77

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**Evaluation of Naked Proviral DNA as a Vaccine for
Ovine Lentivirus Infection**

**J.C. DeMartini, J.O. Carlson, L.E. Pearson, J.K. Young, A. Yaniv,
A. Gazit, K. Perk, A. Friedman, N. Safran**

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Investigators' Names
(Principal listed first)

James C. DeMartini
Jonathan O. Carlson
Leonard D. Pearson
Jamie K. Young
Abraham Yaniv
Arnona Gazit
Kalman Perk
Aharon Friedman
Noam Safran

Investigators' Institutions

Colorado State University
Colorado State University
Colorado State University
Colorado State University
Tel Aviv University
Tel Aviv University
Hebrew University
Hebrew University
Hebrew University

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Signature
Principal Investigator

Signature
Institution's Authorizing Official

TABLE OF CONTENTS

ABSTRACT	3
INTRODUCTION	3
MATERIALS AND METHODS	5
RESULTS	14
Table 1. Summary of Results of Inoculation of Lambs with pKV72 Complexed with Liposomes DDAB/DOPE or Virus Derived from the Molecular Clone KV72	16
Fig. 1. Chloramphenicol acetyl transferase (CAT) expression in serum.	17
Table 2. Summary of Results of Inoculation of Lambs with Ovine Lentivirus Strain KV72 pCMVgag or pCMVenv Constructs and Intravenous Challenge	19
DISCUSSION	19
REFERENCES	22
DESCRIPTION OF COOPERATION	23
EVALUATION OF RESEARCH ACHIEVEMENTS	23
CONCLUSIONS	24
LIST OF PUBLICATIONS	24

FINAL REPORT

ABSTRACT

Ovine lentivirus (OvLV) infection is widespread in sheep of the United States and Israel and is responsible for substantial economic losses. The primary goal of this project was to evaluate naked proviral DNA as a vaccine to induce protective immunity in sheep in endemic areas. Contrary to expectations, inoculation of sheep with proviral DNA derived from the full length OvLV molecular clone pkv72 did not result in detectable OvLV infection, but infectious virus was recovered from transfected ovine cells. Kv72 virus produced by these cells infected sheep and induced antibody responses, and was used as a viral challenge in subsequent experiments. To improve *in vivo* transfection efficiency and compare the viral LTR with other promoters, expression of reporter genes was studied in sheep transfected *in vivo* by injection of cationic liposome-DNA complexes; one formulation produced gene expression in a sheep for 4 months following a single intravenous injection. Since the *pol*-deleted OvLV construct was not stable *in vitro*, twelve lambs were injected with plasmids containing the Kv72 *gag* region (pCMVgag) or *env* region (pCMVenv), or saline. Prior to challenge, no detectable anti-OvLV immune responses were detected. Following homologous challenge with OvLV, no significant differences were found between groups and all sheep became infected with OvLV. Although the naked DNA approach to vaccination holds promise for control of ovine lentivirus-induced disease, further work needs to be done to develop more effective methods of transfecting sheep with DNA.

INTRODUCTION

Ovine lentivirus (OvLV) infection is widespread in sheep of the United States and Israel. Considerable economic losses can result from diseases caused by OvLV and from restriction of export of infected animals (Brodie *et al.*, 1992). Presently, no vaccine exists to protect sheep against OvLV infection in endemic areas. One of the main features of lentiviral pathogenesis is virus persistence and spread in the face of the host-mediated immune response. Lentiviruses are also characterized by a high rate of mutagenesis, resulting in the development of quasispecies within a given host, and genetically diverse field strains. Both cytotoxic T lymphocyte (CTL) responses and humoral responses are believed to be important for protection against lentivirus infection (Girard *et al.*, 1996), but attempts to develop vaccines against other lentiviruses, such as human immunodeficiency virus (HIV), have been disappointing. An ideal candidate vaccine might be viewed as one that is replication incompetent, capable of eliciting immune responses against a wide range of viral epitopes, capable of stimulating both CTL and humoral responses, and of producing a long lasting immune response.

Since the early 1990's, a new vaccination strategy involving the inoculation of DNA has been actively explored against numerous different viruses, including lentiviruses. DNA vaccines potentially offer long term, broad immune responses like modified-live vaccines, yet with the safety of inactivated vaccines or subunit vaccines. Instead of using the actual antigen as the vaccine, the inoculum is plasmid DNA expression vector(s) encoding the antigen(s) of choice. *In vivo* transfection results in expression of these foreign proteins within the host. This *in situ* synthesis allows for protein processing, post-translation modification and presentation of the antigen(s) to the host's immune system in a manner similar to that which would occur during natural infections (Fynan *et al.*, 1995). DNA vaccines have been shown to elicit long term immune responses, often with only a single inoculation, cross protect against heterologous strains of viruses (Ulmer *et al.*, 1993), preferentially elicit a strong cytotoxic T cell responses (Whalen *et al.*, 1995) and to protect against some persistent viral infections ((Martins *et al.*, 1995).

Infectious molecular clones allow one to synthesize various expression constructs, vaccines and homogeneous virus inocula for controlled studies. By serial passage in sheep, Lutley *et al.* selected for an especially neurovirulent substrain KV1772 (Lutley *et al.*, 1985). Using high molecular weight DNA from cells infected with KV1772, Andresson *et al.* constructed the infectious, pathogenic proviral molecular clone KV72-67r from the infectious clone kv72 and clone kv67 which had a complete 3' LTR, unlike kv72. Two constructs, named pkv72-67r and pLGkv72-67r, containing stable, full-length, viral genomes were constructed using low copy number vectors. Plasmid DNA from the above constructs was successfully transfected into various permissive cells, and OvLV was isolated starting 7 days post-transfection. Icelandic lambs were infected by intracerebral inoculation of 4×10^7 TCID₅₀ of KV72 virus derived from transfected cells. Five of five lambs became infected and developed lesions characteristic of OvLV infection. Infections were characterized by strong, rapid neutralizing antibodies (Andresson *et al.*, 1994). Thus, this newly described molecular OvLV clone appeared to be an excellent candidate from which to develop DNA vaccines against OvLV.

The objectives of this work were to:

- 1) Examine the immunological and viral consequences of inoculating sheep with plasmid DNA containing an infectious OvLV molecular clone,
- 2) Construct a non-infectious OvLV molecular clone and produce bacterially synthesized OvLV peptides,
- 3) Examine the immunological and viral consequences of inoculating sheep with plasmid DNA encoding a non-infectious molecular clone,

4) Determine whether sheep inoculated with plasmid DNA encoding a non-infectious molecular clone are resistant to homologous or heterologous OvLV challenge.

As the experimental results became available, we deviated from some of these objectives. Since the full-length OvLV molecular clone was shown not to be infectious for sheep (Objective 1), attempts were made to improve *in vivo* transfection efficiency using plasmid DNA containing reporter genes in cationic liposome:DNA complexes. The *pol*-deleted OvLV (Objective 2) was not stable, so non-infectious *gag* and *pol* OvLV constructs were prepared separately and used to transfect sheep (Objective 3). Once an optimal/minimal OvLV homologous challenge dose was established, the remainder of the work was carried out as planned.

MATERIALS AND METHODS

Seroepidemiology and selection of research sheep

For the U.S. study, blood was collected from animals in two well-managed flocks with a history of minimal OvLV infection. Sera was examined by agar gel immunodiffusion for anti-OvLV antibodies. A seroepidemiology flock survey was performed in Israel (Dr. Perk) by collecting representative blood samples from twenty two flocks distributed throughout the country. One well-managed, isolated flock (E.Ch.) that appeared to be free of OvLV provided the lambs for study.

Cells

For the U.S. research, sheep choroid plexus (SCP) cells were purchased from American Tissue Culture Collection (Rockville, MD). The other primary cell cultures such goat synovial membrane (GSM), ovine cornea cells (OC), fetal ovine lung (FOL), ovine skin fibroblasts (OSF) were established in our laboratory. Cells were routinely grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 5-10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin. In Israel, Himalayan tahr (mountain goat) ovary cells were routinely grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum.

Plasmids

pLGkv72-67r and pACkv72-67r, henceforth referred to as pkv72, both of which contain the full-length proviral OvLV clone were kindly provided by Dr. O. Andresson. Plasmid DNA was purified using anionic exchange columns (Qiagen, Chatsworth, California). pACkv72 Δ RT, pCMV*gag* and pCMV*env* expression vectors were constructed by Drs. A. Yaniv and A. Gazit. pCMV-CAT and pCMV-huG-CSF were kindly provided by Dr. R. Debs. pVIS-LTR-CAT was kindly provided by Dr. J. Clements. Plasmid DNA were purified by alkaline lysis and polyethylene glycol (PEG) precipitation.

Plasmid DNA was quantified using OD₂₆₀ readings, and DNA purity was assessed by OD_{260/280} ratios and restriction endonuclease digestions.

pCMVgag and pCMVenv were constructed in Israel using the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) which utilizes the CMV enhancer-promoter sequences, and the polyadenylation signal and transcriptional termination sequences from the bovine growth hormone gene. The entire gag region (nt 388-2029) or entire env region (nt 5839-7768) were amplified by PCR using the Expand High Fidelity PCR system (Boehringer Mannheim Biochemica, Indianapolis, IN). PCR was performed in the presence of forward and backward specific primers containing appropriate restriction sites to facilitate the insertion downstream of the CMV promoter. The nucleotide sequences were verified by sequence analysis. Highly efficient canine cells were transfected with pCMVgag or pCMVenv, and stably transfected cells were selected by neomycin resistance. Lysates of these stably transfected cells were analyzed by immunoblot using rabbit anti-OvLV anti-serum. pkv72, pCMVgag and pCMVenv were purified by using anionic exchange columns (Quiagen), and provided for *in vivo* studies.

In vitro transfection of ovine cells with pLGkv72-67r and pACKv72-67r

In the U.S., sheep choroid plexus (SCP) cells were grown to 75-80% confluence in 6-well tissue culture-treated plates. Transfection was facilitated with the cationic liposome LipofectAMINE (Gibco BRL, Gaithersburg, Maryland). Plasmid constructs pLG72-67r and, pAC72-67r, and positive control pCMVβgal (provided by Dr. J. Carlson), were diluted in Opti-MEM (Gibco BRL, Gaithersburg, Maryland) and added to diluted LipofectAMINE. Diluted complexes containing 3 or 5 µg of DNA, or diluted lipofectAMINE as a negative control, were added to separate wells of washed SCP cells. SCP cells were incubated with diluted DNA/liposome complexes in serum-free Opti-MEM for 5-6 hours, and then the medium was replaced with DMEM supplemented with 10% FBS, L-glutamine and penicillin/streptomycin.

In Israel, Himalayan tahr ovary cell monolayers, 24 hours after passage, were transfected with 5 µg of pkv72 DNA, using a calcium phosphate precipitation technique. As a positive control, cells were transfected with 10 µg of high molecular weight DNA obtained from OvLV-infected Himalayan tahr ovary cells. 48 hours later, the transfected cells were co-cultivated with polybrene (10 µg/ml)-treated naive tahr cells. Supernatants were harvested 7, 14 and 21 days post-transfection for RT assay.

Morphogenesis of kv72 virus clone

Infected Himalayan tahr ovary cells and ovine fetal corneal cells, at different stages of infection, were examined by electron microscopy in Israel by Dr. Perk.

Liposomes

The cationic lipid dimethyldioctadecyl ammonium bromide (DDAB) (Sigma Chemical Co, St. Louise, MO) and the neutral lipid dioleoyl-phosphatidylethanolamine DOPE (Avanti Polar Lipids, Alabaster, AL) in a 1:1 molar ratio were evaporated to dryness under nitrogen gas. Deionized water was added, and the mixture was sonicated to produce small unilamellar vesicles (SUV). DOTIM-cholesterol multilamellar vesicles (MLV) were provided by Dr. R. Debs.

Preparation of DNA-liposome complexes for inoculation

In the U.S. studies, DDAB-DOPE SUV liposomes, shortly before inoculation, were further diluted in deionized water and mixed with DNA diluted in deionized water (1 μ g DNA/16 nmoles total lipids). A 500 μ g dose of DNA plus liposomes was inoculated in an 8 ml total volume. Two batches of DOTIM-cholesterol MLV liposomes already complexed to pCMV-CAT or pCMV-huG-CSF in 5-10 ml volume were shipped and stored at 4°C prior to use. For the final pCMV-CAT and pVIS-LTR-CAT experiment, a stock of DOTIM-cholesterol MLV liposomes were shipped and stored at 4°C prior to complexing with plasmid DNA prepared in our laboratory. Shortly before inoculation, plasmid DNA was diluted in 5% dextrose and 1 mg of DNA was complexed with 5 ml of lipid stock (1 μ g DNA/16 nmole total lipids). Significant aggregation of DNA/liposome complexes was not noted in any sample prior to inoculation.

Inoculation of sheep with OvLV proviral DNA or virus derived from the molecular clone KV 72

At the U.S. research site, a total of seventeen, 3-8 week old lambs were divided into three groups: 2 negative control lambs (95RS08, 95RS09) received liposomes only, 8 lambs (94RS9, 94RS10, 95RS10, 95RS11, 95RS12, 95RS13, 96RS00, 96RS20) received pkv72 DNA, and 7 lambs (94RS11, 95RS14, 95RS15, 95RS16, 95RS17, 96RS19, 96RS21) received kv72 virus. Each group of sheep was housed separately in raised pens indoors.

Six lambs were inoculated with 500 μ g of cationic liposome-complexed plasmid DNA. Shortly before inoculation, DDAB-DOPE SUV liposomes were further diluted in deionized water and mixed with DNA diluted in deionized water (1 μ g DNA/16 nmoles total lipids). A 250-500 μ g dose of DNA complexed with cationic liposomes was inoculated in an 8 ml total volume. For the '95 lambs and 94RS9, the 500 μ g dose was divided intramuscularly (IM) and intravenously (IV). The '96 lambs received the entire 500 μ g IV, and 94RS10 received 250 μ g dose intratracheally. IM inoculations were given in the semitendinosus/semimembranosus muscles. All IV inoculations were given slowly into the cephalic vein using a butterfly catheter. The negative control lambs received 4 ml liposomes further diluted in 4 ml of deionized water via IV and IM injection.

The KV 72 virus-inoculated lambs received 3×10^5 or 1×10^6 TCID₅₀ kv72 virions grown in sheep choroid plexus cells, in 4 ml of conditioned tissue culture medium via IV injection. The dose of virus was increased after a small pilot experiment determined that 2000 TCID₅₀ kv72 virions did not result in detectable OvLV infection in the California variegated mutant crossbred sheep chosen for this project.

Every 1-3 weeks, blood was collected for sera and isolation of PBMC. Bronchoalveolar lavage (BAL) cells were collected at the time of necropsy (*ex vivo* BAL), and for some lambs, BAL cells were collected at an earlier time point from anesthetized animals via bronchoscopy (*in vivo* BAL). At the termination of the experiment, sheep were euthanized, and BAL cells, and tissues were collected. BAL cells were collected by *ex vivo* lavage using 750-1000 ml of cold Hank's buffered saline solution (HBSS) supplemented with 1% FBS, and then BAL cells were concentrated by centrifugation. Samples of lung, spleen, rib (for bone marrow) were refrigerated for virus isolation and frozen for nucleic acid isolation. Samples of lung, caudal mediastinal lymph node, left prescapular lymph node, thymus, brain, synovium from the left carpus, mammary gland, spleen, liver, kidney, rib (for bone marrow) and small intestine were fixed in 10% buffered formalin for histopathology. The left lung was fixed by insufflation with formalin.

In Dr. Perk's studies in Israel, four lambs were inoculated with pkv72 DNA, 4 lambs were inoculated with kv72 virus, and a total of 6 lambs were inoculated with liposomes only. Blood was collected day 1, 2 weeks, one month, two months and three months post-inoculation.

Inoculation of sheep with DNA reporter gene-liposome complexes

This experiment was designed in the U.S. to test *in vivo* transfection of sheep using expression vectors encoding reporter genes prior to attempting DNA vaccination using constructs derived from the OvLV molecular clone. Two reporter gene vectors expressing chloramphenicol acetyl transferase (CAT) and human granulocyte colony stimulating factor (huG-CSF), pCMV-CAT (p4119) and pCMV-huG-CSF had already been successfully used for *in vivo* transfection studies in mice (Liu *et al.*, 1995). A second reporter gene expression vector, pVIS-LTR-CAT, had been utilized for *in vitro* transfection studies of lentiviral LTR promoters (Hess *et al.*, 1986).

pkv72 complexed with DDAB/DOPE SUV liposomes: Five weanling California variegated mutant-cross lambs (94RS9, 95RS10, 95RS11, 95RS12, 95RS13) were inoculated with 500 μ g of plasmid DNA divided IV and IM. One weanling California variegated mutant-cross lamb (94RS10) was inoculated intratracheally with 250 μ g of plasmid DNA. Two, slightly younger, 5 week old lambs, one Rambouillet (96RS00), one California variegated mutant-cross (96RS20) were inoculated IV with 500 μ g plasmid DNA. At multiple time points, blood was collected for sera and isolation of peripheral blood

mononuclear cells (PBMC). Bronchoalveolar lavage (BAL) cells were collected at necropsy (*ex vivo* BAL), and for some lambs, from anesthetized animals via bronchoscopy (*in vivo* BAL). At necropsy, samples of spleen, lung, and bone marrow were collected for virus isolation or frozen at -70°C for extraction of DNA to be used for PCR.

pCMV-CAT and pCMV-huG-CSF complexed with DOTIM-cholesterol MLV liposomes: four lambs (96RS11, 96RS19, 96RS20, 96RS21) were inoculated at two different time points 4 months apart with inoculum prepared "ready-to-use" and shipped to use by Dr. R. Debs (University of California, San Francisco). Lambs were bled at 24 h and 48 h post-inoculation. Serum and PBMC were collected and frozen at -70°C for analysis of CAT protein levels.

<u>Lamb #</u>	<u>5/8/96 inoculum</u>	<u>9/17/96 inoculum</u>
96RS00	1 mg pCMV-huG-CSF	1 mg pCMV-CAT
96RS19	1 mg pCMV-CAT	3 mg pCMV-CAT
96RS20	1 mg pCMV-huG-CSF	1 mg pCMV-huG-CSF
96RS21	1 mg pCMV-CAT	1 mg pCMV-huG-CSF

pCMV-CAT and pCMV-VIS-LTR-CAT complexed with DOTIM-cholesterol MLV liposomes : Four 4-9 day old Rambouillet or Rambouillet-cross lambs were inoculated IV with either 1 mg pCMV-CAT (96RS27, 96RS28) or 1 mg pVIS-LTR-CAT (96RS26, 96RS30) plasmid DNA. Lambs were bled on day 0, 2, 4, 6, 8, 10 post-inoculation. Sera were collected at all time points. PBMC were isolated from the final sampling. All samples were later analyzed for CAT protein levels.

Serum and PBMC were shipped frozen to Dr. D. Liggitt (University of Washington, Seattle), whose laboratory performed the CAT and G-CSF assays. CAT was quantitated by ELISA and reported as pg/ml. HuG-CSF was also quantitated by ELISA and reported as pg/ml. Samples were run in triplicate.

Inoculation of sheep with plasmid DNA containing non-infectious kv72 constructs

Although pACKvΔRT DNA was the original choice for DNA vaccination, this construct could only be replicated in a very low copy number plasmid. Thus, purification of sufficient quantities of DNA for a vaccination trial would have been difficult. Also, in light of the poor transfection efficiency with pkv72 and the increasing number of articles reporting induction of good immune responses using pCMV expression vectors, it was decided that pCMVgag and pCMVenv were superior choices as DNA vaccines. In research carried out in Colorado, 4 ml of 1 mg/ml pCMVgag or pCMVenv plasmid DNA was mixed shortly before use with 440 μ l of sterile 10x phosphate buffered saline (PBS) to make a 1x saline solution. 1.1 ml of plasmid DNA (1 mg) in 1x PBS was used to vaccinate each lamb. Approximately half of the

dose was inoculated IM into the left semimembranosus/semitendinosus muscles and the remaining dose was inoculated ID into multiple sites immediately caudal to the left ear. The IM/ID route, without the use of cationic liposomes, was chosen based on multiple published reports of success using this technique, whereas IV inoculation liposome-complexed DNA had been primarily used to demonstrate expression of encoded genes (i.e. for the purpose of gene therapy) but immune responses had not been well characterized for this route. The primary DNA vaccination was administered when the lambs were less than 1 week old. Identical booster inoculations were administered 3 weeks and 6 weeks following the primary inoculation for a total of three vaccinations per lamb. Four lambs were inoculated with pCMVenv (97RS9, 97RS19, 97RS11, 97RS12), 4 lambs were inoculated with pCMVgag (97RS5, 97RS6, 97RS7, 97RS8) and four control lambs (97RS1, 97RS2, 97RS3, 97RS4) were inoculated with saline.

In the Israeli studies carried out by Dr. Perk, twelve lambs were divided into three groups: 6 lambs were inoculated with pCMVgag, 4 lambs were inoculated with kv72 virus, and 2 lambs were inoculated with liposomes only. An additional 6 sheep had been inoculated with pCMVgag DNA at an earlier time point. Blood was collected from all animals on day 1 after inoculation, at 2 weeks, and at 1, 2 and 3 months.

OvLV challenge of sheep inoculated with plasmid DNA encoding non-infectious kv72 constructs

All twelve lambs previously inoculated with pCMVgag, pCMVenv or sham vaccinated with saline in Colorado were challenged 5-6 weeks after the last vaccination, by intravenous inoculation with 5×10^6 TCID₅₀ kv72 virions. This challenge dose was considerably higher than the 1000 TCID₅₀ dose originally proposed. In a pilot experiment, an intravenous dose of 2000 TCID₅₀ kv72 virions was not infectious in the one test lamb. Although 6/6 lambs inoculated with 1×10^6 TCID₅₀ kv72 virions all became infected, but it was difficult to re-isolate virus from these lambs. With the small sample number, it was critical that the challenge dose be 100% infectious; therefore, the higher dose was chosen. Blood was collected weekly for the first month, then every 3-4 weeks until euthanasia 4 months after challenge.

In Israel, the second group of six sheep inoculated with pCMVgag and the group of 6 sheep inoculated at the same time with pkv72 were split in two. At 12 weeks post-inoculation, three pCMV-inoculated sheep and three pkv72-inoculated sheep were challenged with 1000 TCID₅₀ kv72 virions (homologous challenge) and three pCMV-inoculated sheep and three pkv72-inoculated sheep were challenged with 1000 TCID₅₀ of a local field strain of OvLV (heterologous challenge). Blood was collected monthly for 4 months.

Agar gel immunodiffusion

Either fresh or frozen sera was used. AGID was performed using a commercial diagnostic kit in the U.S. according to the manufacturer's directions (Veterinary Diagnostic Technology, Inc., Wheat Ridge, CO). Lines of immune complex precipitation were graded 1+, 2+, 3+ or 4+, with 4+ being most prominent and closest to the antigen wells.

Immunoblotting assay

For the immunoblotting assay in the U.S., kv72 and OvLV 85/24 viral antigens were concentrated 200 fold from cell culture supernatants by ultracentrifugation and a 1:1 mixture was used. The kv72 antigen had capsid antigen (CA) levels by immunoblotting, but poor envelope glycoproteins (SU). The OvLV 85/34 antigen contained a lower concentration of CA but had very good SU glycoproteins. Sera from kv72-infected lambs cross-reacted with OvLV 85/34 CA and SU antigen. The antigens were resolved using a 4-20% Tris-buffered gradient gel (Redi-gel, Bio-Rad Laboratories, Richmond, CA) under denaturing conditions, and the separated proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Richmond, CA). The transblotted membrane was blocked overnight in 5% nonfat dry milk (NFDM) and 0.2% TWEEN 20 in PBS pH 7.4 at 4°C. Using a miniblott apparatus (Immunoetics, Cambridge, MA), the blot was incubated for 2 hours at room temperature with sera diluted 1:100 in 3% NFDM. After the membranes were washed in 0.2% TWEEN 20 in PBS pH 7.4, the blot was incubated for 1 hour at room temperature in 1:1000-1:2000 horse radish peroxidase-conjugated anti-sheep IgG secondary antibody, washed again, and reacted with the DAB chromagen substrate. Antibody reactions to SU (gp105), CA (p25) and matrix protein (MA,p16) were detected visually. Generally, a positive immunoblot reading consisted of 2 or more positive bands, one of which was p25.

Enzyme linked immunosorbant assay (ELISA)

Drs. York and Boshoff kindly provided OvLV TM40-p25 recombinant fusion protein antigen-coated 96-well plates, reference sera and some reagents for a previously described ELISA (Boshoff *et al.*, 1997). The assay was performed as described, with modifications in the secondary antibody and reference reagents. A different rTM40 ELISA was performed by F. Mbithi using the rTM40 antigen and a modification of the technique originally described by Kwang *et al.* (Kwang, 1993).

In Israel, an OvLV antigen was purified by isopycnic sucrose gradient centrifugation. Sera was diluted 1:100, and two negative sera (newborn and negative 3 year old doe) and two positive sera (low and high titer) were used as controls in each plate. Serum samples were tested in duplicate. A

horseradish peroxidase-conjugated secondary antibody was used according to recommendation, followed by a 30 minute incubation with ABTS as a substrate. Optical density readings were analyzed as described by Archambault (Archambault *et al.*, 1988).

Virus isolation

Virus isolation was attempted in the U.S. from PBMC, BAL cells, and from samples collected at necropsy. From EDTA-anticoagulated blood, PBMCs were isolated using a 1.077 density gradient (Histopaque 1.077, Sigma Chemical Co, St. Louis, MO). Some BAL cell were collected from the '95 by bronchoscopy of anesthetized sheep. At necropsy, splenocytes and BAL cells were collected. Using 25 cm² tissue culture flasks, 1 x 10⁷ PBMCs, 1 x 10⁶ splenocytes, or 1 x 10⁵ BAL cells were co-cultured with a 50-60% confluent monolayer of OSF in DMEM supplemented with 10% FBS, 20mM HEPES, 5 x 10⁻⁶ 2-mercaptoethanol, 2 mM/L L-glutamine and penicillin/streptomycin. Cells were passaged routinely every 5-7 days, and observed for 4 weeks for cytopathic effects (CPE).

In Israel, PBMC were isolated from the buffy coat of sodium heparin sulfate-anticoagulated blood using Ficoll-Paque (Pharmacia) density gradients. Cells were resuspended in DMEM containing 20% heat-inactivated FBS and seeded in 10 cm² tissue culture dishes. Monocytes were allowed to adhere for 16 hours, after which the non-adherent cells were removed and fresh medium was added to the plates. In some cultures, 1.5 x 10⁶ indicator cells (Himalayan tahr ovary cells) were added. All cultures were monitored by light and electron microscopy and supernatant reverse transcriptase (RT) activity.

PCR amplification of viral nucleic acids

In Colorado, total DNA was isolated from PBMC, co-cultured cells or tissue using either a Puregene kit (Gentra Systems, Minneapolis, MN) or Tri-Reagent (Sigma Chemical Co., St. Louis, MO) per manufactures's directions. Oligonucleotide PCR primer pairs from either the *env* region or the LTR were used. A standard 50 μ l reaction mix contained 1 x PCR buffer (Gibco BRL, Gaithersburg, MD), 1.5 mM MgCl₂, 0.2 mM each dNTP, 50 pM of each primer, 1.25 U *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD). Often the upstream primer would be exchange labeled with ³²P. Using a Perkin Elmer GeneAmp PCR 9600 thermocycler (Norwalk, CT), the samples were initially denatured at 95°C for 2 minutes, and then amplified for 30 cycles, consisting of 95°C denaturation for 30 seconds, followed by 50°C annealing for 30 seconds, followed by 72°C elongation for 30 seconds. The final 72°C elongation was for 5 minutes. Amplified PCR products were detected either by EtBR-stained agarose gel electrophoresis or by polyacrylamide gel electrophoresis and autoradiography using intensifying screens. The LTR primer pair (1097/1098) yielded a 330 bp product. The *env*1 (59/61) primer pair yielded a 350 bp product.

Total RNA was isolated from PBMC stored at -70°C as a dry cell pellet using Tri-Reagent (Sigma Chemical Co., St. Louis, MO) per manufactures's directions. 0.2-0.3 μg of RNA (as determined by OD_{260} absorbance) was reversed transcribed in a 25 μl reaction mix containing 1x PCR buffer (Gibco, BRL, Gaithersburg, MD), 1.5 nM MgCl_2 , 0.2 nM each dNTP, 25 pM of a primer, 0.5 U RNasin (Promega, Madison, WI), 0.5 U AMV-RT (Boehringer Mannheim, Indianapolis, IN) for 45°C for 2 h followed by 90°C for 5 minutes. The entire 50 μl RT product was amplified by PCR using this protocol.

Infectious center assay (ICA)

In the U.S. studies, using glass chamber slides pre-coated with type I collagen, 5×10^4 GSM cells/well were incubated for 24 hours. The media was then aspirated, and the GSM cells were co-cultured with 10^6 PBMC, 10^6 splenocytes or 10^4 *ex vivo* BAL cells, four wells per sample. Negative control wells contained media only. Positive control wells were infected with kv72 virus (MOI of 0.01). After 24 hours co-cultured, non-adherent cells were aspirated and the wells washed two times with media. The GSM cells were re-fed and routinely cultured for 5 more days, then fixed for 10 minutes in cold acetone and frozen at -70°C . Virus-infected foci were identified by immunohistochemistry using a monoclonal antibody (2F, generated by K. Marcom and L.D. Pearson) against OvLV capsid protein.

Lymphoproliferation assay

In the Colorado DNA vaccine study, PBMCs were isolated at 5 different time points: one day prior to challenge (6/10/97), 9 days post-challenge (6/20/97), 5 weeks post-challenge (7/17/97), 10 weeks post-challenge (8/21/97) and 16 weeks post-challenge (10/2/97). Freshly isolated PBMCs were cultured in the presence of antigen or mitogen for a total of 6 days. 18 hours prior to harvest, cells were pulsed with 1 μCi of tritiated thymidine. The antigens used were recombinant p25 (1 μg per well), recombinant TM40 (1 μg per well) and a crude kv72 whole virus antigen concentrated from cell culture media (5 μl per well). Endotoxin-free rp25 and rTM40 were kindly provided by Dr. J. Kwang. All antigens (rp25, rTM40, kv72) were aliquoted from single stocks, and one aliquot was used per assay. Negative control cells were cultured with media only. Positive control cells were cultured with concanavalin A (4 μl per well). Prior to harvesting, all wells were examined grossly and microscopically for evidence of bacterial contamination. Data from the rare well noted to be contaminated were not included in the calculations. Cells were harvested with a 96-well cell harvester onto glass fiber membranes, and beta radiation counts were determined for each sample. Triplicate samples were assayed, and means and standard deviations calculated for each data set. If one of the three values within a data set was substantially different than the other two values, it was not included in the calculation of the mean and standard deviation. Stimulation indices (SI) were calculated by dividing the mean of the sample wells by the mean of the control wells.

Histopathology

In Colorado, lung samples from all three left lobes of formalin-insufflated tissues, in addition to samples of caudal mediastinal lymph node, periscapular lymph node, thymus synovial membrane, mammary gland, brain including choroid plexus at the level of the lateral and fourth ventricles, and any additional lesions noted at necropsy were routinely paraffin embedded, and 5µm sections were stained with hematoxylin and eosin (H&E). In a blinded study, sections were examined by light microscopy and lesions compatible with OvLV infection were scored. In Israel, all animals were necropsied and histopathology performed

RESULTS

Seroepidemiology and selection of research sheep

By AGID, all sheep within the two selected flocks in the U.S. work were seronegative for OvLV. The smaller flock, composed primarily of California variegated mutants crossbred sheep, provided lambs for '94, '95 and '96. This flock was disbanded in 1996. The larger flock composed of purebred Rambouillet sheep provided lambs for '96 and '97. Nineteen of twenty-two flocks studied in Israel contained individuals that were seropositive for OvLV. However during the period of the study, according to the flock managers, no animals with the typical characteristics of OvLV had died.

Plasmids

Drs. Yaniv and Gazit found pACKvΔRT to be stable only when inserted in the low copy number pACYC expression vector. Using an immunoblot assay and anti-visna serum, expression of *gag* or *env* proteins were detected in lysates of pCMVgag and pCMVenv stably transfected cells. pCMVgag expressed a 35 and 22 kda gag-specific proteins.

In vitro transfection of ovine cells with pLGkv72-67r and pACKv72-67r

In the U.S. work, on day 7 post-transfection, confluent cells were trypsinized and expanded into 25 cm² tissue culture flasks. On day 8 post-transfection, a few syncytial cells were observed in all wells transfected with pkv72 DNA. The cytopathic effect (CPE) progressed with increasing numbers of syncytial cells and cytolysis until by day 18 there was nearly complete lysis of the transfected SCP. CPE was similar to that seen after infection of SCP with the closely related visna KV1514 virus. Cell-free supernatants were collected between days 9-18 post transfection, diluted with cell culture media and used to successfully infect other permissive cell cultures including SCP, goat synovial membrane (GSM), ovine cornea (OC), fetal ovine lung (FOL) and ovine skin fibroblast (OSF), thus demonstrating the production of infectious virus. Cells treated with liposomes only continued to thrive in culture without any observable CPE. Seven days post-transfection, cells transfected with pCMV-βgal were reacted with x-gal, and distinct blue cell clumps were evident.

Virus collected from these transfected cell cultured was expanded by passage on other cell cultures, and used for future infectivity studies. A 330 bp sequence PCR amplified from the *env* region had a 98-99% homology with the published kv72 sequence.

In Israel, pkv72-transfected cells failed to develop CPE, although some elevation of RT (11,883 cpm) was detected day 14 post-transfection. Cells transfected with high molecular weight DNA from infected cells developed CPE and elevated RT levels on day 7 (9,682 cpm) and day 14 (26,510 cpm) post-transfection.

Morphogenesis of kv72 virus clone

Examination of kv72 infected Himalayan tahr ovary cells and fetal ovine cornea cells by electron microscopy by Dr. Perk in Israel revealed a similar morphology to field strains of OvLV. The morphogenesis of the kv72 molecular clone was characterized by typical lentivirus budding from the plasma membrane and the presence of intracytoplasmic A particles, which consist of two electron dense shells of which the inner is more dense. The development of intracytoplasmic virus particles in the process of the virus molecular clone infection is of particular interest, since it indicates its integral part in the morphogenesis of the virus and not as a "contaminant" virus when using field isolation viruses, as claimed by some investigators (a manuscript is in preparation).

Inoculation of sheep with OvLV proviral DNA or virus derived from the molecular clone KV 72

A summary of the results of the Colorado study to test the infectivity of proviral DNA and virus derived from the KV72 molecular clone during the 3-5 mo. study period is presented in Table 1. The liposome-inoculated control sheep remained seronegative, and no virus was detected by either virus isolation or by PCR in these animals. All sheep inoculated with pkv72 plasmid DNA remained seronegative by AGID, ELISA and immunoblot for the duration of the experiment except one equivocal result (95RS11). Virus was isolated once from PBMC's collected from one of the pkv72 DNA inoculated lambs (96RS20), but this was not repeatable, and in conjunction with this sheep's seronegative status, this virus is believed to be a contaminant. PCR amplification of proviral DNA was generally unsuccessful from the pkv72 inoculated sheep with the exception of weak positives in two terminal PMBC samples (95RS11 and 95RS12) run at the same time. Although repeatable with these specific DNA samples, PCR amplification of DNA from spleen collected at the same time point was negative, and virus was never recovered from these animals. Once again, this is considered to be a PCR contaminant rather than a true positive result. There were no other data to support that these two lambs were infected, and it is highly unlikely that any inoculated DNA would still be present in sufficient quantities to result in a positive PCR

reaction 3 months after inoculation. The six lambs inoculated with the two higher doses of KV72 virus all became infected by serological criteria or by presence of virus or viral DNA. These sheep became seropositive by 2-4 weeks post-inoculation, although the strongest AGID reading was a 2+. Virus isolation was infrequent, with PBMC and spleen being the tissues of choice.

Table 1. Summary of Results of Inoculation of Lambs with pKV72 Complexed with Liposomes DDAB/DOPE or Virus Derived from the Molecular Clone KV72

Group	n	Dose ^a	Route ^b	AGID ^c	Imblt ^d	VI-spl/BAL ^e	PCR-PBMC ^f	Lesions ^g
Liposomes	2		IV	-	-	-	-	0
pKV72	1	250 μ g	IT	-	-	-	ND	0
"	5	500 μ g	IV/IM	1?	-	-	2	0
"	2	500 μ g	IV	-	-	-	2	0
KV72	1	2 x 10 ³	IV	-	-	-	-	0
"	4	3 x 10 ⁵	IV	3	4	3	4	0
"	2	1 x 10 ⁶	2	2	2	2	2	0

^a Virus in TCID₅₀, plasmid DNA in μ g

^b IV-intravenous, IT- intratracheal, IM- intramuscular

^c No. of lambs with OvLV antibodies detectable by the agar gel immunodiffusion assay

^d No. of lambs with OvLV antibodies detectable by immunoblotting

^e No. of lambs positive for virus isolation by coculture from spleen or bronchoalveolar lavage cells

^f No. of lambs with detectable OvLV DNA (LTR or env) in peripheral blood mononuclear cells by PCR

^g No. of lambs with lesions attributable to OvLV in lung or other organs by histopathology

All lambs inoculated in Israel with pkv72 remained seronegative by ELISA at all time points examined. Virus isolation was unsuccessful.

Inoculation of sheep with DNA reporter gene-liposome complexes in Colorado

Some lambs inoculated intravenously with DNA-liposome complexes experienced complications similar to those reported by others. To prevent known peracute reactions following IV administration, a butterfly catheter was placed in the cephalic vein and DNA/liposome complexes were administered very slowly over 2-5 minutes.

Significant levels of hu-G-CSF were not detected in samples from any lamb. Significant levels of CAT was not detected in any PBMC samples, but were detected in some serum samples (Fig. 1). For the first pCMV-CAT transfection experiment, lamb 96RS21 inoculated with 43 μ g/kg CAT DNA but not lamb

96RS19 inoculated with 56 ug/kg CAT DNA, had serum CAT protein activity detectable by ELISA at 24 hours; serum levels at 48 h were not tested.

The second pCMV-CAT transfection experiment used the same lambs as above, but there was over 4 months between DNA inoculations. Serum was collected at 24 and 48 hours post-transfection. Lamb 96RS00, which received the lower dose of pCMV-CAT, had CAT protein in serum at 48 but not 24 hours. Lamb 96RS19, which received the higher dose of pCMV-CAT, had CAT protein activity in serum at both 24 and 48 hours, and the 48 hour sample had approximately 50% more CAT activity than did the 24 hour sample. Most surprisingly, lamb 96RS21, which was inoculated with pCMV-CAT in the earlier experiment 4 months earlier, and pCMV-huG-CSF in the second experiment, still had measured CAT protein in both the 24 and 48 hour sera. However, in this lamb, unlike the others, there was minimal difference between the 24 and 48 CAT protein levels. No CAT activity was detected in the serum or leukocytes of either lamb injected with the pVIS-LTR-CAT expression vector.

Fig. 1. Chloramphenicol acetyl transferase (CAT) expression in serum of four sheep 1 or 2 days following intravenous inoculation with pCMV-CAT DNA or pCMV-huG-CSF DNA (negative control) on Days 0 and 120.

Inoculation and viral challenge of sheep with ovine lentivirus strain KV72 pCMVgag or pCMVenv constructs

In the Colorado study, anti-OvLV antibodies were not detectable by ELISA or immunoblot prior to viral challenge 5-6 weeks after the last DNA vaccination (Table 2.). Results of the lymphoproliferation assay set up one day prior to challenge revealed that lambs in all three groups had mild proliferative responses to all three antigens: rp25, rTM40 and kv72 whole virus (data not shown). There was no significant difference between study groups. Anti-OvLV antibodies were not detectable by ELISA in any of the 12 sheep inoculated with pCMVgag DNA in Israel.

Within 4 weeks after challenge, all 12 sheep had detectable anti-OvLV antibodies by immunoblotting assay, although one sheep from the control group (97RS1) had a lower titer than the other 11 sheep. All sheep also seroconverted in the AGID assay when the sera were tested 10 weeks after challenge, but three sheep had weak reactions at this time. One sheep from the control group (97RS1) had ELISA values below cutoff for the duration of the experiment, but there were no consistent group differences in anti-OvLV antibody responses, either for the time when antibodies were initially detected or for antibody titers. Lymphoproliferative responses to rp25 and kv72, with stimulation indices greater than 10, were seen 9 days and 5 weeks post-challenge in some sheep from each group. There was no significant group differences in lymphoproliferation to OvLV antigen or to concanavalin A.

Attempts were made to quantitate viral load. Some other studies using DNA vaccines for other OvLV have reported a decrease in viral loads during acute infection. PBMC collected 4 weeks after challenge were co-cultured with OSF. By day 14, cultures from all 12 sheep had CPE compatible with OvLV infection. DNA purified from these co-cultured cells was amplified separately with *env* region and LTR PCR primers, and PCR products of the expected sized were detected by ethidium bromide stained gel electrophoresis.

Using an infectious center assay, the number of virus infected cells per cell sample were estimated. Once again, the viral load was lower than is typically seen with sheep infected with field strains of OvLV. Very few foci of viral antigen, as detected by immunohistochemistry, were detectable, and most samples were negative. No group differences were apparent in the number of foci detectable using 4×10^6 PBMC. Accurate quantitative comparison between sheep was not feasible.

Although a DNA PCR assay employing ^{32}P end-labeled PCR products amplified with the *env* region primers was able to detect 20 or fewer copies of viral DNA in a standardized sample, RT-PCR was less sensitive. A quantitative-competitive PCR (QC-PCR) assay developed in the laboratory only had a sensitivity of approximately 500 copies of viral RNA. Using PBMC collected 2 ½ weeks and 4 weeks after challenge, RT-PCR using ^{32}P end-labeled upstream primers, was 6/12 and 11/12 sheep had PCR product of the expected size, but many of these bands were faint. Since QC-PCR was not attempted on these samples, the significance of these results, if any, is not clear.

Table 2. Summary of Results of Inoculation of Lambs with Ovine Lentivirus Strain KV72 pCMVgag or pCMVenv Constructs and Intravenous Challenge with 5×10^6 KV72 Virus 12 Weeks Later

[---Pre-Challenge---][-----Post-Challenge-----]										
Group	n	ELISA ^a	Imblt ^b	ELISA ^c	AGID ^d	Imblt ^e	VI-spl ^f	ICA ^g	PCR ^h	RT-PCR ⁱ
Saline	4	0	0	0,5,4,4	4	1,3,3,3	4	1	4	2
pCMVgag	4	0	0	9,5,9,5	4	2,2,3,3	4	2	4	4
pCMVenv	4	0	0	9,4,5,5	4	3,2,3,2	4	1	4	0

^a No. of lambs with serum OvLV CA and TM antibodies detectable by ELISA

^b No. of lambs with serum OvLV antibodies detectable by immunoblotting in lambs of group

^c Lambs with serum OvLV CA and TM antibodies detectable by ELISA, week first positive in each lamb

^d No. of lambs with serum OvLV antibodies detectable by the agar gel immunodiffusion assay

^e Lambs with serum OvLV antibodies detectable by immunoblotting: 1=faint CA and SU bands; 2=strong CA or SU band with faint second band; 3=strong CA and SU bands

^f No. of lambs positive for virus isolation by coculture from spleen cells at termination

^g Infectious center assay: No. of lambs with at least 1 focus of viral infectivity per 4×10^6 PBMC

^h No. of lambs positive for OvLV DNA (*env*) by PCR in spleen cells at termination

ⁱ No. of lambs positive for OvLV RNA (*env*) by RT-PCR in PBMC at 18 days post-challenge

After challenge, all sheep injected with these constructs in Israel were seropositive by ELISA at all time points, and there was no statistical difference between groups.

Histopathology

None of the lambs in the Colorado study had lesions that were indicative of OvLV infection, nor where the changes seen in the two vaccinated groups of animals significantly different from each other or from the unvaccinated group. Many sheep had very mild, often multifocal, increased numbers of mononuclear cells within the lung interstitium with some hyperplasia of the bronchus associated lymphoid tissue (BALT). A few sheep had slightly increased numbers of mononuclear cells within the choroid plexus of the brain. In the Israeli study, no significant lesions compatible with OvLV infection could be detected in any of the sheep, even in sheep that had been seropositive for 21 months.

DISCUSSION

Transfection of sheep with plasmid constructs encoding the full length infectious kv72 OvLV molecular clone or non-infectious constructs derived from the kv72 clone did not result in detectable expression of the viral gene(s) encoded by the inoculated DNA. Prior to use in sheep, all constructs had been used successfully to transfect ovine cells in culture with resultant expression of viral genes, and in

the case of pkv72, infectious virus. Ideally, *in vivo* transfection with DNA expression vectors should result in expression of the encoded gene(s) and immune responses against foreign proteins.

In vivo transfection was achieved in sheep using pCMV-CAT but not pCMV-huG-CSF, pCMV-VIS-CAT or pkv72. Thus, there is good evidence that intravenous, liposome-mediated *in vivo* transfection of sheep can result in consistent gene expression. It is unknown which tissue(s) expressed significant levels of CAT, although PBMC did not have significant CAT protein. Although the sample size was small, there appeared to be a dose response, within the 15-55 $\mu\text{g}/\text{kg}$ dose range used in these studies. There are few data available for extrapolating DNA doses for *in vivo* transfection of large animals. These doses are considerable smaller than the 2-3 mg/kg routinely used in mice, but comparable to the few other published reports of successful transfection of non-rodent species (Willems *et al.*, 1992; Cox *et al.*, 1993; Liu *et al.*, 1996; Boyer *et al.*, 1997). Unfortunately, the successful transfection experiments were poorly reproducible. One lamb from the first experiment inoculated with a relatively high "sheep dose" of 56 $\mu\text{g}/\text{kg}$ failed to have detectable CAT protein in the 24 hour serum sample.

After challenge with Kv72 OvLV, all vaccinated and unvaccinated animals became infected with virus, as determined by positive virus isolation, PCR amplification of viral nucleic acids and anti-OvLV immune responses. Thus, vaccination with neither pCMVgag nor pCMVenv was protective against OvLV infection. Due to the lack of detectable pre-challenge anti-OvLV immune responses, and our apparent inability in the past to transfect sheep with other DNA expression vector constructs, the most likely explanation is that the sheep were not transfected by the inoculated DNA, and thus did not express OvLV capsid proteins or envelope glycoproteins prior to challenge. Alternatively, transfected cells may not have expressed sufficient levels of viral proteins to elicit an effective immune response in the host. Failures such as this are one of the most frustrating aspects of *in vivo* transfection. Once the DNA is inoculated, it is very difficult, if not impossible, to document what happens to the inoculated DNA at the cellular level. Transfection is generally documented by detection of the actual foreign protein or by a specific immune response against that foreign protein. When that is unsuccessful, it is difficult to determine at what step transfection and/or expression failed.

The CMV promoter/enhancer region is considered to be one of the most effective promoters for *in vivo* transfection studies, and the pcDNA3 expression vector used to construct pCMVgag and pCMVenv has been successfully used for other *in vivo* transfection experiments. The pCMVgag and pCMVenv expression vectors had been shown to express OvLV capsid and envelope proteins, respectively, following transfection of cell cultures. Thus these expression vectors appeared well designed for *in vivo* transfection. The IM/ID route of DNA inoculation, and the dose of DNA used was modeled after the techniques used by researchers who were successful in inducing immune responses to proteins encoded

by inoculated plasmid DNA. Similar lentiviral *gag* and especially *env* constructs have elicited specific immune responses in other species (Liu *et al.*, 1996; Boyer *et al.*, 1997 ; Cruisnier, *et al.*, 1997). Willems *et al.* were able to transfect sheep with bovine leukemia proviral DNA (Willems *et al.*, 1992), and we demonstrated long term protein expression following transfection of sheep with a pCMV-CAT reporter gene construct, although by a different transfection method than was used for these studies (Mounkes *et al.*, 1997). Thus it is possible to transfect sheep by DNA inoculation. It may be significant that, after inoculation of two lambs, the visna LTR CAT constructs were unable to induce CAT activity in the serum or PBMC.

The pCMV*gag* and pCMV*env* constructs should serve as valuable tools for future studies, since bacterially synthesized OvLV peptides would provide a ready source of standardized OvLV antigen for use in various assays or diagnostic tests. If techniques can be developed that result in a reasonable transfection efficiency in large animals like sheep, these pCMV*gag* and pCMV*env* may still be excellent DNA vaccines, either in combination or separately.

Despite the shortcomings of *in vivo* transfection with OvLV constructs, this study was able to further characterize this infectious OvLV molecular clone. Kv72 is cloned from the sheep-adapted neurovirulent visna strain KV1772 which was derived from prototype visna KV1514. Using U.S. commercial AGID diagnostic kit, sera from kv72 infected lambs produced only subtle 1-2+ reactions, compared to the more robust 3-4+ reactions typically seen with sera from naturally infected sheep or sheep experimentally infected with OvLV 85/34. This is most likely due to less specific cross-reactivity between kv72 and the antigen, prepared from a North American strain of OvLV, used in the U.S. AGID kit. As with sera from sheep experimentally infected with OvLV 85/34, positive AGID reactions could usually be detected by 4-6 weeks post-inoculation in sera from kv72-infected sheep, but unlike sera from OvLV 85/34 infected sheep which typically progressed from a 1-2+ reaction to a 3-4+ AGID reaction within 2-4 weeks, the intensity of the AGID reaction produced by sera from kv72-infected sheep did not change markedly over time, and usually did not exceed 2+.

Using immunoblot, sera from kv72-infected sheep had detectable anti-OvLV antibodies by 2-4 weeks post-infection, as is reported for other strains of OvLV. Interestingly, gp105 antibodies were detected prior to p25 or p16 antibodies. Kajikawa *et al* characterized antibody responses to different OvLV and found that anti-p25 antibodies could often be detected by 2-4 weeks post-infection, but that anti-gp105 antibodies usually developed many weeks later (Kajikawa *et al.*, 1990). The kv72 envelope glycoprotein may induce an especially strong antibody response, or more likely, the conditions of the immunoblot assay, such as antigen used, favored the detection of one antibody or the other.

Assuming the viral titers of the two inocula used by Dr. Perk in Israel and the U.S. group are comparable, the Israeli sheep became infected at a much lower dose than did the breeds used by the U.S. investigators. Dr. Perk was able to infect 100% of the Israeli study sheep with a dose of 1000 TCID₅₀ kv72 virions, and virus was readily recoverable after 4 weeks of infection. In contrast, 2000 TCID₅₀ kv72 virions failed to infect a sheep used in a pilot study in the U.S., and virus was isolated only rarely from California variegated crossbred sheep inoculated with 1 x 10⁶ TCID₅₀ kv72 virions. Virus was more readily isolated from Rambouillet sheep inoculated in the U.S. with 5 x 10⁶ TCID₅₀ kv72 virions. This may simply reflect the higher dose, but together, these data suggest that there may be a distinct breed susceptibility to this OvLV molecular clone. Other studies have previously noted a breed susceptibility to OvLV infection and OvLV-induced disease (Cutlip *et al.*, 1986); (Johnson *et al.*, 1992).

Significant OvLV-specific lesions were not evident in the sheep used in this study. This finding is not unexpected due to the short time period that the study sheep. Natural infection with OvLV may not result in disease for many years, and some persistently infected sheep never develop disease. Since our main interests were immune responses and viral kinetics of the acute phase of infection, disease was never considered an end-point for this study. Previously reported kv72-induced lesions were reported in highly susceptible Icelandic breeds of sheep inoculated intracerebrally with a relatively high dose of kv72 virions.

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DESCRIPTION OF COOPERATION

The U.S. investigators obtained the kv72 molecular clones and provided them to Drs. Yaniv and Gazit. From the original kv72 molecular clones, Drs. Yaniv and Gazit constructed multiple non-infectious OvLV molecular clones which were then provided to the American group. Both the U.S. investigators and Dr. Perk conducted the animal studies. The groups frequently communicated by telephone, email and FAX. Drs. Yaniv, Gazit and Perk personally visited the investigators at CSU at different times to discuss research results and to design future experiments. Dr. Carlson attended the International Congress of Virology in Jerusalem, Israel.

EVALUATION OF RESEARCH ACHIEVEMENTS

With modifications dictated by early experimental results, the research objectives planned in this project were carried out at the U.S. and Israel laboratories. However, neither hypothesis was validated; that is, sheep could not be persistently infected nor were immune responses detected in animals inoculated with plasmid DNA of the Kv72 ovine lentivirus molecular clone; and inoculation of sheep with non-infectious viral DNA segments did not result in expression of viral gag and env gene products or induction of OvLV-specific immune responses and protection against viral challenge under the conditions of this study. Nevertheless, infectious virus was rescued after in vitro transfection of ovine cells, it was

infectious for sheep, and this virus served as a homologous challenge inoculum for the DNA vaccination studies. By using reporter gene constructs, the lentiviral LTR promoters were found to be inferior to the more widely used CMV promoter in directing CAT expression in sheep inoculated with plasmid DNA. The prolonged expression of CAT in PBMC in the serum of one animal for over 4 months was a remarkable finding.

CONCLUSIONS

Under the conditions of the investigations carried out in this project, ovine lentivirus gene expression and immune responses were not detected following injection of proviral DNA into naive sheep. However, using a CAT reporter gene construct under the control of a CMV promoter, expression of CAT protein activity was found in lambs transfected *in vivo* by injection of cationic liposome-DNA complexes and one formulation produced gene expression in a sheep for 4 months following a single intravenous injection. Although the naked DNA approach to vaccination holds promise for control of ovine lentivirus-induced disease, further work needs to be done to develop more effective methods of transfecting sheep with DNA.

LIST OF PUBLICATIONS

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