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**Virologic, Immunologic, Epidemic and
Pathogenetic Studies of Slow Viral Lung
Diseases in Sheep**

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ABSTRACT

The isolation and partial characterization of three distinct retroviruses, all of which cause "slow" diseases in sheep and goats, and one of which is a novel isolate are described in this report. This unique retrovirus was shown here to be the causative agent for pulmonary carcinoma of sheep (SPC).

A purified viral cell-free preparation, from a fresh tumor was inoculated into the lung of 5 sheep; 4 developed focal or widely distributed tumorous lesions characteristic of sheep lung carcinoma (judged by microscopical and ultrastructural criteria), within 1 year. Co-cultivation of primary tumor cell cultures (prepared from an advanced virus-induced tumor) with tahr cells (permissive for retrovirus replication) yielded ample quantities of the same virus, as proven by ultrastructural, and biochemical means. The virus-induced tumor was successfully transplanted into nude mice.

Also, a retrovirus which causes slow progressive proliferation of epithelial cells and smooth muscle cells of the lung alveoli of sheep, a disease known as "maedi" in various parts of the world, was isolated by us in Israel for the first time.

A third retrovirus which causes the caprine (goat) arthritis-encephalitis (CAE) syndrome, with common involvement of the lung, was also isolated from sick goats, and its growth in tissue culture was achieved.

Extensive clinical and histopathological examinations of goats in California with a syndrome reminiscent of the Israeli CAE disease and that found in Florida, showed similarity though not identity. The disease in California lies, histopathologically, between CAE and SPC. Virus isolation and characterization of the California herds are under study. The last two mentioned viruses are classified as lentiviruses, e.g., non-oncogenic retroviruses. DNA-RNA hybridization experiments revealed the

uniqueness of the virus which causes lung carcinoma of sheep by comparison to known oncogenic retroviruses from avian and mammalian species, and to various lentivirus isolates. The Israeli isolates of Maedi virus and caprine arthritis encephalitis virus, the European strain (1514) of visna virus, the U.S. strain of sheep progressive pneumonia virus and the U.S. strain of caprine arthritis encephalitis virus.

The establishment of the conditions for growing these viruses (primarily, the sheep lung carcinoma virus) should enable further studies on their origin, the pathogenesis of the diseases which they cause and the development of serological tests for their detection. Eradication programs and even vaccination protocols, will presumably stem from this project.

The determination of a tissue protein which is released to the serum in increasing quantities - in parallel to the progression of the lung tumor - as established in this project, serves as preliminary test for the identification of sheep suspected of carrying SPC tumors.

INTRODUCTION

Slow virus infections are a fascinating group of entities; an animal or person exposed to these agents becomes diseased gradually over the course of many months or years. Thus the term "slow" refers to the clinical course of the disease, rather than to the inducing agent, which persists in host tissues throughout most of the clinical disease.

The concept of slow virus infections was first introduced by Bjorn Sigurdsson in his classic descriptions of rida (Icelandic form of scrapie), visna (meaning "wasting"), maedi (meaning "shortness of breath"), and infectious pulmonary carcinoma (jaagsiekte), as transmissible chronic diseases of Icelandic sheep (1,2,3,).

Sigurdsson's concept evolved from his observations on the natural history of this novel group of chronic sheep diseases, which were apparently introduced to Iceland in 1933 when 20 Karakul sheep were brought by the Icelandic government from Halle, Germany. This import was intended to improve the productivity of Icelandic sheep, a primitive breed of hill sheep which were originally brought to Iceland by its first settlers more than 1000 years ago, and had subsequently been kept in almost complete isolation. After a 2-month period of quarantine, the imported sheep were distributed to 14 farms throughout the island. Epidemiological studies showed that two of the imported rams were carriers of maedi and one of them was also a carrier of pulmonary carcinoma (SPC). This latter ram showed distinct symptoms of pulmonary disease in the spring of 1934 and never returned from the common summer pasture. The following year SPC started to appear on the farm where this ram had been kept, as well as on neighboring farms, and the following year the disease spread to many other farms, causing

heavy losses - some exceeding 50% of sheep in a flock annually (4).

Maedi and SPC were found in many farms simultaneously in the same flock and sometimes even in the same animal.

This project was started at time when sheep pulmonary carcinoma was known to be a tumor with high frequency in herds of sheep in Israel (5). On the other hand, Maedi, a slowly developing interstitial pneumonia proven to be caused by a virus which belong to the retroviridae family, was considered nonexistent in Israel. However, some time before this project was initiated it was suspected, on the basis of clinical and histopathological examinations and on serological analyses performed in collaboration with Dr. DeBoer (Holland), that Maedi disease was introduced into Israel by the import of sheep (I. Hod, unpublished data).

This contagious disease is known to cause significant economic losses to sheep production in Europe, and therefore a key objective of this project was to examine Israeli sheep for the presence of Maedi and its causative agent (Maedi-virus).

Since virus particles resembling the type-C retroviruses had also been demonstrated by us in SPC tumors (6), and the association of viral RNA and reverse transcriptase were also demonstrated in the tumors (7), it was suspected that a retrovirus is the causative agent for SPC as well. The natural transmission of the disease among sheep in herds in Europe (8), and in Israel (9), and the experimental data obtained by several research teams (10, 11) also supported the high probability of a viral etiology for this disease. The economic impact of this disease and its possible relevance to human oncology were self evident and therefore, the main objective of this project was to isolate and grow the virus from SPC, and to prove its etiological role. Making this virus available in large quantities by finding the optimal

conditions for its growth in culture was necessary for starting a meaningful strategy for the detection and follow-up of SPC in herds of sheep.

Furthermore, clinical, biochemical and virological comparisons between the two lung diseases, one being a malignant tumoral disease, the other being a chronic inflammatory disease, could be made only after isolating and growing the two viruses in tissue culture..

In the course of this study the American cooperating investigator, Dr. Theilen, drew our attention to the presence of another chronic disease which involves the lungs (and the joints and brain) of goats in California. Interestingly enough, this disease was also proved to be caused by a retrovirus. Mortality of goats from a disease which involves brain, pulmonary and arthritic lesions was reported in Israel in recent years, before and at the time this research was conducted in Israel. It was therefore decided to examine cases of these diseased herds, characterize the causative agent, and if indeed a retrovirus could be isolated, to grow it in tissue culture and examine its biochemical relatedness to the other two viruses mentioned above (SPC and Maedi).

The results obtained by us, definitely indicate that the caprine arthritis-encephalitis virus is an additional distinct member of the lentivirus group of the retroviridae family (12). The etiological agent of the syndrome observed in the California goat herds is under study.

The results of these various searches, which were carried out during the three years of this project, will be outlined in the following paragraphs.

MATERIALS AND METHODS

1. Preparation of histology samples

Tissue samples were fixed in neutral buffered formalin and routinely stained with hematoxylin and eosin. Some preparations were tested for the presence of glycogen granules by staining with Periodic Acid Schiff reagent.

2. Preparation of electron microscope samples

Tissue samples were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) and post-fixed in osmium tetroxide. Following dehydration in ethanol and propylene oxide, the samples were embedded in Eppoxy resin and sectioned with a diamond knife. Sections were picked onto copper grids and stained with uranyl acetate and lead citrate. Viewing was performed in a JEOL electron microscope.

3. Hematology and serum proteins

Peripheral venous blood was taken into sodium citrate (to a final concentration of 1%) to prevent clotting. Routine hematological tests included red cell and white cell count, percent hemoglobin (these three parameters determined using a Coulter Counter), hematocrit and differential white cell count.

Serum was separated from peripheral blood samples which were allowed to clot overnight. Sera were electrophoresed on polyacrylamide gels, stained with Coomassie Brilliant Blue and scanned in a densitometer. Total protein concentration in the sera was determined by the Biuret assay.

4. Cell cultures

A number of primary cell cultures and tissue culture lines were established or collected by us for the purpose of retrovirus isolation and propagation (Table 1). Primary cultures were established from explants (minced tissue) or trypsinized samples. Cell cultures were routinely grown in Dulbecco's Modified Eagle Medium with the addition

of 10% fetal calf serum, 2mM glutamine and antibiotics (1 x antibiotics are 100 U/ml. penecillin, 100 ug/ml streptomycin and 2.5 ug/ml. Amphotericin B), in plastic tissue culture flasks (75 cm² area) in a humidified atmosphere of 95% air - 5% CO₂ at 37°C.

5. Assay for reverse transcriptase activity

Two ml. samples of tissue culture medium or plasma were centrifuged at 3,000 x g. for 10 minutes, then centrifuged at 100,000 x g. for 1 hour on a cushion of 20% glycerol in TNE buffer (0.01M Tris, pH 7.4; 0.1 M NaCl; 0.001 M EDTA). The resulting pellet was resuspended in 50 ul Tris (0.01 M, pH 8.1) and NP - 40 was added to a final concentration of 0.2%. After an incubation period of 10 minutes at 0°C, a reaction mixture (50ul) containing: 50 mM Tris - HCl (pH 8.1), 30mM MgCl, 48mM KCl, 0.4mM dithiothreitol, 0.4ug oligo dT: poly rA, 0.2mM ATP, 0.1mM TTP, ³H-TTP (40-50 Ci/mMole, 1000 cpm/pMole) was added. .

Following an incubation period of 30 minutes at 37°C, the reaction was stopped by the addition of 0.5 ml 35% trichloroacetic acid (TCA) and the precipitated nucleic acids collected on nitro-cellulose filters. The filters were washed repeatedly with cold 5% TCA, dried and the bound radioactivity counted in a liquid scintillation counter using toluene based scintillation fluid.

6. Fractionation of sheep lung tumors

All sheep lung tumors were stored at -70°C. Samples were assayed for the presence of retrovirus as follows. Frozen samples (10 - 50 gm) were ground in a Waring blender for 2 minutes, in 10 volumes of TNE buffer at 0°C. The suspension was further homogenized for 2 minutes in a Polytron tissue homogenizer. Cellular debris was pelleted at 2,000 x g. for 10 minutes at 2°C, and the supernatant cleared by centrifugation at 10,000 x g for 10 minutes at 2°C. The supernatant was then layered onto 20% glycerol in TNE and centrifuged at 100,000 x g for 1 hour at 2°C. The pellet was resuspended in 2 ml TNE and layered onto a continuous

gradient of 20% - 55% sucrose in TNE (total volume 70ml.) and centrifuged at 100,000 xg overnight (12 - 14 hours). The gradient was fractionated into 2 ml fractions and the density of each determined using a refractometer. Since the density of retroviruses in isopycnic sucrose gradients is approximately 1.16 g/ml., fractions spanning this region of the gradient were tested for reverse transcriptase activity. Generally, a 20 ul sample of the sucrose fraction was diluted to 50 ul with 0.01 M Tris (pH 8.1) and 0.2% NP-40 and the reaction performed as described above.

7. Inoculation of cell cultures

The density of the fractions exhibiting reverse transcriptase activity corresponded to that of retroviruses. Viruses were collected by centrifugation at 100,000 xg for 1 hour at 2°C, and resuspended in one ml. TNE buffer containing 5x antibiotics. Following an incubation period of one hour at 0°C, this inoculum was placed onto subconfluent monolayers of various cell lines. After approximately 4 hours, 15ml. standard tissue culture medium was added. At intervals of one or a few days samples of the tissue culture medium were removed and assayed for reverse transcriptase activity.

Cultures to be examined by electron microscopy were rinsed twice with PBS and scraped off the plastic flask using a rubber policeman. The cells were suspended in one ml. glutaraldehyde fixative and pelleted for 1 minute in a microfuge. The pellet was further processed according to our routine procedure for electron microscopy.

8. Purification of virus from tissue culture medium

Media from cultures releasing virus were collected every 2 days, clarified by centrifugation at 10,000 xg for 10 minutes and stored at -70°C. Virus was purified from thawed media in one to two liter batches as follows. Ammonium sulfate was added to a concentration of

35% (w/v) and the solution stirred for one hour at 4°C. The solution was then centrifuged at 10,000 x g for 35 minutes and the sediment resuspended in TNE in one-twentieth the original volume. Virus was pelleted at 100,000 x g. for 60 minutes at 2°C, resuspended in 2 ml TNE and placed on a 20% - 55% sucrose gradient. The gradient was centrifuged, fractionated and the reverse transcriptase activity determined as described above. Virus was repelleted, resuspended in TNE at a concentration of approximately 20 mg protein/ml. and stored at -70°C.

9. Extraction of RNA from SPC virions

Sucrose gradient purified virus was suspended in 1 ml TNE buffer to which were added: SDS (1%, final concentration), yeast carrier RNA (10 ug/ml, final concentration), EDTA (10mM, final concentration), and 250 ug self digested pronase. Following incubation (37°C, 30 minutes) the proteins were extracted with phenol-chloroform (1:1). The phenol-chloroform, was reextracted with TNE, the aqueous phases pooled and extracted with chloroform, NaCl was added to 0.4M, 2 volumes absolute ethanol were added and the RNA allowed to precipitate overnight at -20°C. The RNA was pelleted at 10,000 RPM for 30 minutes at 0°C in a Sorvall HB-4 rotor, resuspended in 200 ul TNE and layered onto a 15% - 30% sucrose gradient (in TNE) containing 0.1% SDS. The gradient was centrifuged for 1 hour at 50,000 RPM (in a Beckman 50.1 rotor), with marker RNA (70 S) in an opposite tube. The appropriate fractions were collected and precipitated from ethanol. The RNA was pelleted, resuspended in 5mM EDTA and the U.V. absorbance at 260 nm and 280 nm determined.

10. Preparation of SPCV cDNA

DNA complementary to the SPC viral genome was synthesized in an exogenous reaction using AMV reverse transcriptase. The reaction contained: ATP, GTP, TTP (1mM), 50mM Tris (pH 8.0), 6mM MgCl₂, 40mM KCl, 2mM DTT, 100ug Actinomycin D, 0.01% NP-40, 200uCi ³H-CTP, 4ul AMV

polymerase, 1.5 ug oligo dT₁₂₋₁₈, 3 ug SPCV RNA. After 30 minutes at 37°C, the reaction was stopped by the addition of 0.5% SDS and 0.005 M EDTA. Pronase (self-digested) was added to a final concentration of 200 ug/ml and incubated at 37°C for 30 minutes. NaOH was added to a final concentration of 0.3N and the RNA digested at room temperature overnight. 0.3N HCl was added to neutralize the mixture and the cDNA filtered on Sephadex G-50 and collected in the void volume.

11. DNA-RNA Hybridization

DNA-RNA hybridization in solution was performed in a volume of 10ul in siliconized capillary tubes. Each reaction contained 1000 cpm cDNA, varying concentrations of viral RNA, 0.6 M NaCl, 40mM Tris (pH 7.4) 2 mM EDTA and 0.1% SDS. The capillaries were sealed, boiled for 2 minutes, chilled on ice and incubated for 20 hours at 68°C. The capillaries were opened into NNZ buffer (30 mM Na Acetate, 3mM Zn SO₄, 0.3 M NaCl) and divided into two equal portions. To one of each pair S₁ nuclease was added, the contents incubated for 60 minute at 37°C, the TCA insoluble counts collected on millipore filters and counted in a liquid scintillation counter.

12. Heat denaturation of hybrids

DNA-RNA hybrids were prepared as above at 10 times the total volume (i.e., 100ul). Following a 20 hour, 68°C incubation, the hybrids were opened and divided into 14 tubes containing 200 ul NNZ buffer. Each test tube was heated for 5 minutes to temperature in the range 65°C to 100°C and rapidly cooled on ice. S₁ nuclease digestion was performed as outlined above.

13. Preparation of tissue-associated-protein complex (TAPC) and anti-TAPC antiserum

Serum-free lung carcinoma tissue sediment was prepared by exhaustive extraction of the tissue by acid-glycine buffer as previously

described (13). TAPC was eluted and concentrated from polyacrylamide gels as previously described (14). Antibodies to this protein were raised in rabbits and in turkeys. The hyper-immune sera were absorbed to a protein A sepharose CL- 4B column (15). The immunoglobulin G which was bound to the protein A gel column was then eluted by glycine buffer (pH 2.8). Final protein concentration and pH (7.0) were then adjusted with PBS.

14. ELISA Test

Microtiter polystyrene plates (flat-bottom wells) were used. Serum samples (obtained from healthy sheep and from sheep inoculated with SPC virus) were diluted 1:10,000 with coating buffer (pH 9.6); 200ul of the diluates were delivered into the wells for 2 hours of incubation at 37°C. The unabsorbed sera were washed three times with PBS, pH 7.5, containing tween 20 (final conc 0.05%). Rabbit immunoglobulin G (O.D. 1.4, read at 280mM) reactive against sheep tissue protein complex was diluted 1:100 with polyvinylpyrrolidone buffer (2% PVP in PBS-tween 20 buffer + 0.2% bovine serum albumin or ovalbumin); 200ul were delivered to the wells, for 2 hours incubation at 37°C. The unbound antibodies were washed 3 times with PBS - tween 20 solution. Alkaline phosphatase-labeled goat immunoglobulin-G reactive against rabbit immunoglobulin (Sigma) and diluted 1:1000 in PVP-40 buffer was added in portions of 200ul, for 12 hours of incubation at 4°C. The unreactive antibodies were then washed, as described above. Substrate solution (20mg phosphatase substrate dissolved in 200 ml substrate buffer - Sigma Chemical Company, St. Louis, Mo.) was added in a volume of 200 ul to each well. The plates were then incubated for 45 minutes at 37°C. The reaction was stopped and the intensity of color developed was determined at 405nm. using a Dynatech Microelisa Auto Reader.

RESULTS

I. Field survey and collection of tumor tissue

Sheep from four herds were screened for SPC and Maedi disease. This screening was based on clinical examination, serum protein analyses and, occasionally, chest x-ray examination. Also, immunodiffusion tests against disrupted Visna virus, were carried out showing a high infection rate for Visna-Maedi (Table 2), a situation previously not known in Israel. Animals were selected from these herds and autopsied. From these animals we obtained four SPC tumors and two pairs of lungs affected with Maedi disease. In addition, material was received from the Dept. of Pathology, Kimron Veterinary Institute, thus, a total of 7 SPC tumors and 15 Maedi diseased lungs were obtained. The pathological diagnosis at autopsy was confirmed by histology and electron microscopic examination.

II. In vivo transmission studies

Sheep were immunosuppressed with cyclophosphamide (1.0 or 1.5 gr/animal) prior to inoculation with a gradient purified, cell-free, reverse transcriptase containing fraction of a tumor (AD-29). The extent of the immunosuppression was monitored by standard hematological methods, and by changes in the various serum protein fractions. The animals were inoculated intrapulmonary with 10-15 gram equivalent of tumor fraction. They were examined weekly for one year and showed no deviations from the normal values for the parameters checked (hematology, serum proteins, reverse transcriptase in plasma.).

At the end of a one year observation period, the animals were sacrificed at weekly intervals and autopsied. Tissue samples and blood were taken for histology, ultrastructure, tissue culture and reverse transcriptase determination. The first four animals exhibited SPC in different stages of the disease, ranging from small multiple foci to

tumor involvement of the whole lobi of the lungs, including bilateral involvement. No metastases were found at this stage. As noted below, the SPC agent was successfully reisolated from these tumors. Tumors No. 180 and 207, which showed the most severe signs of SPC were used in further experiments for virus isolation and characterization. Eight lambs infected with a cell-free, reverse transcriptase containing fraction of Maedi tumor (AD-32) at the start of the second year of the project showed no histopathological indication of Maedi disease following a one year observation period.

Transplantation of SPC tumor in Nude Mice

Male ICR nude mice inoculated subcutaneously with miced tissue obtained from tumor 207 developed a transplant which contained cysts lined by a proliferative epithelium. These cysts showed several characteristics of the epithelial tumor cells of origin, such as high glycogen content and secretion of mucin. Microinvasion of epithelial cells from the adenomatous cysts into the stroma was observed. The stromal tissue demonstrated chondroid metaplasia. It was concluded that pulmonary carcinoma of sheep can be transplanted and grow in nude mice.

III. Isolation of retrovirus and in vivo propagation

Noting an increasing frequency in the occurrence of Maedi disease among Israeli sheep, based on: a) our serological screening of selected herds, b) the frequency with which we recieved Maedi diseased lungs from the slaughterhouse and, c) the report of Maedi disease in sheep imported for meat, we thought it relevant to determine whether a retrovirus could be isolated from such cases. This was done in parallel with our attempts to isolate retrovirus from SPC tumors; the successful isolation of an Israeli strain of Maedi virus from tumor AD-32 is described below.

Various techniques were used in our attempts to isolate a retrovirus from sheep lung tumors.

1. Inoculation of reverse transcriptase containing fractions onto various cell lines.

As shown in figure 1, no particle associated reverse transcriptase activity was released into the culture medium of infected cultures. Electron microscopic examination of these cultures showed no production of retrovirus.

2. Cultures of SPC tumor cells

Samples of tumors AD-28 and AD-29 were trypsinized and the cells successfully grown in vitro. These cultures were periodically examined for virus production by electron microscopy and by the reverse transcriptase assay. In spite of the fact that AD-29 contained particle-associated transcriptase activity (Fig. 2), and experimental transmission of SPC was achieved using cell-free fractions of AD-29 tumor homogenate, the tumor cells did not spontaneously release retrovirus particles in vitro. The treatment of these cultures with 5-iodo-2-deoxyuridine (20ug/ml) and 5-bromo-2-deoxyuridine (80ug/ml) also failed to induce virus production.

3. Co-cultivation of SPC tumor cells with cell lines sensitive for retrovirus growth

The observation of particle-associated reverse transcriptase activity in the original AD-29 tumor and the fact that it was a potent inoculum in vivo (and the diminished growth rate of these cells after approx. 15 passages in vitro) suggested that establishing cocultures from first passage tumor cells, with susceptible cell lines, would be advantageous.

Upon receipt of two fresh tumors, AD-180 and AD-207 (see part II) the trypsinized cells were directly plated with an equal number of each

heterologous cell type and the cultures monitored by the reverse transcriptase assay. The results are summarized in Table 3.

Medium from the co-culture of AD-207 tumor cells and tahr cells was collected and used to infect additional tahr cell cultures. Virus-containing media from these cultures were harvested and stored at -70°C .

4. Isolation of retrovirus from Maedi diseased lung

Tumor AD-32, obtained from a Maedi-diseased ewe, was homogenized and the high speed pellet fractionated on a sucrose gradient; the 1.15-1.18 density region was concentrated and inoculated onto various cell lines (as described in Part III 1. for tumor AD-29). Virus release was monitored by the reverse transcriptase assay. Two cell types (OFC and tahr) showed an increase in reverse transcriptase activity (Fig. 3), and the medium from these cultures was used to infect additional cultures of the same cell type. Virus production was observed by electron microscopy and reverse transcriptase activity, and the media harvested and stored at -70°C . This virus was termed Israel-Maedi virus.

5. In vitro growth of retrovirus (SPCV) from tahr-AD-207 co-cultures

Co-cultures of AD-207 tumor cells and tahr cells produced retrovirus particles which was infectious for and grew on tahr cell cultures. After a 2-3 week lag period following inoculation (1 ml of filtered medium containing virus), culture media were harvested every 2-3 days and stored at -70°C . The infected cultures were amenable to a limited number of passages before generalized cytopathic effects (CPE) were seen, followed by cell death. Virus was released into the culture medium continuously from the onset of the CPE until few live cells remained in the culture. This process took approximately 6-8 weeks and the remaining live cells did not produce virus. This viral isolate was termed sheep Pulmonary Carcinoma virus (SPCV).

6. In vitro growth of retrovirus from I. M. V. infected tahr and OFC cells

The retrovirus released from tahr and OFC cells, inoculated with a reverse transcriptase containing fraction of AD-32 homogenate, was routinely grown on OFC cells. In spite of the CPE, the cells could be repeatedly passaged and virus containing media were harvested every 2 days and stored at -70°C .

7. Comparison and nature of cytopathic effects of both retrovirus isolates on cells in culture

The cytopathic effects of the two retrovirus isolates (Israel-Maedi virus and Sheep Pulmonary Carcinoma virus) on cells in culture were monitored by phase contrast microscopy. The infection of tahr cells in culture with SPCV is immediately followed by a lag period of 2-3 weeks in which no virus production is seen (as judged by reverse transcriptase assay or by electron microscopy). The onset of viral release is accompanied by cytopathic changes in the normal fibroblastic appearance of the cells; particularly, stellate shaped cells and a high percentage of vacuolized cells. The increase in cell death is noticeable (by the cloudiness of the culture media), yet the cells can be successfully passaged a limited number of times during this period, until most of the cells die.

The infection of OFC cells with Israel-Maedi virus results in virus production within 1 week. Virus release is accompanied by localized cytopathic effects; particularly, cell fusion into giant syncytia containing clusters of nuclei; vacuolized cells, refractile cells and multinucleate stellate cells.

8. Morphogenesis of retrovirus particles

The SPC virus released by the infected tahr cell cultures is morphologically characteristic of type C retroviruses. Infected cells often have a large number of budding virions on their surface,

which upon maturation and release into the culture medium or extracellular space typify type C particles with an electron dense, centrally located core and an approximate diameter of 120 nm.

The extracellular virions of the Israel-Maedi retroviral isolate are indistinguishable in morphology from the SPC retrovirus. However, some morphogenetic differences have been noted. Israel-Maedi virions can be found budding into small cytoplasmic vacuoles and the rough endoplasmic reticulum, a rarely reported observation.

9. Comparison of the SPC viral genome with other retroviruses

The degree of genetic homology between the SPC virus and other retroviruses was examined by DNA-RNA hybridization. As can be seen in Fig. 4, the SPC virus is unrelated to avian (AMV) bovine (BLV) and murine (Moloney MuLV) retroviruses, yet shares some genomic sequences with the lentivirus subgroup of retroviruses (Progressive Pneumonia Virus of sheep (PPV), Visna Virus of sheep, Israel-Maedi virus, Caprine Arthritis Encephalitis Virus (CAEV)). Viral RNA of CAEV protected approximately 40% of the SPC cDNA while Visna, Israel-Maedi and PPV viral RNA protected 33% of the SPC cDNA. In a separate experiment the Israel-Maedi viral genome was found to differ by 5%-10% from that of Visna virus.

The partial relatedness between SPCV and the lentiviruses was further examined by heat denaturation studies to determine the fidelity of basepairing in the hybrids. As can be seen in Fig. 5, all three heterologous hybrids were 50% resistant to S_1 nuclease digestion at the same temperature (79°C), and the homologous pairing dissociated to an equivalent degree at 93°C . Thus a significant amount of mismatching exists in the hybrids formed between SPCV and other retroviruses affecting sheep and goats.

10. Hybridization studies using Israel-Maedi virus

The genetic relatedness between the Israeli isolate of Maedi virus and Visna virus (strain 1514) was compared using DNA-RNA hybridization in solution.

When Israel-Maedi virus cDNA was hybridized to 70S RNA extracted from Israel-Maedi virions and Visna (strain 1514) virions, a slight difference (approximately 8%) in maximal hybridization was seen (Figure 6). This difference was more pronounced (approximately 20%) in the reverse combination (i.e., Visna cDNA and Israel Maedi virus 70S RNA); the level of hybridization in this pairing paralleled that of progressive pneumonia virus (Figure 7), supporting the pathological distinctions.

11. Search for viral information in SPC tumor and Maedi-diseased lung tissue

Cellular DNA and RNA were extracted from two SPC tumors and one sample of Maedi diseased tissue in addition to normal sheep lung tissue. Hybridization in solution was carried out using SPCV cDNA and Visna (strain 1514) cDNA.

The results of the DNA-RNA hybridization, presented in Fig. 8, show that in none of the tumors could we find SPCV or maedi provirus to the limit of roughly one provirus per cell.

The level of viral expression, in SPC tumors, as determined by DNA-RNA hybridization with total cytoplasmic RNA, was undetectable by this assay, to the limit of approx. 2-5 RNA molecules per cell. Both the visna virus and SPCV cDNA's hybridized to a limited extent (above the background levels with normal sheep RNA) to RNA from the Maedi diseased lung (Fig. 9). The virus related sequences were detected at relatively high concentrations of cellular RNA (Crt = 500).

The ability of SPCV and Visna cDNA's to hybridize to heterologous

but related viral RNA, cytoplasmically located, in Maedi diseased lung tissue, supported the solution-hybridization studies between the cDNA's and viral 70S Israel-Maedi (and PPV) RNA. These studies showed limited genetic relatedness between these various lentiviruses. The detection of viral RNA in Maedi diseased tissue but not in SPC tumors could be the result of quantitative differences in the number of copies of viral RNA per cell and/or the percentage of infected cells within the entire tissue sample. To clarify these points we are performing these hybridizations to much higher Cot or Crt values, thereby raising the sensitivity of the assay by a factor of 5-10.

12. Isolation of the Israeli strain of Caprine Arthritis Encephalitis Virus

The synovial fluid from a swollen, tender front knee joint of an eight month old female goat (obtained from a farm in which mortality from this disease complex was dramatic) was aspirated aseptically and inoculated onto a subconfluent monolayer of tahr cells. The culture medium was sampled every few days for reverse transcriptase activity. After 20 days, when elevated levels of reverse transcriptase were detected in the culture medium, the medium was inoculated onto two more uninfected tahr cell cultures. Ten days later these cultures showed elevated reverse transcriptase activity, and in electron microscope sections, retrovirus particles (extracellular and budding structures) were seen.

13. Hybridization studies using the Israeli strain of Caprine Arthritis Encephalitis Virus (CAEV)

The genetic relatedness of the Israeli isolate of CAEV to other lentiviruses was compared by employing DNA-RNA hybridization in solution. Viral 70S RNA extracted from various lentiviruses was hybridized to cDNA prepared from either CAEV (U.S. strain) or Visna

virus (strain 1514).

Viral RNA from the Israeli CAEV isolate showed greater homology to the CAEV reference (U.S.) strain cDNA than either PPV RNA or visna virus RNA (Figure 10). When compared against a heterologous cDNA (Visna virus), the two CAEV isolates showed similar hybridization kinetics. PPV RNA hybridized to visna cDNA to a greater level than either of the CAEV isolates as expected from its greater genetic relatedness to visna (Figure 11). Therefore, in both homologous and heterologous hybrids, both isolates of CAEV exhibited a high degree of genetic identity.

14. Structural Proteins of Israel Caprine Arthritis Encephalitis Virus

The Israeli isolate of the Caprine Arthritis Encephalitis virus was compared to the reference (U.S.) strain of CAEV by analyzing the viral proteins on SDS-polyacrylamide gels. 80ug of viral protein of each of the two strains was separated on 10% gels according to the protocol of Laemmli(16). The protein profiles were identical, supporting the results of the liquid hybridization studies, which determined the two viral genomes to be generally homologous.

15. Host response to SPCV infection

Since virus isolation was tedious, and large quantities of it could be obtained only towards the termination of the project, no data related to the appearance of antibodies to viral proteins in experimentally infected or naturally SFC- diseased sheep is yet available. However, we have taken another course of investigation, looking for a serum protein fraction(s) that might be useful to monitor the development of SPC in sheep. Indeed it was found that at late stages of the disease in naturally affected animals there was not only a marked elevation in serum IgG immunoglobulins, as previously described

by us (), but that an additional fraction related to the α -globulins was present in the lung of these sheep in high concentrations, as compared to healthy sheep or to sheep with viral pneumonia. An effort was thus made to isolate and purify this tissue protein and to develop an assay to monitor it in the serum. An ELISA test was worked out for this purpose and it was then used to detect changes in this tissue-protein in the serum of sheep prior and at various times (up to one year) after intrapulmonary inoculation with the SPC retrovirus. It was found that the level of this α -globulin was significantly increased within a few days after virus inoculation, that its levels then decreased for a while and then gradually increased again with a magnitude that was proportional to the progression of the lung tumor which developed in the experimentally virus-inoculated sheep (Fig. 12). This was based on clinical histopathological and electron microscopic examinations performed on these sheep one year after virus inoculation. Results of these analyses are given in fig. 12 and table 4. They show that a correlation between the stage of tumor development and the increased concentration of this α -globulin fraction in the serum could be found.

DISCUSSION

Despite the assumption that maedi disease doesn't exist in Israeli herds, our group showed, by serology and histopathology, and most importantly, by the first maedi virus isolation in Israel (Israel Maedi Virus; I.M.V.). This virus' morphology and morphogenesis is identical to the maedi-visna reference virus (strain 1514), however, slight genetic differences can be detected. These differences are presently under study in order to obtain data as to how and when this virus arrived in Israel.

Strains of maedi (or maedi-visna) virus were obtained by us from Germany and Holland, and the virus causing progressive pneumonia in sheep in the U.S.A. was also obtained. This enabled us to compare the Israeli strain of maedi virus to its close or more distant counterparts in other countries, using nucleic acids hybridization techniques. The pathogenesis of maedi, its histological and ultra-structural characteristics were also studied.

The second isolation, the causative agent of sheep pulmonary carcinoma, an internationally novel retrovirus, was identified and partially characterized. In vitro conditions for its propagation were determined for the first time, a task that required a considerable amount of work until finally achieved. It was found that, indeed, a C-type retrovirus which was first detected (by this group) by E.M. in the tumor some 12 years ago (6) [a finding that was confirmed later by others (17)], and was detected in very small amounts in the tumor in 1973 by Perk et. al. (7), using the " simultaneous detection test " for retroviruses, induced tumor foci or even extensive tumoral lesions one year after intrapulmonary injection into one year old healthy sheep. It was found that co-cultivation of primary tumor cell cultures

with " permissive " tahr cells (obtained from the ovary of a himalayan goat) enabled the virus to grow rapidly in the tahr cells. A virus-containing preparation which was sedimented, extracted and purified could then be proved infective, causing the typical tumoral lesions as mentioned above.

This virus, termed SPCV, was found by genetic hybridization techniques to be distantly related to the various retroviruses infecting sheep and goats, and genetically unrelated to retroviruses oncogenic for bovine, avian and murine species. Also, although not part of the planned project (but arising as a new problem prevalent in U.S.A., as pointed out to us by Dr. Theilen, the American cooperating investigator), it was found that there is another retrovirus (CAEV) which causes lung disease as well as arthritis and encephalitis in goats. This disease, as turned out, is prevalent in Israel but its causative agent was hitherto not identified. Goats were selected from herds which exhibited high mortality rates due to this disease-complex. Material was aspirated from swollen joints and a retrovirus grown in tahr cells. Thus, ample quantities of the virus were obtained, thus enabling its characterization and analysis of its genetic relatedness to all the other viruses mentioned above.

Maedi-Visna virus and the caprine arthritis-encephalitis virus belong to the lentiviridae (retroviruses which cause slow progressive diseases but not malignant tumors). The Israel isolate of maedi turned out to be genetically close to the European strain of maedi, and more remote from the American progressive pneumonia virus. The Israeli isolate of CAEV was shown to be more distantly related to all those lentiviruses (except the U.S. isolate of CAEV) and the retrovirus causing lung carcinoma of sheep stood out as a distantly related virus which had, slight (about 20%) nucleic acid homology with maedi virus.

Recent studies showed the presence of maedi viral genomic information in maedi diseased lung tissue, but a similar search for the sheep lung carcinoma virus has proven negative, presumably due to methodological problems to be overcome by increasing the sensitivity of the test.

In summary the outcome of this project was the identification of a new retrovirus as the causative agent of pulmonary carcinoma of sheep, a tumor with relatively high prevalence in Israeli herds; the isolation of an Israeli strain of maedi virus and an Israeli strain of caprine arthritis encephalitis virus, the last two being retroviruses of the lentivirus group.

The genetic relatedness of these 3 viruses, among themselves and to strains of viruses causing similar diseases in other geographic regions, as well as the comparison of the sheep pulmonary carcinoma virus to various distant tumorigenic retroviruses from avian and mammalian species, was made. Today this laboratory is capable of growing these viruses in large amounts, and developing convenient and sensitive serological techniques for their detection in tissues and in the serum. Also, future work on viral proteins and - in the case of pulmonary carcinoma of sheep - or tumor cell membrane protein will, hopefully, enable the design of effective program for the eradication of these diseases.

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TABLE 1

Primary Cultures

<u>Designation</u>	<u>Description</u>	<u>Source</u>
SCP	Sheep Choroid Plexus	Adult ewe, slaughterhouse
FSL	Fetal Sheep Lung	Slaughterhouse
AD - 28	Tumor AD - 28	Kimron Vet. Institute
AD - 29	Tumor AD - 29	Kimron Vet. Institute

Tissue Culture Lines

<u>Designation</u>	<u>Description</u>
Tahr	Himalayan wild goat ovarian fibroblasts
OFC	Fetal sheep corneal fibroblasts
MK	Mink lung fibroblasts
KL	Fetal cat fibroblasts
SIRC	Fetal rabbit corneal fibroblasts
QT - 6	Quail fibroblasts
FRH	Fetal rhesus monkey lung fibroblasts
BL	Bat lung fibroblasts

Table 2
Summary of the pathological findings and the humoral immune response
against Virus-maedi antigen in Awassi sheep in Israel

Age of animals	Origin of animals	No. of animals	Immunology	Pathology
5 years or more	O.H.	11/16 (68.75%)	Positive	Vermittuous pneumonia, lung abscesses, interstitial pneumonia (viral), mediastinal lymphadenitis (C. ovis), nephritis, liver abscesses, toxemia of pregnancy, inhalation pneumonia, pyloric obstructions due to Brunner's glands adenoma.
5 years or more	O.H.	5/16 (31.25%)	Negative	Vermittuous pneumonia, interstitial pneumonia (viral), mediastinal lymphadenitis (C. ovis), lung abscesses, pulmonary adenomatosis (Jaagstekte).
1 to 3 years	O.H.	3/32 (9.67%)	Positive	Normal
1 to 3 years	O.H.	28/32 (87.77%)	Negative	Normal
1½ years	O.H.	1/32	Negative	Pulmonary adenomatosis (Jaagstekte)
1 year or below	M.L.	4/28 (13.97%)	Positive	Normal
1 year or below	M.L.	24/28 (86.03%)	Negative	Normal
1 year or below	K.S.	1/11	Positive	Normal
1 year or below	O.P.	26/26	Negative	Normal

FIG. 1

Reverse Transcriptase Activity in Tissue Cultures
Infected with AD-29 Reverse Transcriptase Containing Fraction

FIG. 2 RNA DEPENDENT DNA POLYMERASE
ACTIVITY ON EXOGENOUS TEMPLATE

TABLE 3

Reverse Transcriptase Activity (cpm) in Tumor Cell Cultures and Co-cultures

te	Control OFC	Control FSL	AD - 180	OFC - AD - 180	Tahr AD - 180	AD-207	FRH AD-207	KL- AD-207	BL- AD-207	FSL- AD-207	Tahr- AD-207	OFC- AD-207	SCP- AD-207
.11.31	868	909	653	870	754							800	769
.11.31				905	968	1,065	1,275	2,417	3,370	1,788	1,358	1,028	852
.12.81			918					1,242	717	675	745		
.12.31	385	205	899	1,020		703	650	661	778		13,664	428	437
.12.81			763	622	547	444	529	740	369	400	135,546		397

Fig.3.- Reverse transcriptase activity in Tahr and OPC cultures infected with AD-32 rev. trans. fraction

Fig 4 - Hybridization of SPCV cDNA to various viral RNA's

Fig 5 - Heat denaturation of hybrids between SPCV cDNA and various viral RNA's

Fig. 8 - Hybridization of SPCV cDNA to DNA from normal and tumorous lung tissue

Fig. 9 - Hybridization of SPCV cDNA to RNA from normal and tumorous lung tissue

Fig. 10 - Hybridization of CAEV (U. S.) cDNA
to various lentiviral RNA's

Fig. 11 - Hybridization of Visna cDNA to various lentiviral RNA's

TEXT-FIGURE 12- Sheep tissue associated protein complex (TAPC) concentration in the serum of animals treated with cyclophosphamide (CP-injection) followed by sheep bronchoalveolar agent inoculation (virus inoculation) which resulted in tumor initiation and development (animals no 03, 032, 180, 207). Note the acute and self-limiting increase in the TAPC concentration as a result of the drug and the virus treatments and the characteristic gradual and infinite increase in the TAPC concentration during the tumor growth. Note also that in animal no. 267 where no tumor could be detected the TAPC concentrations in the serum remain in the normal range.

TABLE 4 — Summary of post-mortem examination of sheep inoculated by the sheep bronchoalveolar agent and sacrificed a year later

Ewe number	Pathologic findings ^a
03	Solitary lung abscess at the site of virus inoculation, surrounded by a zone of lung tissue with extensive alveolar epithelialization reaction and few foci of adenomatous proliferation (early stage of tumor development).
032	Similar pathological findings as in ewe no. 03.
180	Pulmonary adenomatosis at the site of the virus inoculation with metastatic spread to the adjacent lobules of the inoculated lung (more advanced tumor development).
207	Bronchoalveolar carcinoma (pulmonary adenomatosis, jaagsiekte), extensive type, bilateral with adhesion to the thoracic wall (very advanced tumor development).
267	Normal lung tissue.

^a Based on macroscopic, microscopic and electromicroscopic examinations.

Evaluation of research achievements

The main achievement of this project was the establishment of a novel RNA C-type retrovirus as the causative agent of lung carcinoma of sheep, and the establishment of in vitro conditions for its growth and replication. For about 12 years there were several laboratories in different parts of the world, engaged on this topic. Thus, in Scotland, South Africa and U.S.A., attempts were made to identify the virus which causes this contagious neoplastic disease. The wide interest in this disease stemmed from its wide distribution, and its close resemblance to a human neoplasm, namely the broncho-alveolar lung carcinoma. Herpes-virus was suspected by many to be etiologic in this disease, but it is now clear that the disease is caused by an oncogenic RNA virus. This research thus brought to completion the early investigations and findings made by this group concerning the ultrastructural and biochemical characteristics of the viral particles which were observed in the tumor. This new virus was then compared to known and representative oncogenic retroviruses of avian and mammalian species and its uniqueness was thus confirmed.

In addition, in this project two other retroviruses which cause slow progressive non-tumoral but, nevertheless, fatal disease of sheep and goats were isolated, for the first time in Israel, and the conditions for their in vitro propagation were established. This enabled the comparison of these viruses to each other, to the sheep lung carcinoma virus and to European and American strains of related viruses. The project did not yet reach the goal of establishing serological or immunological methods for the monitoring of SPCV infection, but today the basis exists for developing the methodologies necessary for planning an eradication program and, to some extent, the vaccination protocol,

to protect herds of sheep from this contagious malignant disease. Future investigations in this laboratory should enable us to reach these ultimate goals, which are meaningful in terms of animal husbandry, export-import control, etc., and may have important implications in comparative oncology - the highly problematic, mostly incurable lung cancers in man.

COOPERATION

Collaboration between the Israeli team and the American cooperating investigator, Dr. G. H. Theilen, involved joint planning, discussion of results during the three year period, and evaluation of these results. Dr. Theilen's suggestions stimulated our search for the caprine arthritis encephalitis virus in Israel, which was presumably very closely related to the maedi virus, and which caused severe losses in goat herds in California. Serum samples of sheep and goats suspected of carrying maedi or CAE, respectively, were exchanged, for testing for the presence of virus and antibodies.

During a collaborative study at the Davis campus, histopathological material from sheep and goats (lung, brain and joints) from the last three decades was reviewed and clinical observation of several herds was performed by Dr. Perk.

Dr. Theilen paid a visit to the group in Rehovot in September, 1982, to finalize all the findings and to plan future strategies in the direction of the eradication of SPC, and the methods necessary for the initial trials at immunization of sheep to SPC.

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