77

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TABLE OF CONTENTS

A. Cover Page	
B. Table of Contents	1
C. Abstract	2
D. Objectives of the Original Research	3
E. Body of Report	3
I. Introduction	3
II. Main Clinical and Pathological Symptoms	5
III. Isolation of SPCTV and Its Characterization	7
a. Isolation of SPCTV	7
b. Virus Structure	7
c. Classification of SPCTV within the Retroviridae	8
1. Biochemical characterization of SPCTV	8
2. Characterization of the structural polypeptides of SPCTV	10
3. The genomic relatedness of SPCTV to other retroviruses	14
d. SPCTV infection <u>in vitro</u> and <u>in vivo</u>	19
1. Material and methods	19
2. Infection of various cells with SPCTV	20
3. The sequence of morphological changes	21
4. Formation of colonies in soft agar	22
5. Transplantation of SPCTV infected cells into nude mice	22
6. Tumorigenicity of SPCTV in lambs, kids and calves	23
7. SPCTV serological study	25
8. Search for transforming oncogenes in SPCTV	25
9. Search for transforming genes in SPC tumors	26
e. A recent lentivirus isolate from SPC in South Africa	27
IV. Lentivirus Isolates in Israel	27
V. Herpes Virus and Its Possible Involvement in the Aetiology of SPA In Israel	31
VI. The Retroviruses Associated with Sheep Pulmonary Adenomatosis	32
F. Cooperation	34
G. List of Publications	60

C. Abstract

Sheep pulmonary adenomatosis or carcinoma (SPA;SPC) has been shown to be associated in nature with a retrovirus. A retrovirus (SPCTV) has been isolated from SPA by co-cultivation of tumor tissue and HTO cells.

SPCTV morphology and morphogenesis in cell culture is indistinguishable from the ungulate lentiviruses which are a subfamily of the retroviridae. The virus particles contain a reverse transcriptase, Mg^{++} dependent, in association with a 60-70 S RNA. The characterization of the structural polypeptides for SPCTV and the genomic relatedness of SPCTV to other retroviruses revealed that this novel virus is unrelated to representatives of the oncoviruses (a subfamily of retroviruses) - to murine, avian and bovine leukemia viruses, to be exogenous to the ovine species, and have only limited genetic relatedness to the lentiviridae (MVV and CAEV), and on the basis of the shared antigenic determinants between SPCTV, CAEV and OPPV in the heterologous radioimmunoassay and on the basis of the similar molecular weights for the structural polypeptides of SPCTV and CAEV and their similar behavior in homologous radioimmunoassay, SPCTV is classified as a lentivirus and furthermore, SPCTV and CAEV are closely related.

In vitro and in vivo infection studies revealed that SPCTV possesses properties which indicate a transforming oncogenic potential; i.e. the morphological changes induced following infection of HTO and SL cells, the capability of such cells to multiply in soft agar, to form large site colonies and most significantly to induce lymphoid tumors in nude mice and probably PA in lambs. A serological perspective SPCTV study is ongoing.

Lentiviruses isolated by us in Israel were characterized and studied as to their relatedness to SPCTV. A retrovirus isolated from maedi/visna diseased sheep showed to have close if not identical genetic homologous features with the ovine progressive pneumonia virus reported in the US, rather than with those of the MVV of European origin or SPCTV. The Israel CAEV isolate is indistinguishable from CAEV Florida isolate and that the California CAEV isolate is the closest to SPCTV.

In addition to the retrovirus association in SPA a herpes virus was also implicated as a possible aetiological factor in SPA. Serum samples obtained from SPA diseased sheep, or from sheep of infected herds and random samples all tested for herpes antibodies were negative. This serological survey indicated no aetiological significance of herpes virus in SPA in Israel.

Retrovirus particulates extracted from SPA lung fluids from sheep in Scotland, Peru, South Africa and Israel revealed the presence of p25 as determined by Western blotting procedure, employing a goat antiserum to MPMV p25, but p25 was not detected in the retrovirus particles obtained from cell culture supernates of SPCTV. It will be important therefore to further examine the relatedness of the particulates of SPA lung washings and SPCTV.

Cooperation - Prior to the first isolation and characterization of CAEV, numerous goats in California showed clinical symptoms of MVV. However, at autopsy they differed from the classical sheep disease. Our collaborative project revealed that the lung lesions in addition to the typical MVV lymphoid infiltration and hyperplasia, many areas showed histologically and ultrastructurally features indistinguishable to the tumor nodules seen in SPC. Furthermore, the virus isolated from diseased goats was diagnosed as CAE virus and hybridization experiments comparing four CAEV different CAEV isolates with SPCTV, the California isolate was found to be the closest. Serological studies revealed that 53% of 13 large goat dairies had antibodies to CAEV. Different epidemiological and preventive parameters were tested and were reported.

D. Objectives of the Original Research Proposal

Sheep pulmonary adenomatosis (SPA), (also known as jaagsiekte or sheep pulmonary carcinoma (SPC), is a frequent and spontaneously occurring malignant disease, with worldwide distribution.

This project had five major objectives:

1. Further characterization of the etiological agent of SPA, isolated by us and referred to as SPCTV.
2. Examination of biological properties of SPCTV, in particular the determination of the species of origin of the virus, in vitro host range among cells of different species and embryonic origin, and tumorigenicity in domestic animals.
3. Understanding of the pathogenetic and biochemical events accompanying SPCTV infection.
4. The development of routine survey procedures for the rapid and early (i.e., before overt symptoms are observed) detection of sheep pulmonary adenomatosis in the field.
5. The development of schemes to protect animals from the disease (according to the results of the above goals).

E. Report

I. INTRODUCTION

The concept of slow virus diseases evolved from the observations of Bjorn Sigurdsson (1954) and Covorkes in Iceland on a group of progressive chronic sheep diseases formerly unknown in Iceland, and which were characterized by dispnoe or wasting and by lung tumors. These progressive chronic sheep diseases were referred to as maedi, visna and as pulmonary adenomatosis, respectively. They were apparently introduced into Iceland in 1933 when 20 Karakul sheep were brought from Halle, Germany. This import was intended to improve the productivity of the local Icelandic sheep which were originally brought to Iceland by its first settlers more

than 1,000 years ago, and had subsequently been kept in almost complete isolation. After a two month period of quarantine, the imported sheep were distributed to 14 farms. Epidemiological studies showed that two of the imported rams were carriers of maedi and one of them was also a carrier of pulmonary adenomatosis. By early 1940 these diseases reached epidemic proportions. They were only brought under control by applying an extensive slaughter policy (Dungal et al., 1938; Dungal, 1946; Palsson, 1985).

Prior to the Karakul sheep import, the Icelandic sheep population approximated 800,000. It has been estimated that more than 105,000 sheep succumbed to the slow virus diseases and that more than 650,000 had to be killed in order to eradicate the disease. The campaign lasted thirty years.

At present, slow virus infections are reported worldwide and cause considerable economic losses. However, in addition to its importance in veterinary practice, great interest in the sheep slow viruses was evoked from the standpoint of comparative medicine as well. Very recently, interest has even increased considerably with the discovery of Gonda et al., (1985), that the human retrovirus HIV, the etiological agent of AIDS, has many parallels with the ungulate slow viruses (Shaw et al., 1985; Wong-Staal and Gallo, 1985; Hess et al., 1985).

Our project concerns mainly sheep pulmonary adenomatosis.

Although primary neoplasms of the lungs are prevalent in humans, they are not prevalent in the lungs of domestic animals. Sheep are exceptional. Sheep pulmonary adenomatosis (SPA), also called sheep pulmonary carcinoma (SPC) or "jaagsiekte" (from the Dutch jaag-to drive; siekte-sickness) is characterized by tumor cell growth in the lungs with metastases in the pulmonary and mediastinal lymph nodes, and exhibits all the characteristics included in the description of human bronchoalveolar cell carcinoma (Nobel and Perk, 1978). Its symptomatology and macroscopic pathology was apparently first described in great detail by Hutcheon in South Africa in 1891. This disease has probably been known in South Africa since 1850, and it has been suggested that it was introduced to SA from Spain in the early 1800's (De Villiers, 1906). This naturally occurring malignant disease occurs in most breeds of sheep and in most countries the world over. Epidemiologic and experimental evidence indicate that this neoplasm is horizontally transmitted (Tustin, 1969; Martin et al., 1976; Perk, 1982).

II. MAIN CLINICAL AND PATHOLOGICAL SYMPTOMS

Although SPA has been reported worldwide and may cause considerable economic losses, most of the infected animals are probably subclinical, yet persistent and probably cause persistent infections in others. Only some of the infected animals develop the neoplasm. The fate of such a diseased animal is always serious illness and death.

Characteristic for SPA is having a very long initial period of disease latency after infection, lasting for several months to several years. (Usually the "incubation" period is six months to four years.)

The main clinical characteristics of SPA are the presense of abundant bronchial exudate causing moist rales, nasal discharge, particularly in the mornings, and frequent spasmodic coughing, mainly in the later stages. The diseased sheep are very cachetic and usually die 3 to 6 months after the clinical signs become apparent (Figure 1).

The affected lungs in SPA show many randomly distributed light colored nodules and/or large confluent gray-white masses. The lesions have a solid tumor-like appearance and the cut surface is usually very moist. The main characteristic in SPA histopathologically is that the neoplastic cells (cuboida or columnar) are arranged in short rows, layers and acinar or papillary structures or clusters. The neoplastic cells are derived from terminal bronchiolar epithelial cells and from type II alveolar epithelial cells (also called "type B alveolar cells" or "granular pneumocytes") (Perk et al., 1971; Nisbet et al., 1971). Various stages of neoplastic expression, ranging from "alveolar epithelialization" to poorly differentiated adenocarcinoma, can be detected in the same tumorous lung of a clinically ill animal. Most neoplastic cells contain glycogen granules and cytosomes characteristic also of undifferentiated, embryonal lung epithelia. Intensely proliferating cores, sheets or nests of anaplastic epithelial cells are also seen. In summary, the overall tumor architecture in PA and the ultrastructure of the neoplastic cell including the metastasis, warranted the classification of SPA as a bronchoalveolar cell carcinoma, strikingly resembling the human bronchoalveolar carcinoma (Nobel and Perk, 1978). Both diseases originate in the periphery of the lung and tend to grow within the lung for a prolonged period, terminating in a late

inoculum containing the reverse transcriptase producing agent (retrovirus), adenomas were induced in two out of four inoculated animals. When the retrovirus and herpes virus particles were inoculated simultaneously, four of six inoculated animals developed adenomatosis (Martin et al., 1976). This could be interpreted as an increase of malignant expression.

III. ISOLATION OF SPCTV AND ITS CHARACTERIZATION

a. Isolation of SPCTV

Five one year old Awassi sheep were inoculated intrapulmonarily with a cell-free reverse transcriptase and virus particle-containing fraction obtained from a spontaneous field case of SPA. Five mock infected controls were housed separately under identical conditions. After one year, two of the five inoculated sheep had developed massive lung tumors, another two of the inoculated sheep developed small tumor foci in the immediate region of the inoculation site. Neoplastic tissue was taken from the most advanced tumor and co-cultivated with a large number of cell lines which included fetal kitten lung, bat lung, sheep choroid plexus, NIH/3T3- mouse fibroblasts, chick embryo fibroblasts, fetal mink lung, fetal sheep lung, fetal sheep kidney, muscle and a tahr ovary cell line (HTO). After three weeks, the mixed culture of HTO and SPA tumor cells began producing retrovirus. This virus isolate was termed in the past SPAV and is now called SPCTV (sheep pulmonary carcinoma tissue culture virus) and as determined by electron microscopy and by reverse transcriptase assay (Irving et al., 1984).

b. Virus Structure

Morphologically, SPCTV propagated in tissue culture is indistinguishable from the ungulate lentiviruses. Similar to other members of the retrovirus family it buds from the infected cell plasma membrane. The bud is characterized by a crescent electron dense internal structure. The diameter

of the bud is variable and measures approximately 80 - 120 nm. With further maturation, the buds protrude into the extracellular space. The extracellular mature virions show a distinct intermediate layer and condensed electron dense core and possess an envelope with short protruding spikes. The thin sections also reveal extracellular virus particles with bar-shaped nucleoids. As expected from the variability in size of budding virions, the size of mature virions is more variable than other retroviruses. Infected cells often have a very large number of budding virions on their surface (Figures 3 and 4) and within small cytoplasmic vacuoles. In rare cases, virion budding at the membrane of the endoplasmic reticulum may be seen. Furthermore, cytoplasmic A particles which are present in retroviruses morphologically categorized as precursors of B or D, and which consist of two electron dense shells of which the inner is more dense, are also rarely seen in the SPCTV infected tissue culture cells or in vivo in the epithelial tumor cells. In addition, aberrant "particles" in both size and shape and many multilaminate virus-like particles are rarely seen. It is of importance to note that the other members of the lentivirus subfamily, the human immunodeficiency virus (HIV), the equine infectious anemia virus (EIAV) are morphologically indistinguishable from this sheep virus.

c. Classification of SPCTV within the Retroviridae

1. Biochemical characterization of SPCTV

Production of relatively large virus volumes for this project was achieved by its propagation in the Himalayan tahr ovary cell line. Therefore this line served for most of the SPCTV biochemical and biological studies. The infected HTO cells showed large numbers of virion buddings, and free extracellular mature virus particles which contained reverse transcriptase in association with 60 - 70 s RNA .

It should be noted again that numerous induction experiments performed on uninfected HTO cells revealed that no endogenous virus could be induced. No spontaneous release of retrovirus from such cells was detected

(unpublished data from our lab and R. Vigne, France, personal communication.)

In order to fully support the contention that the DNA polymerase activity detected in the medium of the HTO-infected cultures was retrovirally associated, a number of properties known to be characteristic for retroviruses were examined.

The buoyant density of the particles released into the medium was determined by assaying fractions of isopycnic sucrose density gradients. Figure 5 shows a single peak of DNA polymerase activity at a density of 1.16 g/ml--the density on sucrose gradients characteristic for retroviruses.

To insure that the observed DNA polymerase activity was virus associated reverse transcriptase and not a cellular DNA polymerase, sucrose gradient purified SPCTV was examined for its synthetic template preference. The polymerase successfully utilized the ribohomopolymer template-primer, oligo (dT)₁₂₋₁₈: poly (rA) in a reaction which proceeded at a high rate for at least 60 minutes. However, no enzymatic activity was detected in the reaction directed by the synthetic deoxyribohomopolymer oligo (dT)₁₀:poly (dA).

The virion associated DNA polymerase was next examined in an endogenous reaction, in which no template-primer was added, some samples pretreated with RNase A₁ or with one of the four deoxynucleotide triphosphates omitted. The results, presented in Table 2 show that the reaction was dependent on all four deoxyribonucleotide triphosphates and that there was a definite requirement for an RNA polymer, ostensibly serving as a template for the synthesis of DNA.

The dependence of the enzymatic activity on the presence of a divalent cation cofactor (either Mg⁺⁺ or Mn⁺⁺) is a characteristic of retroviral reverse transcriptase. The preference of the virion associated DNA polymerase for Mg⁺⁺ or Mn⁺⁺ was examined in an endogenous reaction. The results show a definite preference for Mg⁺⁺ over Mn⁺⁺. The enzyme displayed an optimum of activity at a Mg⁺⁺ concentration of 10mM, and the maximal level of activity in the presence of Mn⁺⁺ was achieved at a concentration of 0.2 mM. (Figure 6).

2. Characterization of the structural polypeptides of SPCTV

In this section, an analysis of the structural polypeptides of SPCTV was performed by SDS-PAGE. The assignment of viral envelope and core polypeptides was made, and glycoproteins were identified by ^{14}C -glucosamine labeling. Because of the apparent relatedness of SPCTV and the lentiviruses, a comparison was made between the polypeptides of SPCTV, caprine arthritis-encephalitis virus (CAEV) and maedi/visna (MV).

The following methods and results were employed and obtained for this study section.

Cell Culture and Virus

HTO cells were grown in Dulbecco's modified Eagle medium containing 5% fetal calf serum and antibiotics. SPCTV, CAEV (Israeli strain) and maedi/visna virus (strain 1514) were harvested from the medium of infected HTO cells and were purified from the tissue culture medium by isopycnic centrifugation. The virus was resuspended in TNE buffer (0.1 M NaCl; 0.01 M Tris-HCl, pH 7.4; 0.001 M EDTA).

Virus Labeling

SPCTV was metabolically labeled for 48h with 0.8 μCi of D- ^{14}C -glucosamine hydrochloride, (250-350 mCi/mmol; New England Nuclear, Boston, MA) per ml medium, 21 and 25 days after infection of cells, at the stage of near maximal virus release.

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed in 20 cm gels using the buffer system of Laemmli (1970). The gels were stained by the silver stain method of Sammons et al., (1981). Radioactive polypeptides were detected by fluorography (Bonner and Laskey, 1974).

Preparation of Cores

Purified virus particles were treated with 1% Nonidet P-40 in TNE buffer for 10 minutes at 0°C. the cores were purified by isopycnic centrifugal gradient of 15-65% sucrose (Fig. 7).

Gel Filtration of Viral Particles

Purified viral samples were in 0.1 M (w/w) in TNE buffer and applied to a column (4 by 50 cm of Sephacryl S-1000 Superfine (Pharmacia fine Chemicals, Uppsala, Sweden) which was equilibrated with the same buffer. Fractions (10ml) were eluted with the same buffer. Reverse transcriptase activity was examined in each fraction.

Uninfected Cell Homogenate

The culture medium from uninfected HTO cells was decanted and saved and the cells collected by trypsinization. The cells were resuspended in 5 ml ice-cold growth medium and were disrupted by 30 strokes with a rotor driven Potter homogenizer fitted with a glass-teflon piston. Eighty percent of the cells were found to be disrupted by this procedure. The saved culture medium was added to the homogenate and the suspension was clarified by centrifugation for 15 minutes at 10,000 xg. The supernatant was subjected to the same purification procedure as virus-containing medium. After isopycnic centrifugation of the homogenate on a gradient of 20-55% sucrose, fractions of density range 1.14-1.16 g/ml were pooled, diluted with TNE buffer and pelleted by centrifugation for 1 hour at 100,000xg. The pellet was resuspended in TNE buffer.

Radioimmunoassay

Double-antibody competition radioimmunoassays were performed using ¹²⁵I-labeled CAEV p28 (a major structural protein), CAEV rabbit antiserum and disrupted lentivirus and oncogenic viruses as competing antigens in the homologous assay. The relatedness of SPCTV, CAEV and PPV was investigated

by this method, and other retroviruses (bovine leukemia virus and human T-cell lymphotropic virus) were included.

Size Heterogeneity of Virus Particles

Electron microscopic examination of SPCTV particles propagated on HTO cells showed heterogeneity in size. Chromatographic separation of purified virus on a Sephacryl S-1000 column revealed 3 distinct fractions in which reverse transcriptase activity was demonstrated. As a further characterization of the virus, cores were derived from purified viral particles by treatment with NP-40 and isopycnic centrifugation on a gradient of 15-65% sucrose. The density of the cores was in the range of 1.24-1.26 g/ml.

SDS-PAGE Analysis of Viral Polypeptides

The polypeptide profile of SPCTV exhibited 20 bands. To distinguish between viral structural polypeptides and cellular proteins which co-purified with the virus, a homogenate of uninfected HTO cells which was subjected to the same purification procedure was electrophoresed in parallel with the purified virus. Comparison of the cell homogenate with the virus showed that most of the polypeptides detected in the virus sample might be of cellular origin. Polypeptides having molecular weights 16,000, 18,000, 28,000, 67,000, 76,000, 90,000 and 135,000 daltons were identified as viral structural polypeptides. The viral core included polypeptides p16, p18, p28, p76 and p90.

Viral Glycoproteins

The autoradiograph of polypeptides which were labeled with ^{14}C -glucosamine showed the polypeptides p67, p76, p90 and p135 were virus glycoproteins.

CAEV and Visna Virus Polypeptides

Comparison of SPCTV polypeptides with those of the lentiviruses CAEV and visna virus showed that SPCTV was very similar to CAEV. A difference in the high molecular weight region of the two viruses was noted, however, with p135 of CAEV being a single moiety while that of SPCTV migrated as a doublet. The three low molecular weight polypeptides of SPCTV (p16, p18 and p28) differed from the corresponding maedi/visna virus polypeptides (p14, p16 and p27).

Results presented in this project part show that similar to other retroviruses, the number of polypeptides associated with SPCTV exceeds the number that is presumably coded by the viral genome. Thus, in addition to truly structural and functional viral polypeptides, there are polypeptides of cellular origin that are incorporated into the mature virion or co-purify with it. The cellular polypeptides can be accidentally included in the virus during its morphogenesis. Additionally, purified viral preparations may be contaminated with cellular debris. In order to mimic the lytic processes resulting from virus infection of cells in culture, we disrupted, (by mechanical means), uninfected HTO cells and then purified the homogenate in a fashion analogous to the virus purification. There were 7 polypeptides which were not present in the cell homogenate. The presence of identical electrophoretic bands in a purified viral preparation and in a similarly "purified" uninfected cell homogenate does not prove their structural and functional identity and, therefore, the number of viral polypeptides including their precursors and degradative products may be greater than seven (Vigne et al., 1982).

The identification in SPCTV of p16, p18 and p28 as non-glycosylated polypeptides present only in the viral core and of p135 as a glycosylated polypeptide, agrees with previous reports on the structural polypeptides of lentiviruses. The presence in SPCTV of a glycosylated p90 can be explained by it being a partially glycosylated precursor of p135 and the glycoproteins p67 and p76 could conceivably be degradation products of p135, based on their relative amounts. The comparison of SPCTV with CAEV

and maedi/visna virus showed that, in agreement to some extent with the genetic relatedness between SPCTV and CAEV found.

IN SUMMARY, the polypeptide profile of SPCTV was found to be very similar to CAEV, except for a difference in the p135 region. SPCTV differed from visna virus in that the low molecular weight polypeptides of SPCTV had a slower electrophoretic mobility than the visna polypeptides.

3. The Genomic Relatedness of SPCTV to Other Retroviruses

In order to examine the genomic relatedness to SPCTV to other retroviruses, the following methods were used.

Maedi/visna virus (strain 1514), SPCTV, maedi/visna (Israel), CAEV, and PPV were grown on tahr cells, a permanent cell line derived from Himalayan tahr ovary generously provided by Dr. R.C. Cutlip (USDA, Ames, Iowa). BLV was grown on fetal sheep kidney cells. Viral particles were purified by isopycnic sucrose gradient centrifugation and the 70 S viral RNAs were isolated. Representative ^3H -labeled complementary DNAs (cDNAs) were prepared in a primed endogenous reverse transcriptase reaction from purified viral particles, in a reaction mixture containing 2 mg/ml of calf thymus DNA primer, 2 mg/ml viral protein, 0.05% Nonidet P-40, 50mM Tris HCl, pH 8.0, 10 mM NaCl, 6 mM MgCl_2 , 10mM DTT, 100 ug Actinomycin D, 1 mM each of dGTP, dTTP, and dATP, and 1 mCi/ml of $(^3\text{H})\text{dCTP}$ (21 Ci mmol^{-1}). Following 3 hr incubation at 37° the $(^3\text{H})\text{DNA}$ was deproteinized and purified and the RNA template was alkali hydrolyzed. For association kinetic analysis, a total of 1000 cpm of viral $(^3\text{H})\text{DNA}$ was annealed at 68° to increasing amounts of 70 S RNA in 10 ul reaction volumes containing 0.6 M NaCl, 0.04 Tris-HCl, pH 7.2, 0.002 M EDTA, and 0.1% SDS. Hybrid formation was assayed by resistance to S_1 nuclease. Hybridization values are given after subtraction of self-annealing values (approximately 15%). For thermal stability analysis, hybridization reactions were carried to a $C_{\text{r}}t$ of $1 \text{ mol}.\text{sec};\text{liter}^{-1}$ at 68° . Samples were diluted 10-fold in a 0.4 M NaCl, heated at the indicated temperatures for 5 min, and then digested with S_1

nuclease. The temperature at which 50% of the hybridized (^3H)cDNA becomes dissociated (T_m) was determined from these melting profiles.

Association kinetics analysis between SPCTV (^3H)cDNA and cellular RNA or DNA of various cell types were performed as follows: tissues (sheep lung or cat liver) were minced, then homogenized in RSB buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 1.5 mM gCl_2). Nonidet P-40 was added to a final concentration of 0.5%; after incubation at 0° for 10 min, the nuclei were pelleted by centrifugation at 2000 g for 10 min. High molecular weight DNA was extracted from the nuclei. For RNA extraction from the supernatant, sodium dodecyl sulfate and pronase were added to a final concentration of 1% and 200 mg/ml, respectively. Proteins were digested at 37° for 1 hr and phenol extracted, and the RNA was precipitated overnight at -20° with 2 vol of ethanol. The RNA was suspended in TNE and precipitated overnight at 4° with 2M LiCl. After resuspension and precipitation with ethanol, the RNA was suspended in H_2O . HTO cells and HTO cells chronically infected with SPCTV were collected by trypsinization, pelleted, and resuspended in TNE. DNA and RNA were extracted as above for tissue homogenates. Cellular RNA was hybridized to 1000 cpm of SPCTV(^3H)cDNA as described above for viral 70 S RNA. Cellular DNA was fragmented to a length of about 400 bases by boiling for 20 min in 0.3 M NaOH. Cellular DNA was annealed to SPCTV (^3H)cDNA (spec act 2×10^7 cpm/ μg) under DNA excess (80 OD/ml) in buffer containing 0.6 M NaCl, 0.13mM ZnSO_4 , 33mM sodium acetate, pH 4.5), and the extent of hybridization was measured by resistance to S_1 nuclease. Hybridization values were not corrected for salt concentration. The final levels of hybridization were determined, after subtraction of the hybridization values obtained with control heterologous DNA.

To establish SPCTV genomic relatedness to other retroviruses reassociation kinetic analyses were performed between SPCTV (^3H)cDNA and 70 S viral RNAs extracted from representatives of various retrovirus groups: lentivirus group, maedi/visna virus, maedi/visna -I (Israel virus), caprine arthritis-encephalitis virus-CAEV, and ovine progressive pneumonia virus OPPV); murine leukemia viruses (Moloney-murine leukemia virus-MoMuLV); avian leukemia viruses (avian myeloblastosis virus-AMV); and bovine leukemia virus (BLV); Figure 8A demonstrates that among the various

heterologous RNAs, limited sequence homology (maximum hybridization levels 20 to 30%, with $Ct_{1/2}$ values of 5×10^{-1} mol.sec.liter⁻¹) were obtained with the various members of the lentivirus group.

To measure the extent of mismatching within the hybridizable portions of the viral genomes, the thermal stability of the hybrids was measured (Fig. 8B). In contrast to the high T_m value of 93° for the homologous hybrid, the hybrids formed between SPCTV cDNA and the 70 S RNAs of the various lentiviruses exhibited a 14-15° reduction in melting temperatures, suggesting the existence of 9-10% nucleotide mismatching between viral genomes. From the reciprocal hybridization assays in which ³H-labeled DNA probes, complementary to the various retroviruses tested, were annealed to 70 S genomic RNA of SPCTV, the same conclusion could be drawn (Fig 8C); i.e., the only retroviruses that had some nucleotide sequence homology with SPCTV were the lentiviruses MVV, MVV-Israel, CAEV, and OPPV.

Further annealing assays were performed to compare the levels of genetic relatedness between SPCTV and the lentiviruses, to those observed among the various members of the lentivirus group themselves. It was found that the annealing kinetics between maedi/visna virus cDNA (FIG.8D) or CAEV cDNA (Fig.8E) and SPCTV 70 S RNA was similar to the annealing kinetics obtained between each of these two lentiviruses cDNAs and the heterologous 70 S RNAs of maedi/visna virus, CAEV and OPPV.

These data strongly indicate that SPCTV is an additional distinct member of the lentivirus genus of the retrovirus family.

Although SPCTV can be considered a member of the lentiviridae, which are exogenous to the ovine species, its limited genetic homology to the known lentiviruses does not certify that it is exogenous to the sheep. To ascertain whether or not SPCTV is in fact exogenous to both the normal sheep and the tahr genome, additional hybridization analyses were performed.

The results of hybridization reactions between SPCTV (³H)cDNA and cellular RNAs from normal sheep cells, uninfected HTO cells and HTO cells chronically infected with SPCTV (Fig. 9A) show that uninfected HTO cells as well as normal sheep cells do not express any genetic information related to the SPCTV genome. Similarly, DNA-DNA hybridization studies showed no increase in the genetic relatedness between the SPCTV genome and genomic

DNA extracted from uninfected HTO and normal sheep cells over that existing between the SPCTV genome and cat genomic DNA (representing an unrelated species) (Fig 9B). These analyses were performed to a level capable of detecting one viral genome per haploid genome. Additionally, attempts at inducing endogenous retrovirus from normal sheep cells and HTO cells proved unsuccessful (data not shown). These experiments included the cocultivation of HTO and normal sheep lung cells (as well as fetal sheep kidney cells and sheep choroid plexus cells) in addition to the use of the inducing agents BrdU and IdU.

In conclusion, this novel retrovirus, which has been isolated from SPC experimentally induced tumors and is now propagated in culture, was shown to be unrelated to murine, avian, and bovine leukemia viruses, to be exogenous to the ovine species, and to have only limited genetic relatedness to the lentivirus group.

Radioimmunoassay

Double antibody competition radioimmunoassays were employed in an attempt to reveal possible antigenic differences between the major structural proteins of SPCTV and CAEV. In this assay, a purified viral protein is radiolabeled with ^{125}I and reacted with an antiserum prepared against it or against a related protein. The addition of a competing protein (e.g. disrupted virus) will reduce the binding of the antiserum to the labeled protein by an amount relative to the degree of shared antigenicity between the two proteins.

A homologous assay, using ^{125}I -CAEV p28 and anti-CAEV antiserum showed that disrupted SPCTV and CAEV compete equally well, while disrupted ovine progressive pneumonia virus (OPPV) competed poorly (Fig. 10). Two type C retroviruses from the oncornavirus subfamily--bovine leukemia virus (BLV) and human T-cell leukemia virus (HTLV-1)--failed to compete with the binding of CAEV p28 to the anti-CAEV antiserum. In a heterologous assay, using ^{125}I -CAEV p28 and anti-PPV antiserum, SPCTV, CAEV and maedi/visna virus competed equally well, while equine infectious anemia virus (EIAV), an additional lentivirus, did not react at all (Figures 10 and 11).

In summary, this novel SPCTV virus which was shown to be unrelated to murine, avian and bovine leukemia viruses, to be exogenous

to the ovine species, and have only limited genetic relatedness to the lentiviridae (MMV and CAEV), and on the basis of the shared antigenic determinants between SPCTV, CAEV and OPPV in the heterologous radioimmunoassay and on the basis of the similar molecular weights for the structural polypeptides of SPCTV and CAEV and their similar behavior in the homologous radioimmunoassay, SPCTV is classified as a lentivirus and, furthermore, SPCTV and CAEV are closely related. Furthermore, due to the close relatedness of SPCTV and CAEV, the high molecular weight DNA from SPCTV infected OFC and HTO cells, was analyzed for the presence of restriction sites characteristic of CAEV genome. To this end, the high molecular weight DNAs were digested with several restriction enzymes, the digested DNA samples were electrophoresed by the Southern technique, and blotted onto nitrocellulose. The blots were hybridized to cDNA probe prepared from 70 S RNA of the purified SPCTV.

The sizes of the hybridized DNA fragments were as follows (in Kb):

BamHI - 5.0; 4.4; 3.8

Hind III - 17.0; 5.7; 2.5

EcoRI - 4.7; 3.5

Sall - 12

XhoI - 12

These restriction sites are not compatible with the sites of our cloned CAEV genome; but more extensive hybridization analyses under various stringency conditions have to be carried out to determine SPCTV place in the lentivirus subfamily. The SPCTV and CAEV relatedness is of special interest since CAEV is very close to HIV. By using molecular hybridization, heteroduplex mapping and SNA sequence analyses, Gonda and coworkers (Gonda et al., 1986), derived a phylogenetic tree from viral genes of the lentivirus subfamily including also representatives of the retrovirus family as well. In rooting this phylogenetic tree, Moloney murine leukemia virus was considered and found to be the outgroup taxon. The phylogenetic tree examinations clearly indicated that the human acquired immuno deficiency virus (HIV) clusters with the ungulate lentivirus group. Within this group, MMV and CAEV are very closely related (with amino acid homology of 90%).

It was found also that HIV is closer to CAEV and MVV than to EIAV. The next closest virus is Rous sarcoma virus followed by human T leukemia virus-I (HTLV-I).

d. SPCTV Infection in vitro and in vivo

In this part of the project the transforming potential of SPCTV in vitro on the nude mouse and in sheep was examined.

1. Materials and Methods

Cell lines

The following cell lines were used: HTO cells (a permanent cell line derived from Himalayan tahr ovary (*Hemitragus jemalicus*)); OFC cells (ovine fetal cornea); SM cells (sheep muscle) and SL cells (sheep lung). All cell lines were grown in DMEM supplemented with 10% fetal calf serum, antibiotics and glutamine.

Virus

SPCTV was routinely propagated in cultures of HTO and SL cells. The medium of the SPCTV infected cultures was passed through a membrane filter (pore size, 0.22 μ m) before being used to infect subsequent cultures.

Cell morphology

Syncytia and plaque formation following infection with SPCTV were observed on cultures grown in petri dishes. Cells were fixed in absolute methanol for 10 seconds and stained with Giemsa.

Assay for virion associated reverse transcriptase activity

The assay was performed according to Yaniv et al., 1976.

In-situ hybridization

SPCTV virus particles were purified by isopycnic sucrose gradient centrifugation and the 70 S viral RNA was extracted. ³H-cDNA complementary

to the SPCTV genome was prepared as described by Irving et al., 1984. The in situ hybridization was performed according to Fournier et al., 1983.

Electron microscopy

Tissue culture samples were prepared according to Dahlberg et al., 1981, and examined in a JEOL JEM-100 CX transmission electron microscope.

Soft agar assay

SPCTV infected and uninfected HTO and SL cells were suspended in 0.36% agar medium (DMEM containing 20% fetal calf serum and antibiotics). This suspension was layered onto a 0.9% agar base layer at concentration of 10^5 cells per 50 mm dish. The dishes were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Two weeks later, the diameter of the colonies was measured. The number of colonies having a diameter of 0-30, 30-60, 60-90 and over 90nm was determined. Five hundred cells were measured in each of 15 dishes/treatment. The results were expressed as the percentage of colonies within a certain size range out of the total number of colonies examined.

Tumorigenicity of transformed cells

Chronically infected HTO and SL cell cultures (and the corresponding uninfected cell cultures) were tested for their ability to produce tumors in BALB/c (nu/nu) nude mice (obtained from a breeding colony at the Hadassa Medical School, Jerusalem) after subcutaneous inoculation with 5×10^6 cells. The mice were monitored for tumor development for 6 months after inoculation and all the inoculated mice were killed at 6 months, unless otherwise specified. The skin and subcutaneous tissue from the site of inoculation, liver, kidneys, lungs, spleen and lymph nodes were taken for histological examination. Blood smears were prepared from all mice 4 and 6 months after inoculation.

2. Infection of various cells with SPCTV

SPCTV was originally isolated and routinely propagated on HTO cells (Irving et al., 1984). In this study an attempt was made to grow the virus

on cells of ovine origin. The results presented in Table 3 show that HTO and SL cells were permissive for productive infection with SPCTV. The infection of HTO cells had a longer lag phase (approximately 2 weeks) than the infection of SL cells (less than one week); the HTO cells, however, supported a similar level of virus production in SL cells. (SM and OFC cells were barely or not permissive for productive infection with SPCTV)

3. The sequence of the morphological changes in SL cells infected with SPCTV

Figure 12 shows the sequential alterations in SPCTV infected SL cells. Uninfected control cells constantly showed a fibroblast-like morphology (Fig. 12a) throughout the course of this study. Infected SL cells showed foci of enlarged polygonal cells (Fig. 12b) around which syncytia started to form 3 days following infection (Fig. 12c). The syncytia increased in size and in number of nuclei per cell, reaching a maximum at 7 days (Fig. 12c). At this stage extensive syncytial cell death occurred, after which there was regrowth of the culture, consisting of cells with one or a few nuclei (Fig. 12c). The cultures then became chronically infected (Fig. 12d). Quantitation of the above changes is illustrated in Figure 13. Beginning on day 3 after infection, the percentage of uninuclear cells gradually decreased. The percentage of cells with 2-5 and 5-20 nuclei per cell increased, reaching a maximum at day 5. The number of all these cells then decreased concomitantly with a sharp increase in the number of cells with 20-100 nuclei per cell. After day 7 the number of these highly nucleated cells sharply decreased due to cell death. At the stage of culture regrowth (after day 11) the number of cells with one nucleus and 2-5 nuclei (but not of 5-20 or 20-100 nuclei) again increased. Virus-associated reverse transcriptase activity determined in the culture media (Fig. 13) started to increase above background level at day 5 after infection, then rose gradually to high levels with maximum at day 12. The subsequent enzymatic activity remained at a high level for a prolonged period, suggesting a state of chronic infection. SL and HTO cells chronically infected with SPCTV showed foci of piling up. Also, multi-layered growth of cells (in a criss-cross pattern) was observed (Fig. 14). When single cell suspensions

prepared from these foci were plated, polygonal cells and multinuclear cells appeared (Fig. 14) and their morphology was retained for 10 months (15 passages). These results indicate that syncytia are formed by the fusion of virus infected cells. The establishment of more viable chronically infected cultures was accompanied by the presence of syncytia having fewer nuclei and, apparently, these cells sustained the production and release of increased amounts of virus. In order to further clarify the relative distribution and the localization of the viral genome in the uninucleated and the multinucleated cells at the stage of chronic infection in situ hybridization technique was employed. The results, presented in Table 4 and Figure 15, show that 85% of the multinucleated cells contained copies of the viral genome. Additionally, the multinucleated cells had higher grain numbers. The grains were situated in the nucleus and the perinuclear area.

4. Formation of colonies in soft agar

Single cell suspensions of HTO and SL cells chronically infected with SPCTV were plated in soft agar. The results, presented in and Table 5 show that SPCTV-infected HTO cells formed colonies of various sizes. Forty one percent of the colonies had a diameter within the range of 30 to 60 μ m (as compared to 4.7% in the controls), while 1.8% had a diameter of 60-90 μ m and 0.2% had a diameter greater than 90 μ m (as compared to zero in the controls). Virus infected SL cells demonstrated a much smaller degree of colony formation. Thus, 7% of the colonies had a diameter of 30-60 μ m (as compared to 2.4% in the uninfected controls), and no colonies of a greater size were presented.

5. Transplantation of SPCTV infected cells into nude mice

HTO and SL cells chronically infected with SPCTV were inoculated into nude mice. One month later, small nodules were observed at the site of inoculation in 3/9 and 3/5 mice inoculated with SPCTV-infected SL and HTO cells, respectively. At 2 months after inoculation, 1/8 and 2/5 mice still demonstrated these nodules in the two experimental groups. In none of the

control mice (inoculated with uninfected HTO or SL cells) could nodules be observed at the site of inoculation. One mouse inoculated with SPCTV-infected HTO cells, which showed a relatively large nodule at the site of inoculation, was killed 4 months after inoculation. Upon histological examination, the presence of a discrete subcutaneous lymphoid tumor was revealed (Fig. 16). In addition, large and scattered lymphoid cell masses were found in the lung, liver and spleen, mainly clustered around the blood vessels, (Fig. 16). The lymphoid tumors consisted of lymphocytes of various sizes, but mainly of lymphoblasts; mitoses were abundant and there was no indication of an inflammatory reaction. All the mice inoculated with SPCTV-infected HTO and SL cells and their controls were killed 6 months following inoculation. Histological examination revealed the presence of lymphoid cell accumulation in the kidney cortex in 2/5 mice inoculated with SPCTV infected HTO cells. Smaller clusters of lymphoid cells were present in the lungs of these two mice and no inflammatory reaction was evident. Mitoses were not rare. In none of the mice inoculated with SPCTV-infected SL cells were pathological changes observed. The examination of blood smears obtained from all of the mice did not reveal any significant deviation from normal parameters. It should be pointed out that in the colony from which these nude mice were obtained, none of the 200 mice examined have shown any of the pathologic findings described here.

These results show that SPCTV, which was isolated from lung carcinoma of sheep and propagated in a permissive cell line (HTO), possesses properties which indicate a transforming oncogenic potential; i.e., the morphological changes observed following the infection of HTO and SL cells with SPCTV, the capability of such cells to multiply in soft agar and to form large sized colonies, and most significantly, the induction of lymphoid tumors in nude mice.

6. Tumorigenicity of SPCTV in lambs, kids and calves

To examine the tumorigenicity of SPCTV, 4 one month old lambs, 4 one month old kids and 3 two week old calves were inoculated intrapulmonarily with SPCTV (approx. 10^6 ^3H -cpm reverse transcriptase activity per animal).

Four additional lambs, 4 kids and 2 calves were mock inoculated with PBS. Six months after inoculation the animals were sacrificed and autopsied. From all those animals, only one lamb No. 457 developed pulmonary carcinoma. The lobe of the lung surrounding the site of inoculation of sheep 457 had a dense grayish-white appearance with small (approx. 5 mm) white foci radiating into the adjacent areas of that lung. The histopathological results confirmed that only sheep 457 was positive for SPC. In addition to the hyperplastic epithelialization with foci of proliferation filling the air spaces in the alveoli, the terminal part of the tertiary bronchiolar surface epithelium was very uneven, and included the formation of papillary projections into the lumen. Both characteristics are symptomatic of the early stages of SPC. However, SPCTV virus could not be reisolated from this case. Although alignots (10ul) of either DNA or RNA extracted from this tumor tissue and from two maedi/visna diseased lung and negative and positive controls (uninfected HTO cells and SPCTV infected HTO cells, respectively) were spotted onto nitrocellulose filters and hybridized to SPCTV 32 p-cDNA. By comparing the intensity of the autoradiograms, it could be concluded that a greater amount of SPCTV 32 p-cDNA was bound to the lung tumor tissues, in comparison to DNA from maedi/visna lungs and uninfected HTO cells. The positive control, SPCTV infected HTO cells showed the greatest level of hybridization on the autoradiograms. This methodology was employed at that time, since it is known that in vivo infection with lentiviruses such as maedi/visna leads to low copy of numbers of integrated viral DNA (provirus) as well as a restricted level of virus production. The solution hybridization technique would be incapable of detecting less than 2-5 viral genomes per cell. Therefore, the "spot" hybridization technique which permits the loading of larger amounts of DNA or RNA into the hybridization reaction was preferred.

The potential oncogenicity in vivo of SPCTV obtained was further extended. A group of 6 new born lambs was inoculated intrapulmonarily with SPCTV (approx. 10^6 3 H-cpm RT activity per animal). New born animals deprived of colostrum were selected in order to increase the malignancy. All inoculated lambs died from pneumonia during the first month without typical SPA symptoms. Although some lymphoid proliferation and B-type cell

hyperplasia were seen in all cases. No virus could be reisolated from the succumbed animals.

A third group in the SPCTV infection studies is presently under observation. In this group 4 week old animals were inoculated with SPCTV as used in the previous experiments. These animals will be kept as long as possible and are periodically x-rayed and examined.

7. SPCTV Serological Study

We have adapted the ELISA test for SPCTV and have initiated (in the second year, 220 sheep) a perspective pilot epidemiologic survey. The animals are periodically tested. This study is still in progress and we plan to continue it for an additional two years. Although the data of this part of our project are not evaluated as yet, a number of observations could be stressed. No sero-conversions occurred. No clinical maedi/visna disease was found in the flocks examined. Only one case of SPC was diagnosed to date. In a number of suspicious diseased animals in addition to the serological tests, x-rays and other examinations are performed. For example, as noted in our second year report, x-ray singled out a ewe sero positive and with pneumonia having SPC. This animal was autopsied and the diagnosis was confirmed. To the best of our knowledge, no additional animals died with SPC or jaagsiekte found at slaughter in these two herds.

8. Search for Transforming Oncogenes in SPCTV

Based on the mechanisms of oncogenesis of retroviruses, SPCTV might induce tumorigenesis either by a direct mechanism through a viral transforming gene, or by an indirect mechanism involving the activation of a cellular proto-oncogene. Since retroviruses replicate via a provirus integrated in host high-molecular genomic DNA, the host DNA of SPCTV-induced tumor cells, might have transforming ability upon its transfection onto recipient cells due either to the virus transforming gene or to an activated cellular proto-oncogene.

Our strategy was, therefore, to search for the presence of transforming oncogene in SPCTV-infected cells employing the transfection assay on NIH/3T3 mouse cells, which is a routinely used system for assessing transforming capacity of oncogenes. In the first set of experiments high molecular DNA was prepared from OFC and HTO cells infected with SPCTV and transfected by the calcium phosphate precipitation technique onto NIH/3T3 cells. Transfected cells were monitored for 21 days for the appearance of transformed foci. No focus appeared. A second trial of transfection was also negative for transformation.

9. Search for transforming genes in SPA tumors

High molecular weight DNA was prepared from several cases of SPA tumors: 3 samples from Israeli tumors, 11 samples from SPA tumors obtained from Peru, and 1 sample from SPA tumors from a Wyoming case.

DNA samples, 80 ug of each tumor were transfected onto NIH/3T3 cells using the calcium phosphate precipitation technique. Transfected cells were monitored for 21 days for the appearance of transformed foci. One of the Israeli SPA tumors, and one of the Peruvian tumors induced the appearance of transformed foci on NIH/3T3 cells. Due to the possibility of spontaneously appearing foci in NIH/3T3 cultures, the foci were tested by the following approach: individual foci were isolated and grown to mass culture. High molecular weight DNA was prepared, Eco RI digested, and analyzed by the Southern technique, for the presence of newly acquired sheep DNA sequences, suggesting that the foci were spontaneously induced ones.

The second approach to insure the spontaneous nature of the foci was to perform a second cycle of transfection. To this end, high molecular weight DNA (80ug) obtained from the first cycle transformants was again transfected onto NIH/3T3. After 21 days in culture, no foci appeared, confirming the spontaneous nature of these foci.

In the absence of transformation of NIH/3T3 cells following transfection with DNA from SPA tumors indicated that these tumors did not contain any transforming gene which was active on NIH/3T3 cells. This fact does not exclude the still existing possibility that these tumors may contain

transforming genes which can be active on other types of cells but not on NIH/3T3 cells.

e. A Recent Lentivirus Isolate from SPC in South Africa

R. Vigne, G. Querat, V. Barban and N. Sauze from the Laboratoire de Virologie, Faculté de Médecine, Marseille, France and D.W. Verwoerd from the Department of Agriculture, Onderstepoort, Republic of South Africa, very recently isolated a novel lentivirus from sheep lung carcinoma diseased animals. This new virus contains an RNA genome which is weakly hybridized to DNA probes specific for maedi/visna virus and CAEV. Restriction enzymes map of the linear unintegrated SPA provirus is very distinct from those of MVV and CAEV. However, as SPCTV, the South African isolate share internal proteins of similar sizes with some common antigenic determinants as measured by immunoprecipitation of 35-S-methionine labeled viral proteins. Tumor cells naturally infected by the South African isolate contain the viral information in a latent form in a small proportion of the cells. However, once the latent virus is activated, cells present CPE typical of SPCTV and produce extracellular virions. Since the South African isolate shares many characteristics with SPCTV, a collaborative study is presently being undertaken to see if it is indeed the same virus which could be implicated in tumorigenesis.

IV. LENTIVIRUS ISOLATES IN ISRAEL

In order to ascertain that SPCTV is indeed a new and novel virus in the retrovirus subfamily, we extended our studies to additional lentivirus isolates available, emphasizing our local isolates.

Israel has, until recently, been considered free of maedi/visna. However, among sheep imported into Israel from Europe and slaughtered for meat under quarantine, MV was diagnosed. No cases have been observed in the local Awassi sheep, although a serological survey performed in 1979

indicated that over 20% of the Awassi sheep from different herds in Israel had antibodies to MV virus.

In order to improve the production of the local breeds, numerous attempts have been made to cross them with various rams imported from Europe. These rams were kept at an experimental agriculture station where the cross breeding was carried out. The progeny were sent to isolated substations where they were used for further breeding. Since 1979, in the progeny of Finnish (European imported) and Awassi (local) crossbreeds, several cases of MV were clinically and histopathologically diagnosed at both the main agriculture station and at the substations.

Clinical and histopathological studies indicate that ovine progressive pneumonia (OPP), present in the US, is indistinguishable from maedi/visna (MV) in Europe. The etiological agents of MV and OPP are lentiviruses and have been considered closely related by morphological, serological and biochemical criteria. Major differences between MV and OPP have been detected, however, by nucleic acid hybridization and radioimmunoassay. Confirmation of these major differences was needed since OPP could be considered an entity distinct from MV.

We have recently isolated, for the first time in Israel, a lentivirus from MV diseased lung tissue obtained from one of these clinical cases at the agricultural station. This virus isolate was termed Israel maedi/visna (I-MV). Although at no time were any sheep from the USA introduced into the agriculture experimental station, the nucleic acid hybridization data presented here indicate that I-MV is indistinguishable from OPPV (USA isolate) and differs greatly from SPCTV and from the other members of the lentivirus group. This also confirms a previous study that indicated that OPPV differs genomically from MV (Perk et al., 1985).

Materials and methods used in this part of the project are as follows:

Viruses and cell culture, using these viruses: Israel-maedi/visna (I-MV); ovine progressive pneumonia virus (OPPV), generously provided by Dr. R.C. Cutlip, USDA, Ames, Iowa; visna viruses D (strain 1514 and 485 and Icelandic isolates); zwoegerziekte (ZZV; strain 133-lung isolate from the Netherlands, generously provided by Dr. G.F. deBoer, Central Diergenesskundig Institute, Lelystad, Holland); caprine

arthritis-encephalitis virus(CAEV; US isolate); bovine leukemia virus (BLV); Rausher murine leukemia virus (MLV) and avian myeloblastosis virus (AMV). The lentiviruses (I-MV, OPPV, visna, SPCTV, ZZV and CAEV) were grown on himalayan tahr ovary cells,(HTO), fetal ovine cornea cells (OFC) and on sheep choroid plexus cells (SCP); BLV was grown on fetal sheep kidney cells (FSK); MLV was grown on NIH/3T3 cells and AMV was grown on chick embryo fibroblasts. In addition, the infectivity of I-MV was tested on fetal kitten lung cells (KL; ATCC CCL 150), fetal mink lung (ML; ATCC CCL 64) and FSK cells.

Reverse transcriptase assay-- Tissue culture growth medium was assayed for reverse transcriptase activity.

Preparation of ^3H cDNA --Virus particles were purified by isopycnic sucrose gradient centrifugation and the 70 S viral RNA purified. Representative ^3H -labeled complementary DNAs (cDNA) were prepared in an exogenous reaction (0.1ml) containing 1.5 mg oligo $^{\text{dT}}$ 12-18 primer, 3ug viral RNA, 60 units purified AMV reverse transcriptase, 0.01% Nonidet P-40, 50mM Tris-HCl, ph 8.0, 6 mM MgCl_2 , 40mM KXl, 2 mM DTT, 100 mg Actinomycin-D, 1 mM each of dATP, dGTP, dTTP and 200 uCi ^3H -dCTP (21 Ci/mmole). Following a 3 hour incubation at 37°C , the ^3H cDNA was deproteinized, purified and the RNA template was alkali hydrolyzed.

Association kinetic analysis -- a total of 1000 cpm of viral ^3H cDNA was annealed at 68°C to increasing amounts of 70 S viral RNA in a 10ul reaction volume containing 0.6 M NaCl, 0.04 M Tris-HCl, ph 7.2, 0.002 M EDTA and 0.1% SDS. Hybrid formation was assayed by resistance to S_1 nuclease. Hybridization values are given after subtraction of the self annealing values (approx. 15%).

The infectivity of the Israel maedi/visna isolate for different cell types was examined by attempting to infect various cell cultures. Reverse transcriptase levels were measured at different times after virus infection and representative data are presented in Table 6. It is apparent that HTO cells and OFC cells had acquired a high level of supernatant reverse

transcriptase activity (i.e., infectivity) and therefore, most of the comparative studies were performed on these two lines.

The in vitro growth characteristics of I-MV was compared to that of OPPV, SPCTV, and maedi/visna virus. Although all four viruses could productively infect HTO cells, maedi/visna virus infection resulted in rapid (within 7-10 days) and widespread cell death preventing further subculture of the cells. Conversely, HTO cell cultures infected with I-MV or OPPV showed a sustained high level of virus release (at approximately 14 days after infection) and a moderate degree of cytopathic effects (CPE). I-MV infected and OPPV infected HTO cultures could be subcultured for a limited number of times despite the CPE. Similar results were observed in the infection of sheep choroid plexus with I-MV, OPPV and maedi/visna virus.

To compare the genetic relatedness of I-MV to known isolates of viruses from the maedi/visna group, reassociation kinetic analyses were performed between I-MV ³HcDNA and 70 S RNAs extracted from OPPV, visna virus (strain 1514) and zweegerziekte virus (strain 133). For further comparison, the ³HcDNA probe was annealed to 70 S RNAs of other retroviruses (bovine leukemia virus, Rausher murine leukemia virus and avian myeloblastosis virus). The hybridization data show (Fig. 17) that OPPV RNA had close, if not identical genetic homology to the I-MV cDNA, whereas the visna virus RNA had approximately 60% homology and the ZZV RNA had approximately 40% homology to the I-MV cDNA. The reciprocal hybridizations using OPPV cDNA, visna virus cDNA and ZZVcDNA and I-MV 70 S RNA (Fig. 18) supported the observed levels of hybridization with the I-MV cDNA. No nucleotide sequence relationships were apparent with the representatives of the murine, bovine and avian retroviruses. In addition, attempts at the induction of endogenous retrovirus from HTO and OFC cells using the inducing agents BrdU and IdU proved unsuccessful (data not shown).

These hybridization data therefore confirm the classification of I-MV with the lentivirus group. The extensive homology existent between viral genomes of the Israeli maedi virus isolate and the ovine progressive pneumonia virus (USA isolate) extends the range of OPPV, which was found previously only in the US. Furthermore, the results showing that only limited sequence homology exists between I-MV with European maedi/visna

strains is puzzling, since it would be suspected that the Israeli sheep would be exposed to these strains due to the importation of sheep from Europe.

The Israel CAEV isolate isolated by us is indistinguishable from the CAEV Florida US isolate. The California CAEV isolate which is probably the closest to SPCTV will be discussed under the subtitle F - Cooperation.

V. HERPES AND ITS POSSIBLE INVOLVEMENT IN THE AETIOLOGY OF SPA IN ISRAEL

In addition to the retrovirus association in SPA, a herpes virus was also implicated as a possible aetiological factor for SPA (Mackay, 1969; de Villiers 1979). Caprine herpes virus (CHV-1) (Roizman 1982) was first isolated in Scotland from adenomatous lung tissue, from a sheep with SPA (Mackay 1969). Subsequently reports from Yugoslavia, (Neujestic et al., 1971), Kenya (Malmquist et al., 1972) and from South Africa (de Villiers et al., 1975) indicated the isolations of herpes virus from lung tumors of SPA. These observations indicated to us to examine for the presence of CHV-1 antibodies in the diseased and normal sheep in Israel. Four CHV-1 serological survey the following methodology was used.

Hyperimmune serum was raised in rabbits to CHV-1. The rabbits were inoculated with 1 ml CHV-1 1/V once a week for 5 weeks, and by an intramuscular injection of 1 ml CHV-1 in complete Freund's adjuvant.

Neutralisation tests were carried out in microtitre plates. Blood samples (sera) were obtained from approximately 100 sheep in farms where SPA was present, from three SPA diseased animals. In addition 80 random samples were taken from sheep flocks from different farms.

All sheep serum samples examined were found to be negative. In order to verify this important observation, representative serum samples were sent to Dr. O. Straub, who confirmed our negative results. Thus the initial isolations of CHV-1 only from adenomatous tissue from sheep with SPA which led to the belief that this virus is involved in aetiology of SPA is not the case. Since no antibodies to the herpes virus could be detected in the SPA diseased nor in flocks with SPA history. Recently it was also shown that there was no direct association between infection of CHV-1 and SPA

(Scott 1985). Furthermore, antibodies to CHV-1 were found in Australia and Northern Ireland, countries where SPA is not known to occur.

Our serological survey which indicates the lack of association of CHV-1 and SPA is, however, too small to be truly representative for the whole country being free from CHV-1 infection.

VI. THE RETROVIRUSES ASSOCIATED WITH SHEEP PULMONARY ADENOMATOSIS

Retrovirus particles were first observed in SPA tumor cells by Perk et al., (1971) and the association of these particles with the tumor was complemented by biochemical investigations and transmission experiments (Perk et al., 1974; Martin et al., 1976; Sharp et al., 1983; Verwoerd et al., 1980; and Herring et al., 1983).

Although the morphological and biochemical findings of the retrovirus association in the lung tumors in the four countries examined - Scotland, Peru, South Africa and Israel - are in agreement (Perk et al., 1985a), there are differences in virus latency. Furthermore, the only tissue culture virus isolate, SPCTV, differs in its antigenic properties from the particulates processed from SPA lung fluids or lung washes from affected sheep.

Recently, by means of the Western blotting technique, an immunological cross-reaction has been demonstrated between the p27 major core polypeptide of Mason-Pfizer monkey virus (MPMV) and a p25 in the retrovirus present in SPA in Scotland (Sharp and Herring, 1983). This technique therefore presented the opportunity to compare one further aspect of the retroviruses identified in SPA tumors and lung fluids from several countries, and SPCTV.

Retrovirus was extracted from SPA lung fluids or lung washes from affected sheep in Scotland, Peru, South Africa and Israel, or obtained from cell culture supernates SPCTV. The presence of the p25, identified in the retrovirus associated with SPA in Scotland was determined by the Western blotting procedure, employing a goat antiserum to MPMV p27 (Sharp and Herring, 1983). Using this technique, the p25 was demonstrated in the

retroviruses obtained from lung fluids or lung washes from affected sheep in Scotland, Peru, Israel and South Africa but was not detected in the retroviruses obtained from cell culture supernates SPCTV (Perk et al., 1985c; Perk and Irving, 1986; Perk, 1987).

It is clear therefore that the retroviruses present in SPA lung fluid and tumors from these four countries possess a common antigen on the p25 that was taken with their similar biochemical properties. It is equally clear, however, that SPCTV obtained from the tumor cell cultures is different in that it does not possess the same antigen on the p25. It will be therefore important to further examine the two retroviruses types and determine their involvement in the pathogenesis of SPA. The characterization of the lung fluid "isolate" and its comparison and relatedness to SPCTV and the lentivirus isolated in South Africa has been greatly handicapped by the lack of virus isolation and of an in vitro culture system of the lung fluid virus particulate. Furthermore, Sharp and Covorkers (Sharp et al., 1985) recently was able to induce SPA after a very short incubation period. In a number of experiments, by using preparations of lung fluids that accumulate in the respiratory tract, particularly during the terminal stages of the disease, and serially passaged, the latency was reduced to 3 weeks, which means that if it is really cell free it must be an acute defective virus. In nature or in our experimental transmission experiments disease occurred more than after six months.

This important observation of the dramatic reduction in latency of SPC, was tried to reduplicate in our laboratory without success, in three separate, repeated experiments. The lung fluids for the preparation of the inoculum were obtained from typical SPC clinical cases. The lung fluid preparations were processed according to the protocol used by the researchers in Scotland (Herring et al., 1983; Sharp et al., 1985).

The prepared lung fluid particulate was inoculated intratracheal in six newborn colostrum deprived lambs. The lambs according to our protocol were autopsied two months after inoculation. SPA could not be diagnosed macroscopically or histopathologically. Because of its importance, this experiment was repeated two additional times with the same negative results. We do not have data to explain this discrepancy of results.

F. COOPERATION

The collaboration originally planned consisted of the following points:

Joint planning and evaluation, exchange of biological material, study the nature of lung lesions seen in Californai goats, survey for SPCTV of sheep and goats, virus isolation and SPCTV prevention trials.

Numerous goats in California herds showed clinical symptoms reminiscent of maedi/visna in sheep. However, at autopsy they differed from the classical maedi/visna lesions. These observations made by Drs. G. Theilen and N. East were made before the caprine arthritis-encephalitis disease syndrome was recognized and CAE virus was reported. Since the lung lesions were suspicious for lung adenomatosis, this collaborative project was initiated .

1. Pathology

As reported in our first year's report, forty five samples of goat lung lesions reminiscent of SPC were selected in California for a comparative histopathological study.

2. Virology

A retrovirus was isolated from these California diseased goats and was diagnosed as CAEV. Furthermore, goat samples obtained from the California herds also indicate that most of the infectivity is confined to the milk, even though the antibody filters are almost the same as in the synovia, lymphocyte and plasma. Four representative cases illustrate it.

Goat No.	Synovial fluid			lymphocyte extract			milk			plasma		
	Ab	Ag	Virus	Ab	Ag	Virus	Ab	Ag	Virus	Ab	Ag	Virus
11	80	4	(-)	10	1	(-)	40	1	(++)	100	1	-
34	2560	24	(-)	640	24	(-)	40	16	(++)	3000	16	-
101	250	N.D.	(-)	4000	3	(-)	2560	1	(++)	500	2	-
109	10	4	(-)	640	1	(+)				40	1	2

The histopathologic and virologic findings of the California goats are of special interest since in the lentivirus group SPCTV was found to be closest to CAEV. Furthermore, in hybridization experiments comparing four CAEV isolates with SPCTV, the California isolate was the closest. (Manuscript in preparation.)

Histopathologically, the lung sections showed, in addition to lymphoid infiltration, lymphoid hyperplasia as seen in maedi/visna of sheep, alveolar epithelization and very marked proliferations of these epithelial cells which form acini and papillary structures. Many areas in these lesions are histologically indistinguishable from the tumor nodules seen in sheep pulmonary adenomatosis (Perk, 1987 in press; Perk, Theilen and East manuscript in preparation). Furthermore, select samples taken for ultrastructure and histochemistry revealed that the epithelial cells involved are proliferating type II alveolar cells which are the major components giving rise to SPC. However, till today no metastasis of these epithelial cells could be demonstrated in contrast to SPC where metastases are regularly found.

3. Serological Prevalence of CAEV in California, a retrospective study

a. We studied the serologic prevalence of caprine arthritis-encephalitis virus (CAEV) in California goat dairies. Fifty-three percent of 2,826 goats in 13 California goat dairies had antibodies to CAEV, as determined by agar gel immune diffusion test.

b. Those goat dairies that reared kids on pasteurized milk had a lower seroprevalance than those that did not. The relationship between age and

antibodies to CAEV was observed for goats reared on pasteurized or unpasteurized milk, which indicated that continued horizontal contact/transmission may be important on these dairies and limited the effect of a pasteurized rearing program on control of CAEV infection (East NE et al., 1987).

4. Serological prevalence of CAEV, a prospective study. At birth kids were fed cow's colostrum and isolated until 9 months of age. These kids were then placed in contact with the milking herd and 5 percent became infected.

5. Serological prevalence of CAEV, a prospective study, where negative goats are maintained in isolation. Forty-six CAEV negative kids fed cow's colostrum are being reared in strict isolation and separated by 2 meters from the rest of the herd by double fencing. Forty-six negative control goats will be reared in a standard goat dairy. This study will be completed by Fall or Winter of 1987.

6. Immunopathology of CAEV. Two control and 10 CAEV infected goats are being sequentially studied using immunoperoxidase to determine the migration of CAE virus in infected hosts and establish types of cells that become infected. This study will be completed by Fall of 1987.

TABLE 1
RETROVIRUS SUBFAMILIES AND THEIR DISEASE ASSOCIATION IN THEIR NATURAL HOSTS

<u>Subfamily</u>	<u>Disease</u>	<u>Natural Host</u>
Oncovirinae	Cancer: leukemia,	man, animals
	lymphosarcoma, sarcomas	birds, reptiles
	mammary carcinoma	mice
	pulmonary carcinoma (?)	sheep
Spumavirinae	Inapparent Infections	man, animals
Lentivirinae	slow infectious pneumonia	sheep, goats
	meningoencephalitis	sheep, goats
	arthritis	sheep, goats
	immune deficiency	man, cats
	pulmonary carcinoma (?)	sheep

TABLE 2

ENDOGENOUS REACTION REQUIREMENTS OF SPCV DNA POLYMERASE

<u>Reaction Mixture</u>	<u>Enzymatic Activity (^3H-CPM)</u>
Complete	9,065
-dATP	1,010
-dGTP	1,066
-dCTP	1,299
Pretreated with RNase A ₁	2,369

TABLE 3

REVERSE TRANSCRIPTASE ACTIVITY IN THE MEDIUM OF VARIOUS SHEEP AND GOAT CELL
CULTURES INFECTED WITH SPCTV

REVERSE TRANSCRIPTASE ACTIVITY (^3H -CPM)							
CELL	DAYS FOLLOWING INFECTION						
TYPE	5	7	10	17	20	26	34
SL	2,765	3,769	4,154	112,249	50,732	96,799	103,138
SM	342	950	292	908	2,008	1,945	2,120
HTO	506	661	1,863	23,398	230,244	220,037	234,145
OFC	N.D. ¹	494	N.D.	537	3,442	N.D.	N.D.

¹ N.D. = not determined

TABLE 4

IN SITU HYBRIDIZATION DISTRIBUTION OF THE NUMBER OF GRAINS/CELL IN
SPCTV INFECTED SL CELLS

	Number of grains/cell		
	0	1-15	15
MONONUCLEAR	7 ¹	12	17
MULTINUCLEAR	14	22	63

¹ Numbers are percentage of cells with the specified number of grains.

TABLE 5

COLONY GROWTH IN SOFT AGAR OF HTO AND SL CELLS CHRONICALLY INFECTED
WITH SPCTV

Cells	Colony Diameter (um)			
	0-30	30-60	60-90	90
HTO	¹ 95.4+0.40	4.7+0.40	0	0
SL	97.6+0.60	2.4+0.60	0	0
HTO-inf	56.7+1.43	41.4+1.46	1.70+0.37	0.20+0.04
SL-inf	92.7+0.34	7.0+0.36	0.30+0.10	0

¹ -Percentage of colonies with a diameter in the specified range. Numbers are average + SE of 15 dishes.

TABLE 6

REVERSE TRANSCRIPTASE ACTIVITY IN THE MEDIUM OF CELL CULTURES INOCULATED
WITH I-MV

CELL TYPE	<u>REVERSE TRANSCRIPTASE ACTIVITY (^3H-CPM)</u>	
	<u>DAYS FOLLOWING INOCULATION^a</u>	
	8	22
KL	309	285
MK	234	198
SCP	222	287
FSK	310	335
OFC	50,374	198,160
HTO	17,898	100,466
OFC control	276	408
HTO control	527	554

^aThe growth medium was removed from subconfluent monolayer cultures and they were inoculated with approx. 0.5 ml growth medium from an I-MV producing cell culture for 2 hr at 37°C following which the growth medium was replaced.

Figure 1 : A five years old Awassi ewe with pulmonary carcinoma.

Figure 2 : The Retrovirus family.

Fig. 3

Fig. 4

Figure 3 and 4 : Electron micrograph of SPCTV grown on SL cells. (3) stages of budding virions (x75,000) ; (4) extracellular mature virions (150,000).

Figure 5: Buoyant density of SPCV virions on isopycnic sucrose density gradient. O, "reverse transcriptase" activity (^3H -cpm) on synthetic exogenous template-primer of oligo(dT)₁₂₋₁₈:poly(rA); ●, density (g/ml) of sucrose gradient fractions tested.

Figure 6 : Divalent cation requirements of SPCTV associated "reverse transcriptase". $\bullet$, Mg^{++} ; $\circ$, Mn^{++} .

Figure 7 : Determination of the optimal NP-40 concentration for the SPCTV associated "reverse transcriptase" reaction.

Figure 8 : Genetic relatedness of SPCTV and other member of the Lentivirus group to other retroviruses. (A) Association kinetic analysis between SPCTV ³HcDNA and 70S viral RNAs from SPCTV, visna virus, CAEV, PPV, BLV, MoMuLV, and AMV ; (B) Thermal stability profiles of hybrids formed between SPCTV ³HcDNA and viral RNAs from SPCTV, visna virus, CAEV, PPV ; (C) Association kinetic analysis between SPCTV 70S viral RNA and ³HcDNA probes complementary to visna virus, maedi virus, CAEV, PPV, BLV, MoMuLV, and AMV ; (D) Association kinetic analysis between visna virus ³HcDNA and 70S viral RNAs from SPCTV, visna virus, CAEV, and PPV ; (E) Association kinetic analysis between CAEV ³HcDNA and 70S viral RNAs from SPCTV, visna virus, CAEV, PPV. 70S viral RNAs or ³HcDNAs were derived from SPCTV (○), visna virus (●), maedi virus (▼), CAEV (Δ), PPV (▲), BLV (▽), MoMuLV (□), or AMV (■).

Figure 9 : Association kinetic analysis between SPCTV ³HcDNA and cellular RNA (A) or DNA (B) of various cell types. HTO cells infected with SPCTV (●), uninfected HTO cells (○), normal sheep lung cells (Δ), cat liver cells (▲).

Figure 10: Double antibody competition radioimmunoassay for ^{125}I -CAEV p28 with anti-CAEV antiserum. Competing antigens: $\circ$, CAEV; ∇ , SPCTV; Δ , PPV; $\bullet$, BLV; $\square$, HTLV-1.

Figure 11: Double antibody competition radioimmunoassay for ¹²⁵I-CAEV p28 with anti-PPV antiserum. Competing antigens: ○, CAEV; □, SPCTV; △, PPV; ▽, EIAV.

Figure 12 : Cytopathic effects in SPCTV infected SL cells. Giemsa stain.
(A) uninfected SL cells (x180); (B) 3 day post infection (x360);
(C) 7 days post infection, syncytium (x180); (D) 11 days post
infection (x80).

Figure 13 : Quantitation of syncytium formation in SPCTV infected SL cells. Percentage of cells with one nucleus ($\bullet$), 2-5 nuclei ($\circ$), 5-20 nuclei ($\square$), and 20-100 nuclei (Δ); Reverse transcriptase activity determined in the culture medium ($\blacksquare$).

Figure 14 : Morphological changes in SPCTV infected HTO cells. Phase contrast micrograph. (A) foci of piling up and multi-layer growth (x80), (B) polygonal and multinuclear cells (x160).

Figure 15 : In situ hybridization. SL cells infected with SPCTV (x320); inset (x640); note grains in the nucleus and perinuclear area.

Figure 16 : Lymphoid tumors in nude mice inoculated with SPCTV infected HT0 cells. Hematoxylin and eosin stain. (A,B) subcutaneous site of inoculation (x250,x650) ; (C,D) lung (x250,x650) ; (F,G) liver (x250,x650) ; The tumors were 4 months post inoculation.

Figure 17 : Association kinetic analysis between Israel-maedi-visna $^3\text{HcDNA}$ and 70S viral RNAs from Israel-maedi-visna virus (▲); ovine progressive pneumonia virus (△); visna virus (●); zweegerziekte virus (○); CAEV (□); Rausher murine leukemia virus (■); AMV (▣); and BLV (▤).

Figure 18 : Association kinetic analysis between Israel-maedi-visna 70S RNA and $^3\text{HcDNA}$ complementary to Israel-maedi-visna virus (▲); ovine progressive pneumonia virus (△); visna virus (●); CAEV (□).

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