Diseases Caused by Maedi–Visna and Other Ovine Lentiviruses

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Summary

Maedi–visna and other ovine lentiviruses (OvLV) are worldwide causes of slowly progressive diseases of lungs, brains, joints and mammary glands of infected sheep. Infection by these retroviruses is characterized by lifelong persistence, horizontal and vertical transmission, and insidious onset of disease in a proportion of infected adults. In the absence of effective vaccines, OvLV infection is controlled by using test and cull schemes or by prevention of vertical (milk) transmission of the virus. Developing breeding stock that are resistant to OvLV infection or OvLV-associated diseases is an alternative approach. Traditionally, this would involve selection and propagation of individuals that exhibit resistance to OvLV infection or disease. However, identification of such animals is so problematic that a selection programme has never been undertaken. For the same reason, no searches for DNA markers have been undertaken. Based on rapidly developing information on molecular characteristics of a related lentivirus, HIV, and new technologies for introducing genes into the mammalian genome, it is now possible to produce sheep that are transgenic for viral or other genes that may induce resistance to OvLV infection or disease. Candidate genes for introduction include viral structural protein genes such as env, mutated viral regulatory protein genes such as *tat* and *rev*, genes encoding viral antisense RNA, and genes encoding ribozymes capable of cleaving viral RNA within infected cells. Success of these approaches will depend upon optimizing expression of the desired gene in appropriate target cells without adversely affecting cell or host physiological functions. After constructing transgenic sheep that stably express the desired gene, their ability to resist OvLV infection or disease must be assessed by natural or experimental challenge. Currently available tools of biotechnology and knowledge of lentivirus molecular virology and pathogenesis provide a sense of optimism that progress in controlling animal retroviral diseases through these means is imminent.

Introduction

Maedi and visna (maedi synonyms: ovine progressive pneumonia in the USA, la bouhite in France, Graaff-Reinet disease in South Africa, and zwoegerziekte in the Netherlands) are chronic multisystemic diseases of sheep caused by ovine lentivirus (OvLV; Petursson *et al.*, 1992; de la Concha-Bermejillo, 1997). Maedi, an Icelandic word meaning dyspnoea, is used to describe a chronic interstitial pneumonia, whereas the term visna (meaning wasting) refers to a slow, progressive disease of the central nervous system resulting in paralysis; chronic mastitis and arthritis are also caused by OvLV. Ovine lentivirus infection has been reported in most sheep-raising countries of the world, with the notable exception of Australia and New Zealand (Dawson, 1980).

Ovine lentivirus, the cause of these diverse disease syndromes, is a prototype virus of the genus *Lentivirus* within the family *Retroviridae*. Ovine lentiviruses share morphological, genetic and pathogenic characteristics with other lentiviruses, including caprine arthritis encephalitis virus (CAEV), bovine immunodeficiency virus (BIV), equine infectious anaemia virus (EIAV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) (Narayan and Clements, 1989). Because of very similar genomic organization, pathogenicity and epidemiology, OvLV and CAEV are often grouped as small ruminant lentiviruses; these viruses can be transmitted between their respective hosts (Banks *et al.*, 1983).

Retrovirus infections have proven difficult to control in domestic animals because of their capacity to integrate into the genome of the host, to induce a persistent infection and to vary the antigenicity of envelope glycoproteins (Pearson *et al.*, 1989; DeMartini *et al.*, 1991; Brodie *et al.*, 1992a). Since effective vaccines have not been developed, small ruminant lentivirus diseases are primarily controlled using test and culling schemes to eliminate infected animals. In this chapter, we integrate new knowledge concerning the biology of lentiviruses and their interaction with the host with approaches based on selective breeding, genetics, embryo manipulation and molecular biology to describe novel strategies for the control of lentivirus-associated diseases of sheep.

Characteristics of Ovine Lentiviruses and their Replication

The OvLV genome, as in other lentiviruses, consists of two identical positive-sense single-stranded RNA subunits of 9.4 kb containing structural and regulatory genes (Fig. 14.1; Sonigo *et al.*, 1985; Narayan and Clements, 1990; Clements and Zink, 1996). The structural genes of the virus are *gag*, *pol* and *env*. The *gag* gene of OvLV is highly conserved and encodes information for one major and two minor core proteins that are proteolytically processed from a 53 kDa gag precursor protein by the virion-encoded protease. The major core protein, referred to as capsid protein (CA, p24–p27), elicits a strong antibody response and has been used to develop sensitive diagnostic assays. The two small core proteins, the matrix protein (MA, p16) and the nucleocapsid
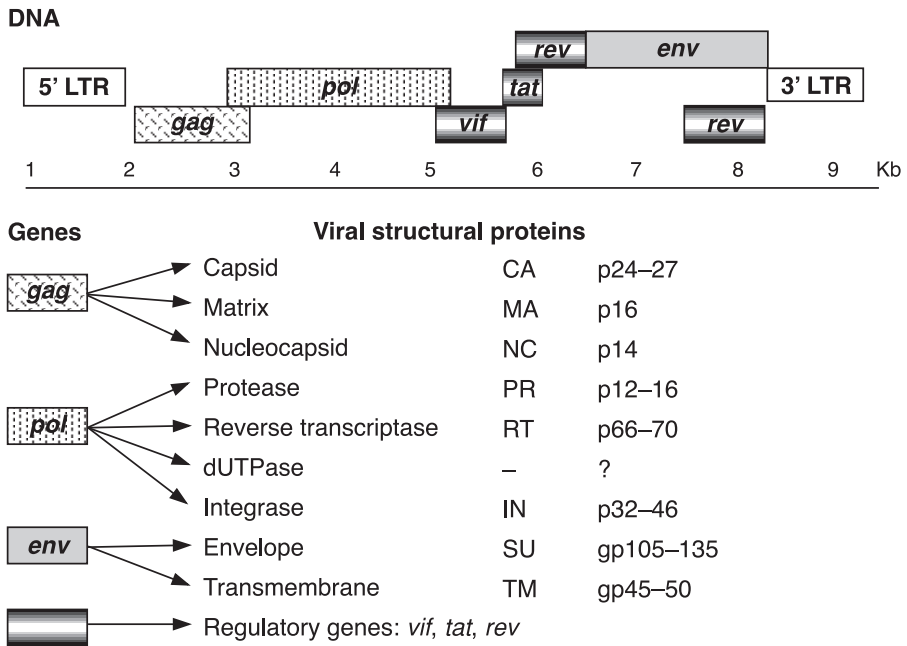
DNA

Fig. 14.1. Ovine lentivirus proviral genomic organization, regulatory genes, and structural genes and their protein products.

protein (NC, p14) are poorly immunogenic. The *pol* gene of OvLV encodes information for reverse transcriptase (RT), a protease (PR), an endonuclease/ integrase (IN) and a dUTPase. These proteins play important roles in viral nucleic acid transcription, protein synthesis and integration into host-cell DNA, during replication. The dUTPase is the only OvLV gene that is not essential for replication, and visna virus lacking a functional dUTPase gene can replicate well in sheep macrophages *in vitro* (Turelli *et al.*, 1996). The *env* gene encodes the information for the envelope surface glycoprotein (SU, gp105) and the transmembrane protein (TM), both of which are used in sero- logical assays along with CA (Juste *et al.*, 1995). The envelope proteins are synthesized as a large precursor protein that is cleaved by a cellular protease into the SU and the TM glycoproteins. The SU protein carries the neutral- ization and fusion epitopes. Antigenic variation within the SU protein confers biological and serological properties of different isolates (Braun *et al.*, 1987; Mwaengo *et al.*, 1997; Cheevers *et al.*, 1999). As with other lentiviruses, the proviral DNA of OvLV is flanked on both ends by long terminal repeats (LTRs) that contain enhancer–promoter elements for the initiation of DNA transcription and play a role in tissue tropisms (Small *et al.*, 1989). A unique genetic feature of all lentiviruses is the presence of small regulatory genes located between the *pol* and *env* genes and the 3′ terminus, *vif*, *tat* and *rev*, that encode proteins that regulate viral replication.

An important characteristic of lentiviruses is that their replication cycle includes the integration of a DNA intermediate (the provirus) into the host

cell's chromosomes. After virus infection, RT transcribes the genomic viral RNA into a double-stranded DNA copy. After circularization, the enzyme IN inserts proviral DNA into the host-cell DNA. The viral genome thus becomes part of the cellular DNA and is duplicated during cell division. As a result, OvLV infection of sheep is potentially lifelong and the virus can be isolated from seropositive sheep years after the original infection (Haase, 1986).

Ovine lentivirus replicates primarily in monocytes and macrophages of the blood, lung, spleen, bone marrow and brain of infected animals (Brodie *et al.*, 1992a; Narayan *et al.*, 1993; Clements and Zink, 1996). *In vitro*, macrophages and primary choroid plexus, lung, trachea, cornea and synovial membrane cells are permissive. Cytopathic effects include the formation of multinucleated syncytial cells and cell lysis, and genetic variants of these have been described (Woodward *et al.*, 1995).

Clinical Features, Pathogenesis and Immunology of Ovine Lentivirus Infections

The most common clinical manifestations of OvLV infection are progressive respiratory failure and cachexia in mature sheep (Bulgin, 1990). Some animals may develop indurative mastitis, swollen joints, or paresis or paralysis. Affected ewes often give birth to small, weak lambs. Once clinical disease becomes apparent, sheep die within 6–8 months, usually due to anoxia or secondary bacterial infections.

The lungs of sheep with OvLV-associated lymphoid interstitial pneumonia (LIP) are non-collapsing and heavy, and there may be 1–2 mm diameter grey foci scattered throughout the parenchyma; red–grey consolidation may involve the cranioventral lobes, particularly if secondary bronchopneumonia is present (de la Concha-Bermejillo, 1997; Brodie *et al.*, 1998). The microscopic features of LIP include lymphocyte hyperplasia surrounding airways and blood vessels, infiltration of the interalveolar septa and alveoli by mononuclear cells, bronchiolar smooth muscle hyperplasia and fibrosis. CD8+ and CD4+ T cells are increased in lung compartments of infected sheep (Watt *et al.*, 1992). Pulmonary lymph nodes are markedly hyperplastic due to increased numbers of B cells and T cells in lymphoid follicles and germinal centres and paracortical zones (Ellis and DeMartini, 1985).

More than 60% of the ewes in OvLV-affected flocks have chronic lymphocytic mastitis and resultant reduction in milk production (Dawson, 1987). Lambs from ewes with OvLV-induced mastitis have reduced weaning weights (Pekelder *et al.*, 1994). Lentivirus-associated arthritis typically begins insidiously 2–3 years after infection, with weight loss and swelling of the carpal and tarsal joints (Kennedy-Stoskopf, 1989). Microscopically, there is infiltration of mononuclear cells and synovial villous hyperplasia, which may progress to chronic osteoarthritis. Sheep affected by OvLV-associated neurological disease (visna) show aberrations of gait followed by progressive paraplegia of the rear limbs, quadriplegia and wasting (Petursson *et al.*, 1990).

Spinal taps reveal mononuclear pleocytosis in the cerebrospinal fluid. Microscopically there is periventricular encephalitis characterized by ependymal necrosis, widespread demyelination and prominent perivascular lymphocytic cuffing (Petursson *et al.*, 1992; Georgsson, 1994). Although CNS manifestations of OvLV infection are uncommon in the USA, a study showed that 18% of naturally infected sheep had subclinical mononuclear cell infiltration in the leptomeninges, cerebral white matter, choroid plexus or cervical spinal cord. Histiocytes in these lesions contained lentiviral CA protein or RNA (Brodie *et al.*, 1995).

The pathogenesis of OvLV-induced disease depends on host, viral and environmental factors. Among the viral factors, the virus load in infected animals, which may be influenced by host genetics, seems to be the most important (Brodie *et al.*, 1992b; de la Concha-Bermejillo *et al.*, 1995). Infected macrophages in the lungs and lymphoid tissues express viral proteins on their surface in close association with MHC antigens. This dual signal is recognized by T cells, leading to the production of inflammatory cytokines that recruit other inflammatory cells, leading to chronic inflammation (Lairmore *et al.*, 1988a; Narayan, 1990; Luján *et al.*, 1994).

During the incubation period, there is an acute viraemia followed by an immune response that restricts virus replication to low levels but fails to eliminate the virus completely (Juste *et al.*, 1998). Seroconversion, detectable by ELISA (DeMartini *et al.*, 1999) or Western blotting assays, usually occurs between 2 and 8 weeks post-infection (Kajikawa *et al.*, 1990; Brodie *et al.*, 1993; Juste *et al.*, 1998, DeMartini *et al.*, 1999). The ensuing decline in viraemia coincident with seroconversion suggests that viraemia is under immune control. However, despite the presence of neutralizing antibodies (Cheevers *et al.*, 1999) and cell-mediated immunity (CMI), OvLV persists in cells of the macrophage lineage of infected animals, often as integrated provirus. Since viral gene expression is activated only when the monocytes mature into macrophages, monocytes form a reservoir of latently infected cells which escape immune surveillance and perpetuate infection (Haase, 1986). This state of cellular latency appears to be controlled by an interaction of cellular and viral transcription factors that regulate viral RNA expression (Gendelman *et al.*, 1985; Staskus *et al.*, 1991; Clements and Zink, 1996).

Cell-mediated immunity is thought to play a more important role in protection against lentivirus infections than humoral immunity. OvLV-specific T-cell proliferation responses to both purified virions and recombinant p25 have been demonstrated (Reyburn *et al.*, 1992; Bird *et al.*, 1993). Using T cells from OvLV-infected animals, it has been shown that OvLV-infected macrophages can induce cytotoxic T-cell (CTL) activity; in turn, these CTLs can kill infected macrophages (Lee *et al.*, 1994; McConnell *et al.*, 1996). This cytotoxic activity is mediated by CD8^{+} T cells (Blacklaws *et al.*, 1994). However, because a pool of latently infected cells that is inaccessible to T-cell-mediated killing remains in lymphoid organs, CMI fails to eradicate the virus from infected animals. This pool of persistently infected cells gives rise to bursts of virus replication, leading to chronic inflammation.

Role of Virus Strains, Host Genetics and Cofactors in OvLV Pathogenesis

The outcome of lentivirus infections is the result of complex interactions among genetically diverse virus strains, the host genome and the environment. Initial studies showed that OvLV strains that grow slowly and produce little cytopathic effect *in vitro* caused little disease in lambs but more lytic virus strains often produced severe disease (Lairmore *et al.*, 1987, 1988b). Although OvLV strains show a predominant phenotype *in vitro* (lytic or non-lytic), more recent experiments indicate that in an infected individual, lentiviruses are present as a 'quasispecies', a genetically heterogeneous viral population (Woodward *et al.*, 1994; Pieniazek *et al.*, 1995; Clements and Zink, 1996).

The influence of host genetics on the outcome of lentivirus infections has attracted a great deal of attention in recent years. Several major histocompatibility complex (MHC) genes or haplotypes appear to influence disease progression, although the effects are complex and may depend on interactions with other host genes. For example, in humans infected with HIV, the haplotype A1B8DR3 appears to be associated with faster progression to AIDS. In one study, eight of 11 haemophiliacs infected in 1984 who had developed AIDS displayed this haplotype, whereas none of seven HIV+ asymptomatic people carried it (Peixinho and Mendes, 1994). In HIV-infected children, the *DR3* allele is associated with accelerated progression to AIDS while *DPB1* is associated with survival (Just *et al.*, 1995). Similarly, disease progression in simian immunodeficiency virus (SIV)-infected monkeys appears to be influenced by MHC genotype. In SIV-infected monkeys, the lack of the *Mamu-A26* allele correlates with a more rapid progression to SAIDS (Bontrop *et al.*, 1996). In the case of CAEV, a lentivirus closely related to OvLV, susceptibility to arthritis has been associated with differences in frequencies of certain caprine leucocyte antigens (CLAs). Thus, animals of the Saanen breed carrying the CLA Be7 specificity are less prone to develop CAEV-induced arthritis than are goats lacking this specificity (Ruff and Lazary, 1988).

Breed differences in susceptibility to OvLV infection and disease supports the notion that host genetic factors influence the outcome of OvLV infection. Reports suggest that Finnish breeds have a greater tendency to become infected by OvLV than the Ile de France, Rambouillet or Columbia breeds (Gates *et al.*, 1978; Houwers *et al.*, 1989). Breed-related resistance to OvLV-induced disease was suggested by studies in Iceland indicating that progression of lung lesions was relatively delayed in crosses between Icelandic sheep and Border Leicester rams (Palsson, 1976). In contrast to these findings, Border Leicester sheep were found to be more likely than Columbia sheep to develop multisystemic lesions in response to experimental or natural OvLV infection (Cutlip *et al.*, 1986). Furthermore, the Awassi breed of sheep is highly susceptible to infection with OvLV, but in a 20-year study, no pure Awassi sheep has developed disease (Perk *et al.*, 1996). Since interpretation of breed-susceptibility studies is complicated by variation in viral strains and host genetics, further rigorously controlled research would be required to confirm a particular breed or group of sheep as resistant to OvLV infection or disease.

More recent experiments using artificially created isogenic twin lambs have confirmed that host genetic factors may play an important role in determining the extent and severity of OvLV-induced pulmonary lesions (de la Concha-Bermejillo *et al.*, 1995). In this study, the degree of pulmonary LIP at necropsy was independent of the virus strain used for inoculation, and the amount of OvLV proviral DNA in alveolar macrophages correlated with the degree of LIP. This genetic basis for susceptibility or resistance of the host to lentivirus-induced disease could explain many of the differences in disease progression in OvLV-infected sheep.

It is well known that a proportion of sheep in an OvLV-positive flock remain seronegative for life. Although such animals may have never been exposed to OvLV or may not have responded immunologically to infection, it is also possible that they possess innate resistance to OvLV infection. In the case of HIV, recent experimental evidence indicates that in addition to the primary CD4 receptor found on both monocytes/macrophages and lymphocytes, chemokine receptors play a crucial role in virus entry and susceptibility to infection. Macrophage-trophic strains use the CCR5 chemokine receptor while T-cell-trophic strains utilize the CXCR4 chemokine receptor (Bjorndal *et al.*, 1997). A 32 bp deletion has been found in the CCR5 gene, and being homozygous for this deletion appears to confer resistance to infection (Fauci, 1996; Samson *et al.*, 1996; Rana *et al.*, 1997). In one study, 4.5% of highly exposed seronegative individuals were homozygous for this deletion, but none of the HIV-infected people were homozygous for the deletion. Moreover, although heterozygosity did not appear to confer resistance to infection, it did appear to delay the progression to AIDS (Zimmerman *et al.*, 1997). Since only 4.5% of the highly exposed seronegative people were homozygous for the CCR5 mutation, it is probable that other genetic factors influencing infection remain to be discovered. Furthermore, polymorphisms in other chemokine receptor genes may affect HIV disease progression. The role of other cofactors in lentivirus pathogenesis cannot be ignored. In one study, pulmonary abscesses due to *Corynebacterium pseudotuberculosis* infection were present in 53% of OvLV-seropositive sheep, all of which had some degree of LIP, but were only present in 30% of OvLV-seronegative animals, none of which had LIP (Ellis *et al.*, 1990). A synergistic basis for this was suggested by data indicating that *C. pseudotuberculosis* induced the secretion of tumour necrosis factor-α (TNF-α) by ovine macrophages which, in turn, enhanced OvLV replication (Ellis *et al.*, 1991). Mycoplasma agents also have been associated with LIP in OvLV-infected sheep (DeMartini *et al.*, 1993). These findings suggest a role for intercurrent bacterial infections in the pathogenesis of OvLV-induced disease.

Transmission of Ovine Lentivirus and Traditional Methods of Control

Transmission of OvLV commonly occurs via lactogenic or aerosol routes, but the importance of each route in the epizootiology of the disease is debatable.

In flocks where the infection is enzootic, the primary route of infection seems to be from ewe to lamb through infected colostrum (Petursson *et al.*, 1992); cell-associated OvLV can be detected in the milk of infected ewes (Ouzrout and Lerondelle, 1990; Brodie *et al.*, 1994). On the other hand, serological surveys throughout different sheep-producing areas of the USA show a wide range of OvLV prevalence, indicating that other factors and routes of transmission may play a role in the epidemiology of the disease. Because a positive correlation has been found between the proportion of sheep seropositive to OvLV and age of infected animals, it has been speculated that OvLV spreads laterally, probably by the respiratory route (Cutlip *et al.*, 1977, 1992). However, in a recent study in Texas in which eight OvLV-free lambs were placed in close contact in open pens with 32 experimentally inoculated lambs for 8 months, none of the contact controls became infected (de la Concha-Bermejillo, unpublished observation). In addition, OvLV prevalence in Texas, where 20% of all US sheep are raised, is less than 1% compared to a 26% national average (Cutlip *et al.*, 1992; de la Concha-Bermejillo *et al.*, 1998). In Texas, most sheep are raised under extensive conditions in a mostly dry and hot climate, which suggests that housing and close contact during lambing in cold weather may play an important role in OvLV transmission. In a European study of flocks with mixed infected and uninfected ewes, approximately 37% of the offspring of infected ewes and about 20% of the offspring of uninfected ewes became OvLV-infected within 1 year (Houwers *et al.*, 1989). Close confinement in winter housing, perhaps combined with genetic susceptibility of Icelandic sheep breeds, was suspected to have been a factor contributing to the explosive OvLV outbreak in Iceland (Palsson, 1976). Although less common, vertical transmission from mother to fetus has also been reported (Cutlip *et al.*, 1981; Brodie *et al.*, 1994). Venereal transmission of OvLV has not been reported; however, recent information indicates that OvLV-inoculated animals co-infected with *Brucella ovis* can shed the virus in semen (de la Concha-Bermejillo *et al.*, 1996).

To date, the only effective means for controlling OvLV-induced disease has been through removal of infected sheep or prevention of spread of the virus. This approach was undertaken on a large scale in Iceland in the 1950s when over 600,000 sheep in infected flocks were slaughtered and the farms were repopulated with unexposed sheep (Palsson, 1976). More recently, OvLV infection has been eliminated from flocks by removing lambs from seropositive ewes before nursing and rearing them in isolation, or by repeated testing and culling of seropositive ewes and their progeny (Houwers *et al.*, 1983, 1984, 1987; Cutlip and Lehmkuhl, 1986). Voluntary eradication programmes based on these methods have been established in The Netherlands, Great Britain and Belgium, and have achieved considerable success. However, since such methods are expensive and are not readily adopted by farmers, they may not be as successfully employed in countries with larger sheep populations where the seroprevalence of OvLV infection is high. This necessitates the development of alternative strategies for control of OvLV (Blacklaws *et al.*, 1995).

Strategies for the Control of Ovine Lentivirus Infections in Individual Animals

Presently, there are no effective vaccines or treatments against OvLV infections. Developing a vaccine to protect against OvLV infection or disease has been a challenging problem because of antigenic variation, latency and the complex interactions that lentiviruses have evolved with their host. Studies of unsuccessful vaccination against OvLV in sheep have involved heat-, formalin- or ethyleneimine-inactivated vaccines, with or without Freund's incomplete or aluminium hydroxide adjuvant (Cutlip *et al.*, 1987; Pearson *et al.*, 1989). In one study, sheep immunized with purified virions homogenized in Freund's complete adjuvant developed more severe CNS lesions than the controls, suggesting a role for cell-mediated immunity in the development of CNS lesions (Nathanson *et al.*, 1981). Alternative novel methods of vaccination against OvLV, such as the use of naked proviral DNA, are presently being studied (Perk *et al.*, 1996).

Several compounds, including phosphonoformate, 2′,3′-dideoxynuclosides and α-interferon, have been shown to inhibit OvLV replication *in vitro* (Frank *et al.*, 1987). In addition, recombinant ovine τ-interferon has been shown to have antiviral activity against OvLV (Juste *et al.*, 1996). However, due to the irreversible nature of the infection, secondary to the integration of the virus genome into the host cell's chromosomes, drug-based approaches for the treatment of OvLV infection would appear to have no practical application.

Cytokine-expressing viruses have been envisioned as a novel approach for the development of safe and efficacious live-attenuated vaccines against retroviruses (Giavedoni *et al.*, 1992, 1997). Retroviral vectors efficiently transfer gene sequences into cells and promote their stable expression (Naldini *et al.*, 1996). Some simple retroviruses that have been engineered are replication-competent; others need helper packaging cell lines. A replication-competent SIV with a deletion in the nef gene ($SIV_{\Delta nef}$) that expresses high levels of human γ-interferon (γ-IFN) (SIV_{HyIFN}) has been constructed (Giavedoni and Yilma, 1996). Rhesus macaques vaccinated with SIV_{HyIFN} had a lower viral load than macaques similarly immunized with $SIV_{\Delta nef}$. Viral loads remained low in the SIV_{HyIFN}-vaccinated group and the animals remained healthy for more than 32 weeks after challenged with virulent SIV_{mac251}, suggesting that the modified virus expressing γ-IFN provided effective protection from subsequent infection by virulent strains of virus (Giavedoni *et al.*, 1997).

Gene therapeutics, using replication-defective lentiviruses and packaging cell lines, is the latest addition to a multitude of approaches that could be used to combat lentivirus infections. Replication-defective lentiviruses are seen as a safer approach for gene delivery than replication-competent retroviruses. Multiplasmid expression systems can be used to generate lentivirus-derived vector particles by transient transfection that result in stable *in vivo* gene transfer into terminally differentiated cells (Naldini *et al.*, 1996).

Selection and Breeding of Ovine Lentivirus-resistant Animals

During the past few decades, animal breeders have been very successful in breeding animals that are superior in the production of milk, eggs, meat and wool. However, selection for disease resistance has been largely ignored (Gavora and Spencer, 1983). Selection for resistance to infectious disease requires the identification of specific genes or genetic markers linked to resistance either to infection or to disease.

Establishing a selection programme for resistance to ovine lentivirus infection is difficult because the OvLV viral receptor is unknown. However, one way to identify animals that may be resistant to OvLV infection would be to study the small percentage of animals in a highly seropositive flock that remain seronegative for life. Although such animals may never have been exposed to OvLV or may have become infected early in life with development of tolerance to the virus, they also may possess innate resistance to OvLV infection, perhaps because of a lack of specific viral receptors. If this can be documented, the progeny of animals that remain seronegative in an infected flock should be selected for breeding stock. Of course, production characteristics of the foundation flock must also be taken into account.

Another selection strategy would be to propagate progeny of sheep that become infected by OvLV (and seroconvert) but do not develop disease. In flocks naturally infected with OvLV, a proportion of infected animals do not develop disease, suggesting that there are factors that make individuals resistant to OvLV-induced disease (DeMartini *et al.*, 1991). Previous research indicates that resistance or susceptibility to OvLV is the result of host genetic factors (de la Concha-Bermejillo *et al.*, 1995). Therefore, the identification of genetic markers that could be used to recognize disease-resistant animals would be a powerful tool for the development of resistant breeding stock. To identify genetic markers that correlate with disease resistance, families of sheep that have segregating alleles for resistance or susceptibility to OvLV-induced disease would need to be created. One approach would be to inoculate sheep that have different genetic backgrounds with OvLV and to evaluate their response, to estimate variation within these animals. Because signs of OvLV-induced disease are non-specific, usually develop late in life, and because animals would have to be killed to evaluate severity of disease, a marker for prediction of OvLV-induced disease would need to be used. Presently, virus load is considered the best predictor of the severity of disease (Brodie *et al.*, 1992b; de la Concha-Bermejillo *et al.*, 1995). In this scenario, the male with the highest response (high virus load) could be mated to the females with the lowest response (low virus load) and the male with the lowest response could be mated to the females with the highest response. The resulting F_1 progeny of these matings would each have alleles for resistance, from the low-response parent, and susceptibility, from the high-response parent. F_1 males then could be mated to unrelated females. The resulting paternal half-sib families of progeny would then be a useful resource for linkage analysis because there will be segregation of putative alleles. Genes that could be targeted for analysis in the created families include tumour necrosis factor-α (TNF-α), interleukin-1

(IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6) and MHC genes. All these genes have been shown to play an important role in the immune response against lentiviruses. A similar approach could be used to select animals that are resistant to infection.

Strategies for Developing Ovine Lentivirus-resistant Transgenic Sheep

Controlling an infectious disease by genetic modification of the host requires identification or development of a 'resistance gene' and methods for introducing that DNA into the germ line of animals such that it will be appropriately expressed in the required target cells. Ultimately, hemizygous founder animals bearing such transgenes must be amplified to generate populations of resistant animals by production of homozygotes, possibly through the use of embryo transfer and artificial insemination, or by deriving offspring from fetal or adult cells.

Methods for producing transgenic sheep

Production of transgenic mice has become a widely used and powerful technique for studying normal and abnormal gene expression (Camper, 1987; Hanahan, 1989). Most commonly, a sequence of DNA containing both regulatory and coding regions is cut from a plasmid and purified, then microinjected into one pronucleus of a recently fertilized, one-cell embryo. In a fraction of surviving embryos, the transgene has been integrated into the genome and is carried in both somatic and germ cells. Assuming the transgene integrates into a site that does not silence transcription, expression is dependent largely on the regulatory region of the transgene. For example, the visna LTR has been shown to direct expression of a reporter gene in macrophages, lymphocytes and the central nervous system of transgenic mice (Small *et al.*, 1989).

Microinjection of DNA has been used by several groups to produce transgenic sheep (Pursel and Rexroad, 1993; Clements *et al.*, 1994; Janne *et al.*, 1994). Success, as measured by the number of transgenic lambs obtained per injected ova, has been much lower than that obtained with mice, generally less than 1%. As a result of this low efficiency and the expense of animal maintenance, production of transgenic sheep is very much more costly than production of transgenic mice.

Another approach to production of transgenic animals is infection of the embryo with a recombinant retrovirus. Infection is followed at very high frequency by integration of the corresponding provirus, which typically contains a transgene flanked by retroviral LTRs. This technique has been used to produce transgenic mice (Van der Putten *et al.*, 1985; Soriano *et al.*, 1986), and infection of early bovine embryos with such viruses resulted in a high percentage of transgenic embryos and fetuses (Haskell and Bowen, 1995). Retroviral infection of early embryos is clearly an efficient means of producing

transgenic animals, but several important limitations exist, among which are the high incidence of mosaicism in transgenic offspring and the limited capacity of the virus for insertion of foreign sequences. None the less, this technique may be of value for production of transgenic sheep, particularly for research purposes.

A final strategy for production of transgenic sheep is to introduce the transgene into cells in culture, then use those cells to reconstitute a whole animal. Remarkable progress has recently been attained in cloning sheep by fusion of enucleated oocytes with fibroblasts. Use of fetal fibroblasts appears most useful, but one cloned sheep appears to have derived from fusion of an oocyte with a cell taken from an adult ewe (Wilmut *et al.*, 1997). If a transgene is introduced into the fibroblasts prior to fusion with oocytes, the resulting animals will be transgenic. Already this technique has been applied to production of sheep transgenic for human factor IX (Schnieke *et al.*, 1997), and initial studies suggest that it may be significantly more efficient than DNA microinjection for production of transgenic ruminants.

Several additional constraints must be considered with regard to producing populations of any type of transgenic sheep. First, even with the use of modern reproduction technology, such projects will take considerable time, particularly if homozygous breeding animals are desired. Secondly, one must expect that a fraction of transgenic animals produced will not express the transgene at levels sufficient to alter phenotype, and an additional number will have some type of defect, due to problems such as insertional mutagenesis. Finally, a disease-resistant sheep will be of marginal value if production characteristics are sacrificed or neglected during the years required to generate a population of transgenic animals.

Approaches to engineering lentivirus resistance

A number of gene therapy approaches have been developed against HIV. Most of these involve expression of genes that interfere with some aspect of the infectious process. This has sometimes been called intracellular immunization. Although it seems unlikely that gene therapy will ever be economically feasible in sheep, some of the intracellular immunization strategies might be the basis for transgenic strategies to modify the germ line to develop sheep that are resistant to ovine lentiviral infections.

Virus structural protein genes

One of the prominent strategies for developing virus resistance has been to develop transgenic organisms that express a critical structural protein of the virus. The rationale for this approach is that many viruses show interference to superinfection. That is, infection with one virus will prevent subsequent infection with related viruses. For example, expression of viral envelope glycoproteins has been used to protect chickens against avian leucosis retroviruses. Robinson *et al.* (1981) showed that some strains of chickens that carried defective endogenous proviruses with a type E envelope were resistant to

infection with other type E retroviruses. This resistance correlated with expression of the envelope glycoprotein of the endogenous virus. Transgenic chickens have been developed that express the type A envelope from a defective provirus (Crittenden *et al.*, 1989; Salter and Crittenden, 1989). These chickens are resistant to infection by subgroup A but not subgroup B avian leucosis virus. The molecular basis of this is thought to be due to interaction of the envelope protein with receptor proteins either on the surface of the cell or in the endoplasmic reticulum (Federspiel *et al.*, 1989; Delwart *et al.*, 1992). This interaction blocks the binding of the virus to receptor protein and therefore interferes with entry of the virus into the cell.

Replication interference between OvLV subtypes has been described (Jolly and Narayan, 1989). OvLV strains related by phenotype and genotype cross-interfered whereas different subtypes did not, thus permitting superinfection of cultured cells or animals by different lentivirus subtypes; the interference seemed to occur at the level of binding of the virus to the cell. By analogy with the avian leucosis virus system, transgenic sheep expressing the gene for OvLV envelope glycoprotein could be protected from OvLV infection. Three transgenic sheep containing the visna virus envelope gene under the control of the visna LTR promoter have been constructed (Clements *et al.*, 1994). The transgenic sheep were healthy, and no deleterious effects or clinical abnormalities from the transgene were observed. Expression of Env glycoprotein was observed in *in vitro* cultured differentiated macrophages and lymphocytes from the animals. Expression was also observed in a number of tissues including lung, spleen, brain and several others (Clements *et al.*, 1996). Two of the animals developed antibodies against the Env glycoprotein. Although lentivirus glycoproteins tend to cause cell fusion and syncytium formation *in vitro*, this did not seem to be a problem *in vivo*. To date, challenge studies to determine the susceptibility of these animals or their offspring to OvLV have not been reported.

Virus regulatory protein genes

A second strategy might be to express a viral non-structural protein in the transgenic animal. As discussed above, lentiviruses have regulatory proteins that modulate viral gene expression during an infection. Generally these are transacting factors that control the temporal order and tissue specificity of expression of the viral genes at either a transcriptional or post-transcriptional level. Cell culture studies suggest that expression of certain types of mutant regulatory proteins can interfere with the normal course of a viral infection. These mutants must be transdominant and thus able to disrupt the function of normal regulatory proteins in an infection. In both OvLV and HIV, the regulatory proteins of the *tat* and *rev* genes are necessary for a successful infection (Neuveut *et al.*, 1993; Toohey and Haase, 1994). The Tat protein is a transacting transcriptional regulatory protein required for efficient transcription from the LTR promoter. Rev protein seems to be necessary for transport of singly spliced and unspliced viral RNAs from the nucleus to the cytoplasm where they can act as mRNAs. During the course of an infection, the Rev protein must accumulate in order for the synthesis of viral structural proteins to

proceed. It seems to function by binding to a site, the Rev responsive element (RRE), in these RNAs to mediate their transport into the cytoplasm for translation. Certain HIV *tat* and *rev* mutants are deficient in their functions and are transdominant and thus prevent either synthesis or transport of RNA from the nucleus (Green and Ishina, 1989; Malim *et al.*, 1989). Thus cells and perhaps transgenic animals that express these mutant proteins would not allow the normal growth of the virus because of aberrant regulation at either the transcriptional or post-transcriptional level.

The visna virus Tat protein influences transcription from the viral LTR promoter and cellular promoters (Neuveut *et al.*, 1993). Thus the effect of dominant mutants on the expression of both viral and cellular genes must be considered. Transgenic mice that express the wild-type visna Tat protein have been constructed (Velutini *et al.*, 1994). These mice show lymphoproliferative disorders in several tissues, including lung, spleen, lymph nodes and skin. Whether transdominant *tat* mutants would cause these lesions is unknown. Domains of the Tat protein associated with transcriptional and pathogenic effects have been defined (Carruth *et al.*, 1994; Philippon *et al.*, 1994), and careful attention to these properties may allow the design and construction of mutant proteins with the desired properties.

The visna virus Rev protein may be an even more attractive target for intracellular immunization strategies. The Rev protein shares its amino-terminal 48 amino acids with the Env protein precursor, and the exon encoding the remaining 119 amino acids is contained within the *env* gene, though in a different reading frame from the Env protein. The transgenic sheep with the visna *env* gene described above (Clements *et al.*, 1994), therefore, carried a functional *rev* gene, and Rev protein expression was detected in the sheep. Indeed Rev protein is necessary for expression of Env protein (Schoborg and Clements, 1994; Toohey and Haase, 1994). Since the transgenic sheep did not show any obvious pathological effects, it does not appear that *rev* expression is deleterious to the sheep, and it seems unlikely that the expression of transdominant *rev* mutants would cause problems. Two other anti-Rev strategies have been devised for intracellular immunization against HIV (Inouye *et al.*, 1997). Single-chain antibodies have been constructed that bind Rev protein and interfere with its function in the cell. Also an RNA decoy consisting of the minimal Rev-binding domain of the RRE was developed. When either of these elements was expressed in cultured cells, HIV replication was significantly reduced, and when both were expressed together, HIV production was almost eliminated. Transgenic sheep expressing similar constructs targeting the visna Rev protein might be resistant to OvLV infection.

Viral antisense RNA

Another strategy for interfering with viral infections is to express antisense RNA representing portions of viral RNA. Antisense RNA presumably forms a double-stranded RNA with the target RNA which can disrupt normal function of the target in the cell. Several naturally occurring antisense mechanisms have been described.

1. Antisense RNA can prevent the normal folding of an RNA molecule. The frequency of initiation of plasmid Col E1 DNA replication is controlled by an antisense mechanism (Polisky, 1988). In this case, the RNA primer for replication must be folded into a certain conformation to act as a primer. An antisense RNA to a portion of the primer prevents this folding and thus regulates the frequency of initiation.

2. Antisense RNA can interfere with translation of an mRNA. The frequency of translation of the mRNA for the Tn10 transposase is controlled by an antisense RNA that binds to the ribosome-binding site of the mRNA and prevents initiation of translation (Simons and Kleckner, 1983).

3. Antisense RNA can target an RNA molecule for modification and perhaps degradation. In *Xenopus* oocytes, a portion of the gene for basic fibroblast growth factor is transcribed in both directions. This allows association of the two transcripts in this region, which is then a substrate for a modifying enzyme that converts adenine residues in double-stranded RNA to inosines. This disrupts the double strands and results in inactivation of the mRNA (Kimelman and Kirschner, 1989).

The design of artificial antisense mechanisms for altering the course of virus infections has been attempted for several viruses, including HIV. Vectors that express antisense RNA to various portions of the HIV genome have been designed and used to stably transform cultured cells (Liu *et al.*, 1997; Veres *et al.*, 1998). Although these cells are resistant to challenge by HIV to various degrees, there is still considerable room for optimization and refinement of the expression constructs.

Ribozymes

A variation on the antisense strategy is to design and express ribozymes that cleave viral RNA within the infected cell. Certain RNA molecules have been shown to be capable of performing enzymic reactions. Among these are several plant satellite virus and virusoid and viroid RNAs that cleave their own replicative form RNAs during replication to generate progeny genomes. Based upon studies to locate and define the active sites of these self-cleaving RNAs (Forster and Symons, 1987; Hampel and Tritz, 1989), it has been possible to design ribozymes that bind RNA targets by base pairing and carry the active site for cleaving the RNA at the binding site (Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Hampel *et al.*, 1990). Since these ribozymes are RNA molecules themselves, they could be expressed in cells and would cleave any substrate RNA that they encounter. Cleavage of HIV-1 genomes by ribozymes has been reported in HIV-infected cell cultures (e.g. Sarver *et al.*, 1990; Gervaix *et al.*, 1997; Smith *et al.*, 1997), and it seems likely that ribozymes could be designed against ovine lentiviruses as well. Once again there is considerable opportunity for refinement of ribozyme strategies. For example, targeting the ribozyme to the appropriate intracellular compartment is critical to successful knockout of viruses by ribozymes (Sullenger and Cech, 1993).

Other strategies

An additional strategy to develop resistant transgenic animals would be to design a toxin gene which would be expressed only in virus-infected cells (e.g. under control of virus-specific transactivating factors). Such cells would commit suicide by expressing the toxin before allowing the virus to multiply. This would prevent the spread of the virus to other cells. Another possibility would be to express a soluble form of the virus receptor which would circulate in the bloodstream and bind virus before it could infect cells, and thus protect the animal. This receptor could also be coupled to antibody Fc regions to assist in destruction of virus or infected cells by the immune system (Byrn *et al.*, 1990). These types of experiments are highly speculative and little has been done to test these concepts at the organism level.

Acknowledgements

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