy to eighty-five percent of weanling cats developed viremia, while only 15-30% of mature cats (≥ 4 months old) developed persistent viremia.[122] In those cats older than 4 months, an immunizing effect was shown without antigenemia or viremia being detected.[59]

Resistance to FeLV infection in adult cats usually is characterized by restriction of virus replication in lymphohematopoietic cells, transient lymphopenia, and induction of immunity to FeLV and freedom from FeLV-related disease. The age-related resistance to viral infection has been shown to be macrophage associated. Macrophages from kittens replicate more infectious FeLV *in vitro* than do macrophages in adult cats.[120] Infection with FeLV is predominantly centered in the myelomonocytic precursors of the bone marrow. Young cats have three times as many lymphoid precursor cells in the bone marrow and twice as many peripheral blood lymphocytes as do adult cats.[84] These cells could represent an increased population of potential target cells for FeLV.[121]

One study showed that 54% of feline lymphosarcomas were positive for FeLV virus by immunohistochemistry, and 74% of the tumors were positive for FeLV DNA using the

PCR.[67] A resistance to the development of FeLV-induced lymphomas, leukemias, and myeloproliferative diseases is thought to be dependent on the development of antibodies to FOCMA. The FOCMA may actually represent endogenous FeLV antigens expressed on the surface of hematopoietic and lymphoid cells transformed by FeLV. The FOCMA also has been associated with feline lymphosarcoma, myelogenous leukemia, and multicentric fibrosarcoma cells.[49] It has been suggested that these endogenous antigens are related to FeLV-B *env* glycoproteins, whereas others believe that FOCMA is more closely related to FeLV-C *env* glycoproteins.[122] A positive FOCMA antibody titer has been thought to be beneficial because it may protect the cat against FeLV-associated neoplastic processes. However, persistent antibody titers to FOCMA have been linked with ongoing low-activity FeLV infection in nonviremic cats.[138]

A correlation between high FOCMA antibody titers and the resistance to lymphosarcoma (LSA) development has been shown in FeLV inoculated cats. All cats that developed LSA had low or nonexistent FOCMA antibody titers. Many healthy cats have low titers of antibody to FOCMA indicating that they were exposed to or transiently infected with FeLV at some time in their lives. Many of these cats, however, may

not have protective titers of FeLV neutralizing antibody. The FeLV-infected cats with protective FOCMA antibody titers are resistant to LSA but are still susceptible to all of the non-neoplastic FeLV diseases.[53]. The only group of cats which exclusively have negative FOCMA antibody are specific pathogen free (SPF) cats. However, positive antibody titers to FOCMA tend to wax and wane and have been associated with an increased prevalence of disease.[138]

Another form of FeLV infection may be one in which a focus of infection is present in the cat resulting in the presence of serum FeLV p27 antigen without GSA in peripheral blood leukocytes and platelets. These cats also often have a high anti-FOCMA antibody titer.[90]

Testing methods for FeLV

The p27 antigen is a 27,000-dalton protein present in the core of FeLV viruses and is the major antigen detected by the enzyme-linked immunosorbent assay (ELISA) test.[106] Because the p27 antigen is present in blood cells, using blood rather than serum may result in larger amounts of antigen detected in the assay.

The ELISA test may be able to detect transient infections because in this case the virus grows in lymphoid tissue, but not in the bone marrow. Therefore, the ELISA test can detect free antigen while there are no neutrophils containing viral antigen.[70]

Other ELISA assays for viral antigen may use serum, saliva[87,89], or tears[54]. Serum has been shown to be positive earlier than saliva because salivary glands become infected only after viremia is established. Cats without FeLV antigen in saliva may not be viral shedders yet and may still potentially eliminate the infection.[87] A 95.5% agreement was shown between serum and saliva results.[89] The saliva ELISA is not as sensitive as the serum ELISA, but it may be used in some cases to determine virus shedding.[87]

Because of the specificity of most ELISA kits, a negative test may be a good predictor that a cat is not infected, but a positive test should be interpreted with caution.[68] One study reported that there is an 86.9% probability that a negative ELISA result is correct, while there is only a 46.3% probability that a positive ELISA result is correct.[51] The most reliable ELISA test results are obtained when serum or plasma is tested. Slightly less

reliable results are obtained when blood is tested, and least reliable results occur when saliva or tears are tested.[106,144] The sensitivities and specificities of the various FeLV ELISA tests are uniformly high (95-100%) when using serum or plasma. The specificity decreases when testing saliva or whole blood.[5]

In contrast with the ELISA assay which detects p27 antigen in the fluid of blood, saliva, or tears, the indirect fluorescent assay (IFA) test detects cell-associated antigen within leukocytes in blood smears. Although the IFA test may be negative during a transient or latent infection, once the infection enters the bone marrow, the IFA test becomes positive with infected neutrophils and platelets released into circulation.[51] A positive IFA result is highly predictive of persistent FeLV infection. The IFA test is performed on whole blood or buffy coat smears which are examined using fluorescent dye-labeled anti-p27 monoclonal antibodies. Infected cells fluoresce when stimulated by light of the appropriate wave length.[5] The IFA test has been considered the reference standard FeLV test.[50,51,80]

The assay for FOCMA antibodies is an indirect membrane immunoflourescence assay in which the test cat serum serves as the source of primary antibody, a fluorescein-labeled anti-cat antibody serves as a secondary antibody, and an FeLV-infected, feline lymphoblastoid cell line (FL74) serves as the target cell. The test for antibody against FOCMA is used to identify the presence of antibodies in plasma and is indicative of exposure to the virus. Cats that develop FOCMA antibody titer >1:8 are thought to be protected against tumor development.[12] Healthy persistently viremic cats do not have detectable amount of antibody to the viral structural proteins, but can develop protective antibody titers to FOCMA in some cases. The amount of humoral antibodies to FOCMA present in cats is inversely correlated with tumor progression.[88]

An association between cats with positive FOCMA antibody titers and a history of disease has been reported. Lower FOCMA antibody titer in young diseased cats was thought to be related to immunosuppression resulting from transient, latent, or low-activity FeLV infection.[138] More specifically, FOCMA was reported to have specific reactivity to the envelope glycoprotein (gp70) FeLV C.[133,150] The FOCMA is thought to be virus encoded and not a tumor-

specific antigen. It binds to nascent but not mature virus particles.[150]

One study reported antibodies to FOCMA in kittens that nursed from mothers who previously nursed kittens injected with FeLV. This showed that the mothers were infected with FeLV but were able to mount an immune response which resulted in the production of humoral antibody.[31]

The polymerase chain reaction test is a powerful technique in which as little as one copy of a gene in chromosomal DNA can be amplified to yield as many as 10^6 or 10^9 copies that can be easily detected.[32] The test can be particularly useful in instances where infectious virus particles are nonreplicating or are present in very low numbers. The use of the PCR has been useful in identifying FeLV proviral DNA in many lymphosarcomas in cats that are otherwise antigen negative.[65,66]

A positive PCR result on a blood sample indicates the presence of FeLV proviral DNA in peripheral blood leukocytes irrespective of the transcriptional activity of the proviral DNA. Therefore a positive result by the PCR test does not necessarily indicate viremia. Although the

PCR has been shown to correlate well with the ELISA assay, discordant results have been found.[101]

One study compared the results of detection of FeLV antigen by ELISA and detection of FeLV DNA by PCR in peripheral blood samples. The study did not find a significant difference in detection of infected cats. Therefore, the authors suggested that the PCR may be more suitable to explore the question of a latent or replication-defective FeLV infection in an antigen-negative cat using tissues other than peripheral blood.[66]

BONE INCORPORATION AND VIRUCIDAL EFFECTS OF A 98% SOLUTION OF GLYCEROL OR ETHYLENE OXIDE STERILIZATION ON BONE ALLOGRAFTS IN CATS

MATERIALS AND METHODS

Harvesting and preparation of bone allografts

Four weeks before implantation, 5 metatarsal bones from an uninfected SPF cat plus 3 groups of bones (n = 5 bones/group) from 5 SPF cats (3 bones from each cat) infected with the Rickard strain of FeLV were aseptically harvested and stored fresh-frozen at -70^0 C. The Rickard strain was chosen to expand upon previous studies using this strain[8,9] and because most cats infected with this strain will not exhibit disease during the 8 week study period.

Bones harvested from the uninfected SPF cat (negative-control allografts) were thawed, placed into separate sterile plastic conical tubes, and refrozen at -70^0 C (Figure 1). The 3 metatarsal bones from each of the FeLV-infected cats were assigned to 1 of 2 FeLV-infected

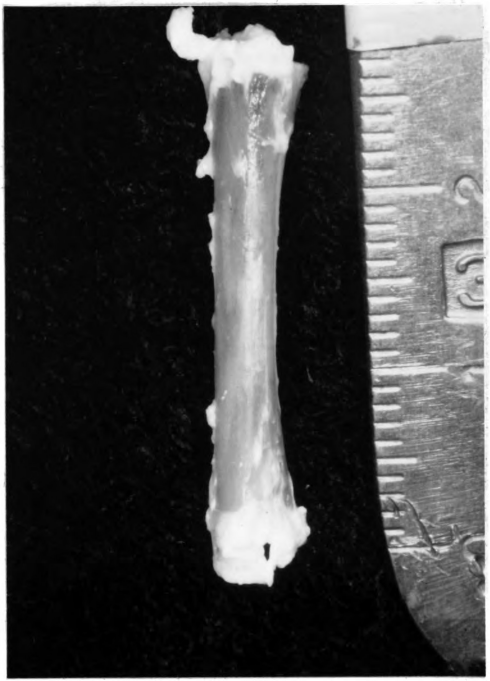

Figure 1: Photograph of a harvested metatarsal cortical bone allograft. The metaphyseal segments of the bones were removed before implantation and saved for quantification of FeLV provirus.

treatment groups or to the positive-control group. After thawing, intact bones for the ETO group were separately double-wrapped by use of heat-sealed plastic and sterilized with 100% ETO. After ETO sterilization, bone samples were aerated at 50^0 C for 12 hours (relative humidity, 30%) and refrozen at -70^0 C. Bones in the glycerol group were placed separately into conical centrifuge tubes that contained a 98% solution of glycerol, then were stored in the dark at room temperature (22^0 C) for 4 weeks.[97] Bones from the positive-control group did not undergo treatment and were placed separately into sterile plastic conical tubes and refrozen at -70^0 C. In addition, a small piece of the diaphyseal portion of the ulna of a FeLV-infected cat was aseptically harvested, placed into a sterile plastic tube, and stored at room temperature (22^0 C) for 4 weeks to test the effects of ambient temperature without treatment.

Recipient Animals

Twenty 8-week-old SPF cats were randomly allocated to 4 groups (n = 5 cats/group). Young cats were used because of their documented age-related susceptibility to FeLV-infection.[121] The animal-use protocol was approved by a University Animal Care and Use Committee. Cats were housed

separately in cages for 1 week to enable them to acclimate before implantation. Each cat in group 1 (negative-control group) was implanted with 1 cortical bone allograft from an uninfected cat. Each cat in group 2 (ETO group) received 1 ETO-sterilized cortical bone allograft obtained from a FeLV-infected cat. Each cat in group 3 (glycerol group) received 1 glycerol-preserved cortical bone allograft obtained from a FeLV-infected cat. Each cat in group 4 (positive-control group) received 1 untreated cortical bone allograft obtained from an FeLV-infected cat. Before and after implantation surgery, all cats were handled separately in accordance with a SPF protocol (coveralls, gloves, hats, masks, and booties), and infected cats were handled last. Gloves were disinfected with bleach between cats within the same groups and were changed between groups of cats.

Surgical technique

Each cat was weighed, medicated with a combination of acepromazine maleate (0.03 mg/kg of body weight, IM) and butorphanol tartrate (0.1 mg/kg, IM), and given antibiotics (cefazolin; 22 mg/kg, IV) prophylactically. Cats were induced using a mask, intubated, and maintained throughout

surgery with halothane in oxygen. Fluids (lactated Ringer's solution; 10 ml/kg/h, IV) were administered during anesthesia.

The right or left ulna of each cat was randomly selected for implantation with a diaphyseal segment of bone allograft. A routine approach to the caudal ulna was performed,[112] and a periosteal elevator was used to elevate the periosteum from the exposed mid-diaphysis. A 1-cm mid-diaphyseal ostectomy was performed on the selected ulna, using Lembert roungeurs. The open wound was lavaged and packed with saline (0.9 % NaCl)-soaked 4 X 4 gauze sponges during preparation of the cortical bone allograft. During surgery, the donor allograft was placed in sterile saline solution to equilibrate it to ambient temperature. The graft was then cut to a length of 1 cm, using a No. 10 scalpel blade (Figure 2). A 0.07-cm in diameter piece of Kirschner wire was driven retrograde into the proximal segment of the ulna through the olecranon until the end of the pin apposed the edge of the proximal ostectomy site. After the bone graft was oriented and positioned in the ostectomy site, the Kirschner wire was driven normograde through the bone graft and into the distal metaphyseal segment (Figure 3). The Kirschner wire was cut close to

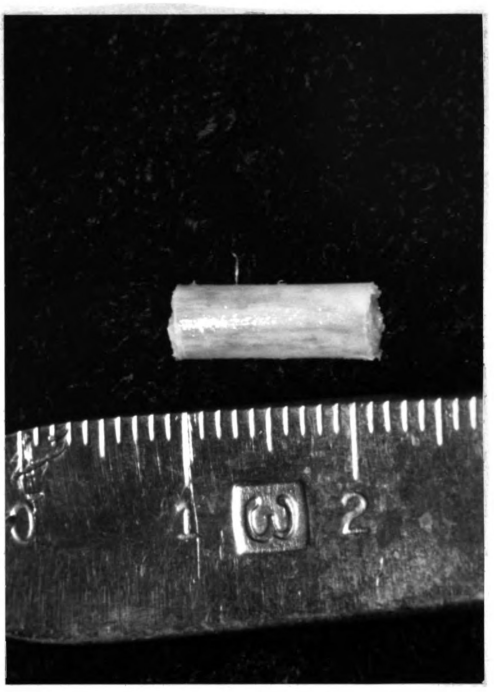

Figure 2: Photograph of a metatarsal cortical bone allograft ready for implantation. The metaphyseal segments were removed with a No. 10 scalpel blade, producing a 1 cm segment of bone.

Figure 3: Intraoperative photograph of a cortical bone allograft implanted into the middiaphyseal segmental ulnar ostectomy site.

the skin, using wire cutters, and the skin was pulled over the end of the wire. Fascia and skin were closed in a routine manner, using 4-0 polydiaxanone and 4-0 nylon, respectively.

The remaining pieces of bone graft were saved for subsequent quantification of FeLV provirus. Radiographs were obtained after surgery to assess placement of the graft and Kirschner wire (Figure 4). Butorphanol (0.1 mg/kg, IM) was given to each cat for analgesia after surgery. Cats were monitored for 8 weeks after transplantation to allow for virus replication within the host, then were euthanatized by use of an overdose of pentobarbital (87 mg/kg, IV).

Collection of blood samples

A blood sample was collected from each cat before surgery and placed into an EDTA-containing tube. Samples were tested to verify an FeLV negative status. Serial blood samples (3 ml) were collected weekly from each cat starting 2 weeks after surgery. When necessary, cats were sedated with a combination of ketamine hydrochloride (10

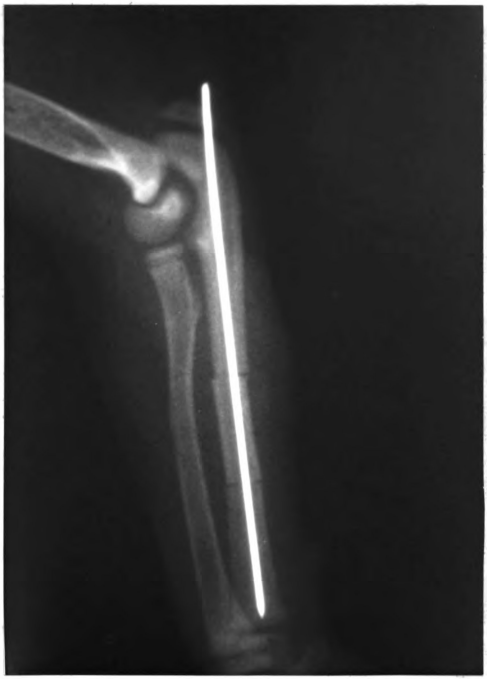

Figure 4: Postoperative lateral radiograph of a radius and ulna. The cortical bone allograft was stabilized into the ulnar ostectomy site using a 0.028 inch Kirschner wire.

mg/kg, IM), midazolam (0.2 mg/kg, IM), and butorphanol (0.1 mg/kg, IM).

FeLV p27 antigen

The enzyme-linked immunosorbent assay (ELISA) was used to test for FeLV p27 antigen in plasma samples obtained at weeks 0 and 2 through 8. An ELISA microplate reader was used to quantify antigen on the basis of optical density (OD). The sample-to-positive control ratio (S/P) was calculated, using the following formula:

$$\frac{\text{(OD of sample)} - \text{(OD of negative-control sample)}}{\text{(OD of positive-control sample)} - \text{(OD of negative-control sample)}}$$

Calculated S/P values of ≥ 0.1 were considered positive.[101]

FeLV antibody

Antibody titers to feline oncornavirus cell membrane-associated antigen (FOCMA) were measured in plasma samples at weeks 0 and 2 through 8, using a live-cell immunofluorescence assay, as described elsewhere.[101] Antibody titers of ≥ 1:16 were considered positive.

Extraction and quantification of DNA

The DNA was extracted from buffy coats of anticoagulated blood samples collected from each cat at weeks 0, 4, and 8, using a DNA extraction kit. Remaining sections of bone graft stored at the time of surgery, as well as the bone sample stored untreated at ambient temperature, were ground to powder, using a freezer-mill. The DNA was extracted from each sample of bone powder by use of a phenol-chloroform technique[126]; extracted solutions were electrophoresed through a 1% agarose gel and stained with ethidium bromide.

The DNA extracted from blood and bone samples was quantified, using a DNA flourometer. Samples of DNA were digested with the restriction endonuclease EcoR1, electrophoresed through a 3% agarose gel, and stained with ethidium bromide to confirm detection and uniformity of DNA in each sample. Digested DNA samples were stored at 4^0 C in sterile microfuge tubes.

Although all bone grafts underwent the same process of DNA extraction, a low quantity of DNA was obtained from

ETO-sterilized bone grafts, impeding quantification of FeLV provirus. Consequently, larger bone samples were collected from the ulna of the corresponding limb of each donor cat. These samples were subjected to ETO sterilization, DNA extraction, and quantitative polymerase chain reaction (QPCR).

FeLV provirus

A QPCR assay was used to quantify FeLV proviral DNA in 100 ng of digested DNA extracted from blood and bone samples. Negative and positive calibration standards were assayed in parallel with test samples. Other PCR assays for detection of FeLV provirus have been reported.[66,101]

Radiographic and histologic evaluation of implanted limbs

After recipient cats were euthanatized, each implanted ulna was harvested and placed in neutral-buffered 10% formalin (Figure 5). High-resolution radiographs were taken of each harvested ulna, using a faxitron and kodalith-ortho film. Each ulna then was decalcified, using 5% nitric acid, and sections were prepared and stained with H&E for histologic examination. Subjective histologic

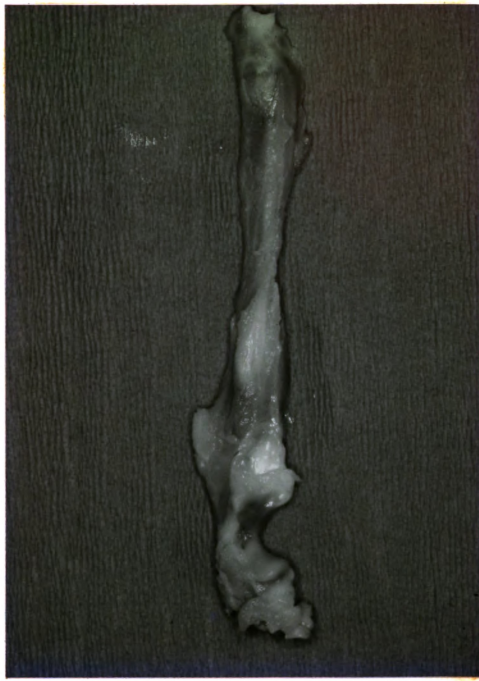

Figure 5: Photograph of a harvested implanted ulna after euthanasia 8 weeks after implantation. All grafts had completely healed in all of the groups.

analysis was performed to evaluate host-graft interfaces, healing of the bone graft, and incorporation of the bone graft by the host. Analysis was concentrated at the proximal and distal host-graph interfaces. Amounts of periosteal overgrowth, dead graft bone (identified as empty lacunae), incorporation of host bone into graft bone, and inflammatory cells were evaluated.

RESULTS

Cats

At the time of implantation, cats were 6.4 to 8.0 weeks old (mean, 7.2 weeks) and weighed from 0.45 to 0.90 kg (mean, 0.68 kg). At the time of euthanasia, cats weighed from 1.36 to 1.80 kg (mean, 1.52 kg).

Surgical outcome

All cats recovered from anesthesia without complications. Nine cats had bone allografts implanted in the left ulna, and 11 cats had bone allografts implanted in the right ulna. On the basis of examination of radiographs taken immediately before and after surgery, all bone grafts

had good apposition within the ostectomy site, and the position of the Kirschner wire was satisfactory, except for 2 cats (1 in group ETO and 1 in group glycerol). The pin was placed medially to the distal portion of the ulna in the cat in group ETO. That cat recovered well and was not lame. The cat in group glycerol was reanesthetized, and the pin was repositioned because it had passed into the carpus. That cat and 2 other cats were lame in the affected limb for 1 week after surgery but subsequently used the limb appropriately. Complications with nonunion or malunion were not observed, and all grafts were incorporated into host bone. All 20 cats eventually used the grafted limb well. One cat in the positive-control group had early signs of carpal valgus at week 2; this condition slowly progressed during the duration of the study. One cat in the glycerol group developed a seroma at the olecranon area of pin placement at week 7, which subsequently resolved over a few days. Another cat in the glycerol group developed acute lameness of the implanted limb at week 8. That cat did not exhibit signs of pain during manipulation of the limb; however, a transverse fracture of the radius and ulna at the distal graft site was discovered when the limb was examined after the cat was euthanatized. We hypothesized that fractures of the radius and implanted ulna were

attributable to trauma from the cage, because adequate incorporation of the graft was evident at proximal and distal host-graft interfaces, and a bent Kirschner wire was evident on radiographs of the limb.

Health status of cats

Six cats developed transient vomiting or diarrhea during the study (2 cats from the negative-control group, 3 from the ETO group, and 1 from the positive-control group). Five cats developed transient sneezing or coughing (1 from the negative-control group, 1 from the ETO group, and 3 from the positive-control group). All but 1 of the cats were in good general health throughout the study. One of the cats in the positive-control group was given antibiotics because of a respiratory tract infection at week 4. That cat initially responded to treatment, but relapsed and died at week 6. We hypothesized that the cat was immunocompromised as a consequence of FeLV infection.

FeLV p27 antigen

All cats had negative results when tested for FeLV p27 antigen just before surgery (week 0). Cats in the negative-

control and ETO groups had negative results for FeLV p27 antigen throughout the entire 8 week study. One cat from the glycerol group had positive results for FeLV p27 antigen at weeks 5 and 6, then reverted to negative results at weeks 7 and 8. All positive-control cats had positive results for FeLV p27 antigen at weeks 2 and 3. Two of these cats reverted and had negative results for the remainder of the study, whereas the other 3 positive-control cats had positive results throughout the remainder of the study, including the cat that died at week 6 (Table 1).

FeLV antibody

All negative-control cats were seronegative to FOCMA at week 0 and remained seronegative for the remainder of the study (Table 2). One of the cats in group ETO had low titers (1:16) from weeks 3 through 8, but all other cats in that group were seronegative to FOCMA. One cat in the glycerol group had moderate antibody titers to FOCMA (1:32 to 1:128) at weeks 6 through 8. Four of the cats in the positive-control group had positive titers (range, 1:16 to 1:128) at week 2. All cats in the positive-control group had moderate to marked antibody titers to FOCMA from week 3

through the remainder of the study (range of titers at week 8, 1:128 to 1:2,096).

FeLV provirus in blood samples

All cats had negative results when tested for FeLV proviral DNA by QPCR before surgery (week 0). All cats in the negative-control and ETO groups had negative results for FeLV proviral DNA throughout the study. One cat in the glycerol group had positive results for FeLV proviral DNA at weeks 4 (257 copies) and 8 (8,729 copies), whereas all other cats in the glycerol group had negative results for FeLV provirus. All positive-control cats had detectable FeLV proviral DNA at weeks 4 (range, 2,772 to 468,021 copies; mean, 204,428 copies) and 8 (range, 661 to 521,192 copies; mean, 243,178 copies). Three cats in the positive-control group had decreased proviral loads, and 1 cat had an increased proviral load, from weeks 4 to 8. The remaining cat in the positive-control group died at week 6 (Table 3).

Table 1: Positive FeLV p27 antigen results in blood samples from cats after implantation with a cortical bone allograft.

Number of Cats with Positive FeLV p27 Antigen Results								
	Week after implantation							
Group	0	2	3	4	5	6	7	8
Negative - control (n =5)	0	0	0	0	0	0	0	0
ETO (n = 5)	0	0	0	0	0	0	0	0
Glycerol (n= 5)	0	0	0	0	1 (0.05)	1 (0.11)	0	0
Positive-control (n = 5)	0	5 (0.27)	5 (0.56)	3 (0.60)	2 (0.39)	3 (0.55)	2* (0.62)	2* (0.84)

* n = 4. Negative-control group = untreated allografts from uninfected SPF cats. ETO group = ethylene oxide-treated allografts from FeLV-infected cats. Glycerol group = 98% solution of glycerol-treated allografts from FeLV-infected cats. Positive-control group = untreated allografts from FeLV-infected cats. Values reported as number of blood samples within a group with positive results (and mean S/P ratio).

Table 2: Antibody titers to feline oncornavirus cell membrane-associated antigen (FOCMA) ≥ 1:16 in blood samples from cats following implantation with a cortical bone allograft.

Number of Cats with Antibody Titers to FOCMA ≥ 1:16								
	Week after implantation							
Group	0	2	3	4	5	6	7	8
Negative control (n=5)	0	0	0	0	0	0	0	0
ETO (n=5)	0	0	1 (1:16)	1 (1:16)	1 (1:16)	1 (1:16)	1 (1:16)	1 (1:16)
Glycerol (n=5)	0	0	0	0	0	1 (1:32)	1 (1:128)	1 (1:128)
Positive control(n=5)	0	4 (1:16-1:128)	5 (1:64-1:128)	5 (1:64-1:128)	5 (1:64-1:512)	5 (1:128-1:2096)	4* (1:128-1:1024)	4* (1:128-1:2096)

Titers ≥ 1:16 were considered positive. *See* Table 1 for key. Values reported indicate number of cats (range of titers).
* n = 4.

Table 3: Quantitative polymerase chain reaction test results in blood and bone graft samples from cats that received a cortical bone allograft.

Group	Copies of FeLV Provirus			
	Blood samples (week after implantation)			Bone samples*
	0	4	8	
Negative Control (n=5)	0	0	0	0
ETO Group (n=5)	0	0	0	3 (5,733 ± 3,400)
Glycerol Group (n=5)	0	1 (51 ± 51)	1 (1746 ± 1746)	5 (47,120 ± 11,690)
Positive Control (n=5)	0	5 (204,428 ± 107,716)	4** (243,178 ± 140,668)	5 (49,459 ± 15,760)
Ambient temperature† (n=1)				3,750

Values reported indicate the number of cats or bones with positive copy numbers for FeLV provirus (mean copies ± SE). A value of 22 or greater copies was considered positive. *Bone samples were obtained from sections of bones implanted into recipient cats. ** n = 4. †Results (recorded as copies) for a bone sample stored at ambient temperature for 4 weeks.

Agarose gel electrophoresis

Following agarose gel electrophoresis and ethidium bromide staining, DNA from bones of negative-control, glycerol-treated, and positive-control groups appeared intact and had distinct bands. In contrast, DNA from the ETO group bones did not have distinct DNA bands; it was smeared throughout the length of the gel, appearing denatured and suggestive of reduction or elimination of intact virus or provirus.

FeLV provirus in donor bone

All negative-control allografts implanted into cats in the negative-control group had negative results when tested for FeLV provirus. Three (range, 2,157 to 20,318 copies; mean = 9,555 copies) of 5 (range 0 to 20,318 copies; mean ± SE, 5,733 ± 3,400 copies) ETO-treated bones had positive results for FeLV proviral DNA. All five glycerol-treated bones had positive results for FeLV proviral DNA (range, 4,459 to 80,464 copies; mean ± SE, 47,120 ± 11,690 copies). In contrast, blood samples from only 1 of the recipient cats in the glycerol group had positive results when tested for FeLV proviral DNA. All untreated positive-control bones

had positive results for FeLV proviral DNA (range, 7,322 to 87,113 copies; mean ± SE, 49,459 ± 15,760 copies), as did recipient blood samples. The untreated bone graft stored at ambient temperature for 4 weeks had less FeLV proviral DNA (3,750 copies) than glycerol-treated or untreated bones (Table 3).

Radiography

High-resolution radiographs taken of implanted limbs after cats were euthanatized revealed moderate callus formation at proximal and distal host-graft interfaces in all cats. Host bone appeared incorporated into graft bone at all host-graft interfaces, and graft bone was almost indistinguishable in most cats. Subjectively, differences in healing were not observed among groups (Figure 6).

Histologic examination

Good incorporation of the donor graft by host bone was observed histologically, and no appreciable differences were seen between groups. Active remodeling and incorporation was detected in all bones along proximal and distal host-graft interfaces. At the graft interfaces, dead

graft bone was surrounded by new woven host bone. Bone grafts were identified by empty lacunae within bone surrounded by new woven host bone (Figures 7 and 8). Moderate periosteal proliferation was observed bridging the host-graft interfaces. Moderate infiltration of neutrophils was evident at the graft sites, but differences in inflammatory or toxic changes were not observed between groups.

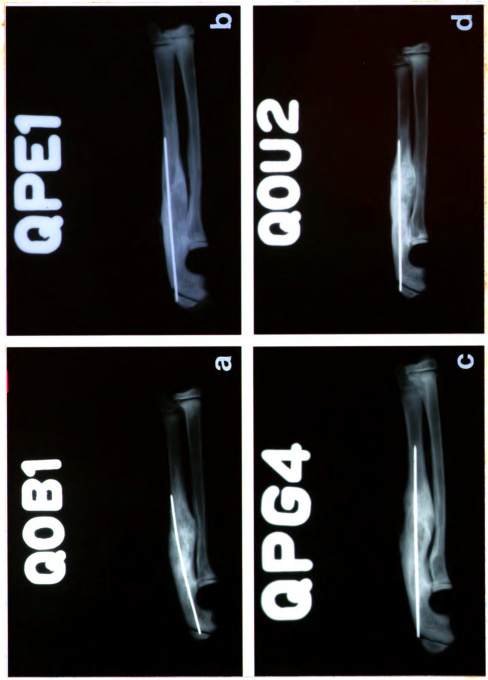

Figure 6: High-resolution radiographs of a harvested radius and ulna 8 weeks after implantation with a cortical bone allograft. Moderate callus formation was present around the graft site in all cats. a) Negative control, b) Positive control, c) Glycerol group, d) ETO group.

Figure 7: Photomicrograph of the host-graft interface in the ulna of a cat that received a cortical bone allograft. Dead bone graft can be visualized as empty lacunae surrounded by new woven host bone.(10X)

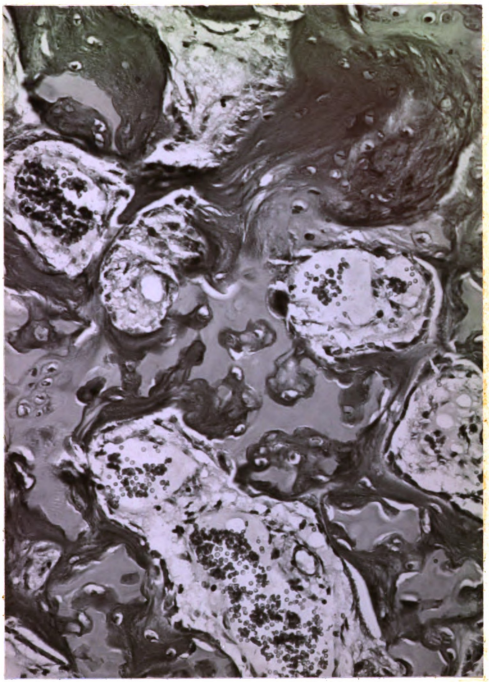

Figure 8: A magnified photomicrograph of the host-graft interface as seen in Figure 7.(20X)

VIABILITY OF RETROVIRUS (FELINE LEUKEMIA VIRUS) IN CORTICAL BONE GRAFTS AFTER ETHYLENE OXIDE STERILIZATION OR 98% GLYCEROL PRESERVATION

MATERIALS AND METHODS

Harvesting and preparation of bone allografts

Four weeks before cell inoculation, 3 groups of bones (n = 5 bones/group) from 5 SPF cats (3 bones from each cat) infected with the Rickard strain of FeLV were aseptically harvested and stored fresh-frozen at -70^0 C in a sterile plastic conical tube.

One of the 3 metacarpal bones from each of the FeLV-infected cats was assigned to 1 of 2 FeLV-infected treatment groups (ETO-treatment or glycerol-preservation) or to the FeLV-infected positive-control group (no treatment). After thawing, intact bones for the ETO group were separately double-wrapped by use of heat-sealed plastic and sterilized with 100% ETO. After ETO sterilization, bone samples were aerated at 50^0 C for 12 hours (relative humidity, 30%) and refrozen at -70^0 C. Bones in the glycerol group were placed separately into

conical centrifuge tubes that contained a 98% solution of glycerol, then were stored in the dark at room temperature (22^0 C) for 4 weeks.[97] The positive-control group of bones did not undergo treatment and were placed separately into sterile plastic conical tubes and refrozen at -70^0 C.

Cell culture

Confluent cells from a feline fibroblast cell line (FEA) were passaged at a 1:3 dilution into 25 separate sterile flasks containing 10 mls of media the day before test samples were added. Media consisted of Dulbecco's modified eagle medium with 15% fetal bovine serum, 2% glutamine, 2% NaHCO3, 1% Na-Pyruvate, 10 μg/ml gentocin, and 10 μg/ml enrofloxacin. Samples of stock media and FEA cells were saved prior to inoculation to confirm FeLV negative status. Media was aspirated from the cells and replaced with 10 ml of FEA cell media containing a 0.03 mg/ml diethylaminoethyl dextran for 30 minutes, then replaced with 10 ml of FEA cell media when test samples were added. Each untreated or treated bone was individually minced using lembert rongeurs, and 250 mg of minced bone was immediately introduced into a separate flask of FEA cells.

Each flask was then placed into a 37^0C, 5% CO_2, humidified incubator.

An additional 0.5 ml of media was added to five flasks of FEA cells (negative control). In addition, five flasks of FEA cells were inoculated with 0.5 ml of supernatant fluid from a productively FeLV-infected cell line, FL-74 (positive control) cells. Cells were allowed to grow until confluency, seen as a homogeneous layer of FEA cells covering the dependent wall of the flask when viewed using an inverted light microscope. When cellular confluency was reached, media was individually saved from each flask. Cells in each flask were trypsinized and centrifuged to pellet the cells. The supernatant fluid was discarded, and the remaining cells were resuspended in 1 ml of media. A 1:15 dilution of resuspended cells was then introduced into new flasks containing 10 mls of media. The remaining resuspended cells were saved for DNA extraction and quantification of FeLV provirus. This process was repeated for a total of four passages.

FeLV p27 antigen

The ELISA was used to test for FeLV p27 antigen in culture media samples obtained prior to inoculation and at each passage. An ELISA microplate reader was used to quantify antigen on the basis of optical density (OD). The sample-to-positive control ratio (S/P) was calculated, using the following formula:

$$\frac{\text{(OD of sample)} - \text{(OD of negative-control sample)}}{\text{(OD of positive-control sample)} - \text{(OD of negative-control sample).}}$$

Calculated S/P values of ≥ 0.1 were considered positive.

Extraction and quantification of DNA

A DNA extraction kit was used to extract the DNA from FEA cells obtained preinoculation and at each of the 4 cell passages. The DNA extracted from the cells was quantified, using a DNA flourometer. Samples of DNA were digested with the restriction endonuclease EcoR1, and digested DNA samples were stored at 4^0 C in sterile microfuge tubes.

FeLV provirus

A real-time quantitative polymerase chain reaction (QPCR) assay was used to quantify FeLV proviral DNA in 100 ng of digested DNA extracted from FEA cell samples. Negative and positive calibration standards were assayed in parallel with test samples.

Statistical Analysis

To compare the mean amount of DNA provirus measured between groups preinoculation and at passages 1, 2, 3, and 4, a one-way ANOVA was performed. A Bonferroni multiple comparisons test was performed at each passage to test for significance between groups. Differences were considered statistically significant at $p<0.05$.

RESULTS

FeLV p27 antigen

All media samples in the negative control, ETO-sterilized, and glycerol-preserved groups were negative for FeLV p27 antigen throughout the entire study. Due to

laboratory error, one sample from the ETO-sterilized group from the second passage was excluded. Four of five media samples from the positive bone control group were positive for FeLV p27 antigen at the first and second passages. At the third and fourth passages, all samples in this group had positive results. All samples in the positive virus (FL-74 cell) control group were positive for FeLV p27 antigen at all four passages (Table 4).

FeLV provirus

All cell culture samples in the negative control and ETO groups had negative results throughout the study for FeLV proviral DNA by QPCR. Four of 5 samples in the glycerol group had positive FeLV proviral DNA at the first passage (range, 42.6 to 581.5 copies). At the second passage, 3 of 5 samples in this group were positive for FeLV provirus (range, 1.58 to 27.97 copies). All of the glycerol group samples were negative for FeLV provirus at the third and fourth passages. One sample in this group was negative at all 4 passages.

All samples in the positive bone and positive virus (FL-74 cell) control groups were positive for FeLV provirus

throughout the study. The mean for the positive bone control group at the first passage was 8,142 copies. The mean for this group peaked at the second passage (254,487 copies) and declined at the third (107,019 copies) and fourth (104,486 copies) passages. The highest mean copy number for the positive virus (FL-74 cell) control group was at the first passage (87,064 copies). The means steadily decreased at the second (85,677 copies), third (59,8945 copies), and fourth (38,357 copies) passages (Table 5).

No statistically significant differences were observed between the negative control and ETO groups throughout the study. A significant difference was present between the glycerol group and the negative control and ETO groups at the first passage. However, no significant differences between these groups throughout the remainder of the study were seen. No statistically significant differences between the positive bone and positive virus (FL-74 cell) control groups were present throughout the study.

Table 4—Positive FeLV p27 antigen results for cell culture media from negative control, ETO-sterilized, glycerol-treated, and positive bone and virus control groups.

	Cell Culture Media Samples with Positive FeLV p27 Antigen Results			
	Passage			
Group	1	2	3	4
Negative - control (n =5)	0	0	0	0
ETO (n = 5)	0	0*	0	0
Glycerol (n = 5)	0	0	0	0
Positive-control bones (n = 5)	4 (0.22)	4 (0.39)	5 (0.34)	5 (0.50)
Positive-control virus (n = 5)	5 (0.55)	5 (0.45)	5 (0.31)	5 (0.44)

See Table 1 for key. Positive-control virus group = inoculated with supernatant fluid from a FeLV-infected cell line (FL-74). Values reported as number of samples within a group with positive results (and mean S/P ratio).

* Due to laboratory error, one sample from the ETO-sterilized group from the second passage was excluded.

Table 5—Results of a quantitative polymerase chain reaction test to detect the number of copies of FeLV provirus in negative control, ETO-sterilized, glycerol-treated, and positive bone and virus control group cell cultures.

Group	Copies of FeLV Provirus Cell samples (Passage)			
	1	2	3	4
Negative Control (n=5)	0	0	0	0
ETO Group (n=5)	0	0	0	0
Glycerol Group (n=5)	4 (187)	3 (9)*	1 (0.63)*	0
Positive Control Bone (n=5)	5 (8,142)	5 (254,486)	5 (107,019)	5 (104,486)
Positive Control Virus (n=5)	5 (87,064)	5 (85,677)	5 (59,895)	5 (38,357)

See Table 4 for key. Values reported indicate the number of cell cultures with positive copy numbers for FeLV provirus (mean copies). * Considered negative. Copy numbers of 22 or greater were considered positive.

DISCUSSION

The feline leukemia virus cat system has been used as an effective model to investigate transmission of a retrovirus via bone and connective tissue allografts.[104,105] Although extrapolations must be made to determine whether sterilization methods exhibiting virucidal activity against FeLV would be effective against HIV, FeLV is a safe and efficient model. The SIV is the lentivirus most closely related to HIV. However, purchase, housing, and maintenance costs as well as the risk of injury to investigators are higher for monkeys than cats. Surgical procedures on cats are relatively inexpensive, yet it is possible to perform intricate treatment and transplant operations that would not be possible using a murine system.

There are limitations with other lentiviruses as a model for HIV as well as advantages for using FeLV to investigate transmission of retroviruses following transplantation. Many serologic tests to detect FeLV infection are available. Investigators can detect early and late stages of FeLV infection using assays to detect FeLV

antigen, antibody, and provirus. Similar assays are available for FIV, but detection of provirus is limited to specific research strains. Cell types that FIV reportedly infects are restricted to lymphocytes, macrophages, astrocytes, microglial cells, and endothelial cells, and have not been shown to include bone tissue.[108] In contrast, transmission of FeLV through bone and connective tissue allotransplantation has been documented, making FeLV the best model.[104,105]

A limitation of an *in vivo* study is the expense and availability of animals, which in turn restricts the number of animals per group. A larger number of cats per group may have increased our power of confidence for results of the *in vivo* study. Importantly, transmission of FeLV occurred in one cat in the glycerol group, but was not documented in any of the cats that received ETO-treated bone grafts.

Various techniques have been reported for preservation and sterilization of canine cortical bone allografts.[27,33,73,119] However, their use in most veterinary practices has been restricted by the cost and inconvenience of allograft collection, processing, and storage. Typical

methods utilized include sterile graft harvesting followed by low-temperature storage (-20 to -40 C) or lyophilization (freeze-drying). Frozen bone grafts have a shelf life of only 6 to 12 months. Freeze-drying grafts has the advantage of allowing storage at ambient temperature, but this technique reportedly decreases the mechanical strength of bone.[33]

Secondary sterilization methods can be used to eliminate the need for aseptic harvesting of bone grafts, thereby reducing cost and increasing convenience. Gamma irradiation is an effective sterilization technique for deep penetration of thick tissues, but requires instruments that may not be available in most veterinary hospitals. In addition, high doses of irradiation have been associated with decreased osteoinductive and biomechanical properties of bone grafts.[33] Ethylene oxide has been widely used for sterilization and reportedly is effective for removing surface contamination, but decreases bone induction and incorporation properties as well as reducing mechanical bone strength.[3,142,152] An optimal storage and sterilization technique would eliminate the need for special equipment, sterile harvesting of grafts, or both.

Reports of viral transmission to people following transplantation of soft-tissue or bone grafts have raised concerns regarding sterilization and storage of these grafts.[4,21,23,25] Although ETO reportedly is an effective bactericidal agent, its virucidal properties for tissues have not been documented. In contrast, virucidal activity of an 85% solution of glycerol against HSV-1 and polioviruses was reported.[97] Soft-tissue grafts stored in an 85% solution of glycerol at 20^0 C had complete inactivation of HSV-1 and poliovirus after 8 or 22 days of storage, respectively. Because that was an *in vitro* study, data was not available regarding the effects of transmission of these viruses in an *in vivo* system. Investigations of the virucidal properties of glycerol on transplanted bone grafts have not been described. However, treatment of canine femoral cortical bone allografts with a 98% solution of glycerol at ambient temperature was adequate for storage and resulted in good healing when a bone plate was used for stabilization.[27] Complete periosteal bridging was seen at the graft sites with a continuity of cortices at the host-graft interfaces 90 days after graft implantation.

In the studies reported here, results of ETO sterilization were promising, because all recipient cats had negative results when tested for viral antigen and provirus with no evidence of viral transmission. Although 3 of 5 ETO-treated donor bone grafts had positive results for FeLV provirus, none of the recipient cats in this group became infected after transplantation. Lack of viral transmission to recipients of ETO-treated bone grafts may be attributable to a decrease in the infectious viral load. Additionally, the 3 bones from the ETO group that had positive results (mean, 9,555 copies) had less FeLV provirus than bones for the glycerol (mean, 47,120 copies) or positive-control (mean, 49,459 copies) groups (Table 3).

Antibody titers to FOCMA ≥ 1:16 may result from exposure to infectious virus or, possibly, viral antigens. A low-positive antibody titer to FOCMA was detected in 1 cat in the ETO group despite lack of viral antigenemia. This may have resulted from a viral antigen(s) in the bone graft stimulating an antibody response in the recipient, despite lack of intact infectious virus. Alternatively, antigen concentrations may have been too low to detect a positive reaction in blood samples.

Cats in this study did not have evidence of ETO toxicosis. Toxic byproducts of ETO sterilization include ethylene glycol and ethylene chlorhydrin. Apart from the toxic effects of these ethylene oxide residuals, another possible explanation for reduced incorporation may be ETO-induced alkylation of amino acids.[142] In the *in vivo* study reported here, equivalent bone incorporation was observed in cats in the ETO group, compared with cats in the untreated negative- and positive-control groups. Although there were undoubtedly toxic byproducts, lack of difference in incorporation in this model may be partly attributable to the young age of the cats and their high propensity for healing. A study that uses adult cats would likely reveal differences in incorporation.

Transmission of FeLV to one of the cats in the glycerol group may have been associated with immunocompetence of the recipient, infectious virus titer of the graft, or both. Because all donor bones in the glycerol group had more FeLV provirus (mean, 47,120 copies) than the untreated bone stored at ambient temperature for 4 weeks (3,750 copies), it is possible that the 98% solution of glycerol enhanced viral preservation. Unfortunately, infectivity could not be assessed in the untreated bone

because it was not implanted in a cat. Despite the fact that ambient temperature storage of untreated FeLV-infected bone appeared to decrease viral load, the resultant putrefaction precludes its use.

Donor bones in the positive-control group had the highest amount of FeLV provirus (mean, 49,459 copies). All cats in the positive-control group had positive results when tested for FeLV p27 antigen 2 weeks after transplantation. Although 2 cats in the positive-control group subsequently had negative results for FeLV p27 antigen (Table 1), these cats had positive results for FeLV provirus at weeks 4 and 8 (Table 3). Transient antigenemia has been reported in cats with natural FeLV infection or infection resulting from experimental implantation of FeLV-infected bone and connective tissues.[104,105] However, investigators in those studies did not assess FeLV status by use of the more sensitive quantitative PCR technique. All cats in the positive-control group were seropositive to FOCMA, confirming exposure to FeLV. Furthermore, all cats had positive results for FeLV provirus in blood samples, documenting infection with FeLV.

In the *in vitro* study, a higher proviral load in glycerol-preserved compared with ETO-treated bone grafts was observed at passage 1. However, cultures containing both glycerol-preserved and ETO-treated bones were negative for FeLV provirus at passages 2 through 4. Results for FeLV p27 antigen were consistently negative in media samples from cultures containing both glycerol-preserved and ETO-treated bones. It appeared that FeLV viral particles in these bone grafts were noninfectious. This was in contrast with results of the *in vivo* study, where one of the recipient cats that received a glycerol-preserved bone graft became infected with FeLV.

During the first passage, cells in the glycerol group took approximately 5 days longer to achieve confluency. Following the first passage and a change in media, cells in the glycerol group achieved confluency at the same rate as cells in other groups. It is likely that residual glycerol present on the bone samples affected the media environment by lyophilization. The resultant slowing of cell replication was less than optimal for viral replication. Detection of the FeLV provirus segment in the glycerol group at passage 1 may have been due to viral particles that were either noninfectious or unable to replicate in

this environment. In contrast, the environment presented to the glycerol-preserved bones in the *in vivo* study was highly cellular with diverse cell types and good blood flow, providing optimal opportunities for replication. Differences observed in transmission of retrovirus in glycerol-preserved bones to cells versus cats highlight the importance of conducting both *in vitro* and *in vivo* studies, respectively.

Whole, intact FeLV provirus or smaller amplifiable regions of FeLV provirus may be detected in DNA samples analyzed by QPCR. Detection of the small segment of provirus amplified by QPCR does not establish infectivity of donor bone grafts. In contrast, detection of provirus or antigen in blood following allotransplantation or passage in cell culture does prove transmissibility of the retrovirus. The fact that all cats in the positive-control group had positive results for FeLV antigen and provirus in blood samples confirms that freezing does not effectively impair viability of the retrovirus or prevent transmission after implantation. This was substantiated by the *in vitro* study that showed positive results for FeLV provirus in cultures that contained untreated bone from FeLV-infected cats at all 4 passages.

The studies reported here documented that ETO sterilization appeared to denature DNA and had effective virucidal activity against the retrovirus FeLV. In contrast, use of a 98% solution of glycerol was inadequate for viral sterilization of cortical bone allografts. Comparison of the virucidal effects of glycerol-preserved with untreated positive-control grafts did not reveal a reduction in the quantity of amplifiable FeLV provirus in donor grafts treated with a 98% solution of glycerol. Although transmission of FeLV was decreased in recipients of glycerol-treated bone grafts, suggesting decreased infectivity, a 4-week duration of glycerol treatment for bone allografts cannot be recommended for virucidal sterilization. Additional studies may be warranted to examine the effect of prolonged (eg, 6 months) glycerol treatment on virus-infected bone grafts.

Histologically, no differences in incorporation at the host-graft interface were observed between groups. It may be concluded from this study that bone allografts sterilized with ETO or a 98% solution of glycerol had comparable incorporation of host bone, compared with untreated-control groups in this model using young cats.

However, ETO sterilization had superior virucidal activity against the retrovirus FeLV.

Ethylene oxide sterilization abrogated transmission of FeLV infection, possibly by denaturing the DNA. However, quantities of provirus detected by QPCR in ETO-treated donor bone grafts were reduced but not eliminated. Additional studies to determine whether provirus in ETO-treated bone was intact infectious virus or smaller noninfectious segments of proviral DNA are warranted. Lack of transmission of FeLV to recipients of ETO-treated bone grafts suggests that the veterinary community may be cautiously optimistic regarding the safety and efficacy of this widely available treatment.

CONCLUSIONS

The *in vitro* and *in vivo* studies reported here documented that ETO sterilization appeared to denature DNA and had effective virucidal activity against the retrovirus FeLV. Quantities of amplifiable provirus detected by QPCR in ETO-treated donor bone grafts were reduced but not eliminated. We hypothesized that virus particles and provirus in bone were rendered noninfectious following ETO treatment. In contrast, although treatment of cortical bone allografts with a 98% solution of glycerol appeared effective in *in vitro* studies, it was inadequate for viral sterilization in an *in vivo* model. Comparison of the virucidal effects of glycerol-preserved with untreated positive-control grafts did not reveal a reduction in the quantity of amplifiable FeLV provirus in donor grafts treated with a 98% solution of glycerol. Although transmission of FeLV was decreased in glycerol-treated compared with untreated bone graft recipients suggesting decreased infectivity, 4-week glycerol treatment for bone allografts cannot be recommended for virucidal sterilization.

While the glycerol-preservation protocol used in this study does not show adequate antiviral effects, additional studies may be warranted using different concentrations (eg, 85% glycerol solution), storage temperatures, or prolonged preservation times (eg, 6 months). Moreover, additional ETO sterilization protocols may be investigated to further document its antiviral effect.

It also may be concluded from these studies that bone allografts sterilized with ETO or a 98% solution of glycerol had comparable incorporation of host bone, compared with that for untreated-control groups in this model using young cats.

These paired studies are a good example of the use of both *in vitro* and *in vivo* studies to investigate the same question. Results of the *in vitro* study were encouraging for both ETO and glycerol and warranted additional investigation. However, the *in vivo* study was a more complete model to test transmission of the retrovirus and demonstrated that although ETO appeared to have adequate antiviral activity, 98% glycerol was ineffective. This points out that both *in vitro* and *in vivo* studies are

required to reach a reliable conclusion regarding antiviral efficacy of treatment protocols.

Results of these studies may be applied to both human and veterinary medicine. The orthopedic community should continue to be vigilant regarding the potential of infectious viral particles present in bone sterilized with ETO. Screening procedures of candidates for donation of allografts are the most important aspect of preventing implantation of infected bone. Dogs are frequent recipients of bone transplants, and results of these studies may be used as a starting point for investigations of the efficacy of ETO for preventing transmission of canine diseases through allotransplantation.

RECOMMENDATIONS

Lack of transmission of FeLV to recipients of ETO-treated bone grafts suggests that veterinary and human communities may be cautiously optimistic regarding the safety and efficacy of this widely available treatment. Intense screening of potential allograft donors should be continued.

Four week 98% glycerol preservation of cortical bone allografts cannot be recommended for retroviral sterilization.

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