Biological Characterization of the Virus Causing Leukoencephalitis and Arthritis in Goats

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SUMMARY

This study describes the biological properties of a strain of virus isolated from tissues of a goat with leukoencephalomyelitis–arthritis. The agent is a retrovirus, having a virion-associated reverse transcriptase enzyme and an antigenic determinant(s) which cross-reacts with the p30 of visna–maedi viruses. Morphogenesis of the virus is also similar to visna virus in terms of virus assembly and the multinucleated giant cell formation which accompanies replication of the latter virus. Despite its cytopathogenic property the goat agent was not lytic in goat cell culture, causing instead a productive infection which persisted through multiple subcultures of the cells. The virus replicated incompletely in sheep cell cultures but could be rescued from the latter, weeks after inoculation, by co-cultivation with goat cells. Our data suggest that this strain of goat leukoencephalitis virus is a variant of the ovine retroviruses with a host range limited to the goat.

INTRODUCTION

Leukoencephalitis, arthritis and interstitial pneumonia have recently been recognized as a multifaceted disease complex of goats in several areas of the U.S.A. (Cork et al. 1974a). The clinical diseases evolve slowly, lead to paralysis and lameness and/or dyspnea. This disease complex has been transmitted to other goats by inoculation of cell-free brain suspensions from naturally affected animals (Cork et al. 1974b), and more recently by the virus described in this report.

Although the aetiological agent of the goat disease complex has not been described, the lesions in the central nervous system (CNS) and lungs of affected animals bear a striking resemblance to those of visna–maedi of sheep. The latter two sheep diseases are classic examples of slow virus diseases (Thormar & Palsson, 1967). Visna is a chronic and progressive, paralytic disease characterized by inflammatory and demyelinating lesions in the CNS (Sigurdsson et al. 1957). Maedi, known as progressive pneumonia in the U.S.A., is a progressive dyspneic condition characterized by chronic interstitial pneumonitis with intense infiltration of mononuclear cells in the affected lung (Sigurdsson et al. 1952). Visna and maedi are caused by a group of serologically related retroviruses (Thormar & Helgadottir, 1965) which have only been recovered from sheep.

This report outlines the biological properties of the goat leukoencephalitis virus (GLV) and establishes its antigenic relationship to the visna–maedi group of sheep viruses with infectivity restricted to the goat.
METHODS

Viruses. The goat leukoencephalitis virus (GLV) used in this study was derived in this laboratory from a thymus explant culture of a goat which had been inoculated 6 weeks previously with an infectious brain suspension from a naturally affected animal (Cork et al. 1974b). The virus was propagated routinely in goat synovial membrane cultures (GSM) (see Results). Visna virus, strain 1514 which was originally obtained from a sheep with experimentally induced visna in Iceland (Petursson et al. 1976), was used in this study. This virus strain has been studied extensively in this laboratory. Progressive pneumonia virus (PPV) was isolated from an American sheep with chronic interstitial pneumonia and kindly provided by Dr John Gorham, USDA, Pullman, Washington.

Cell cultures. GSM cultures were prepared from the membranes dissected from carpal joints of a newborn colostrum-deprived goat. The tissue fragments were minced and explanted in tissue culture flasks using minimum essential medium (MEM; Gibco) supplemented with glutamine, 10% fresh foetal bovine serum (FBS; Gibco) and 10 μg/ml gentamicin. All cultures were incubated at 37 °C in a 5% CO₂ atmosphere. Cell outgrowths were dissociated with 0.5% trypsin/EDTA and expanded through two subcultures. The cells were then suspended in medium containing 10% FBS and 7% dimethyl sulphoxide (DMSO) and stored frozen in liquid nitrogen (Narayan et al. 1977). These cells were used for up to 10 subcultures for growth of the GLV. Sheep choroid plexus (SCP) cultures were prepared and were used for propagating visna and progressive pneumonia viruses as previously described (Narayan et al. 1977).

Virus titrations. The GLV suspensions were assayed for infectivity in GSM cell cultures using MEM plus 1% FBS as diluent; visna and PPV were assayed for infectivity in SCP cultures using MEM plus 1% lamb serum as diluent. Quantal assays in microtitre plates and plaque assays for all three viruses were performed in the respective cell types by techniques described previously (Narayan et al. 1978).

Infectious centre assay. Cells in infected monolayer cultures were dispersed with trypsin/EDTA into single cell suspensions, washed and diluted in growth medium. Four dishes of confluent indicator cell cultures were then inoculated with 2 ml containing 25, 50, 100 and 1000 cells, respectively, and incubated at 37 °C. Four h later, when cell attachment had occurred, the medium was removed gently and replaced with the same agarose medium used for virus plaque assay. Virus-induced plaques were stained and counted as described for plaque enumeration (Narayan et al. 1978).

Reagents for immunodiffusion. Approx. 2 l of supernatant fluids from cultures infected with GLV, visna or PPV were concentrated 100-fold by precipitation with ammonium sulphate followed by sedimentation in the ultracentrifuge (Haase & Baringer, 1974). These virus concentrates were treated with NP40 at a final concentration of 1% in STE buffer (Haase & Baringer, 1974) for 30 min at 37 °C and used directly as antigen or stored at −70 °C for future use. The antisera used in the tests were pre- and post-inoculation sera from two goats which were infected with GLV and a goat antiserum to the purified p30 of visna virus 1514. The p30, prepared from purified virus by dissociation and chromatography on a column of guanidine hydrochloride and purified by electrofocusing (Stowring et al. 1979), was kindly provided by Dr Ashley Haase. Pre-inoculation serum was taken from a goat which was then immunized with the protein, emulsified in Freund's complete adjuvant. Intradermal injections of 25 μg of p30 in adjuvant were given four times 4 to 6 weeks apart. The animal was then given 50 μg of the protein intravenously 1 month after the last intradermal injection and bled for serum 7 and 10 days later. Pre- and post-inoculation sera were tested by immunodiffusion against 5 μg of the protein. A precipitin line was obtained only with the immune serum.
Glass slides were pre-coated with a thin film of 0.5% SeaKem agarose in water. After drying, the slides were overlaid with 5 ml of 1% agarose in 0.14 M-saline. Wells were punched out 7 mm apart and the holes filled with 5 µl of reagent. The slides were incubated in a humid chamber at room temperature. These wells were refilled two or three times before precipitin lines appeared. The slides were then washed in saline followed by water, stained with amido black, dried and photographed.

**Polymerase assay.** Suspensions of virus were activated with non-ionic detergent NP40, final concentration 0.1%, for the polymerase assay. After incubation at 4 °C for 10 min, a reaction mixture was added that contained, at final concentration: 10 mM-tris-HCl, pH 8.0, 8 mM-MgCl2, 20 mM-KCl, 20 mM-dithiothreitol, 5 µCi 3H-TTP (46 Ci/mmol) and 5 µg of either poly(A).oligo(dT)12 or poly(dA).oligo(dT)12 (Boehringer-Mannheim, New York). Zero-time samples were withdrawn from the reaction mixtures immediately after the addition of the substrates. The reaction mixtures were incubated at 37 °C and samples were withdrawn at timed intervals and added to a mixture of 100 µl of 2 mg/ml salmon sperm DNA and 100 µl of 1:1:1 solution of 100% trichloroacetic acid (TCA):saturated sodium pyrophosphate:saturated sodium dihydrogen phosphate. The precipitates were collected on glass-fibre filters, washed extensively with 5% (v/v) TCA and then with 95% ethanol to facilitate drying. The radioactivity was quantified in a Beckman liquid scintillation counter.

**Electron microscopy.** GLV-infected cells at the fourth subculture level were selected for electron microscopic studies when c.p.e. was extensive. The cells were scraped from the culture flasks and lightly centrifuged to form cell pellets which were then fixed for 90 min in 2% glutaraldehyde at 4 °C. They were then washed and post-fixed in ~0.0% osmium tetroxide, dehydrated in graded ethanols and embedded in Spurr low viscosity embedding medium. Sections were cut at 1 µm and stained with toluidine blue for light microscopic examination. Selected blocks were sectioned at 60 to 90 nm with a diamond knife, stained with uranyl acetate and lead citrate and examined in the AFI1801 electron microscope at an instrument magnification of 1000 to 100000 times.

**RESULTS**

**Isolation of the goat virus**

A newborn goat kid was inoculated intracerebrally with 1 ml of brain suspension from a goat with natural leukoencephalitis. Six weeks later the goat was killed and various tissues including brain, choroid plexus, spleen, thymus and prescapular lymph node were cultivated by explant techniques. All of these cultures developed foci of multinucleated giant cells (syncytia) within 2 months of cultivation. The c.p.e. was most extensive in the thymus culture and supernatant fluids from this culture were stored at −70 °C.

**Selection of cell culture for propagation of goat virus**

Primary cell cultures were derived from various tissues of a 1 day-old colostrum-deprived goat. These tissues were choroid plexus, thyroid, thymus, testis, kidney and synovial membrane. Subcultures of each tissue outgrowth were seeded in microtitre plates and all were inoculated with a series of 10-fold dilutions of the thymus supernatant described above. Typical syncytial c.p.e. appeared in all the cultures inoculated with dilutions up to 10−3 except in the GSM culture in which c.p.e. was detected in wells receiving up to 10−6 dilutions. Because these latter cells seemed more sensitive than the other cells, new GSM cultures were inoculated with thymus virus at an m.o.i. of 0.1 and supernatant fluids harvested when c.p.e. became advanced. These fluids were clarified by centrifugation at 5000 g for 15 min and stored at −70 °C as stock GLV.
Sensitivity of the goat virus assay system

GSM cell cultures in microtitre plates and Petri dishes were inoculated with 10-fold dilutions of stock virus to determine the optimum measure of infectivity as measured by c.p.e. at endpoint dilutions (TCD₅₀), reverse transcriptase (RT) activity in rows of wells 2 weeks after receiving the different virus dilutions and by plaque formation under agarose. The two cell culture techniques proved equal in efficiency since the stock virus had a titre of a $5 \times 10^5$ TCD₅₀ and $1 \times 10^6$ p.f.u. per 0.2 ml of virus suspension. RT activity was found in all wells in which c.p.e. had occurred but was not detectable in wells in which inocula failed to induce c.p.e. This indicated that GLV was virulent for GSM cultures, had a high plaquing efficiency and did not replicate without causing c.p.e.

Persistent infection by goat virus

During plaque formation under agarose, virus c.p.e. appeared initially as foci of syncytia which lasted as long as 10 days before degenerating. In contrast, infected monolayers of GSM cells did not undergo total degeneration even after maximum virus production with its attendant c.p.e. (Fig. 1). Subcultivation of these infected cultures yielded new cultures with viable syncytial cells from the previous culture as well as a new crop of cells.
Goat leukoencephalitis virus

Fig. 2. Levels of reverse transcriptase activity of GLV in supernatant fluids of persistently infected GSM cells, monitored for over 150 days. P indicates passage number. Severity of c.p.e. is indicated by 1+ to 4+: 1+, syncytium formation in a rare field; 2+, syncytia in every two to three fields; 4+, syncytia in every field (Fig. 1).

Fig. 2 shows virus production in a culture inoculated at an m.o.i. of 1 and monitored at various intervals for RT activity and c.p.e. during the following 150 days after five cultivations. The cultures were grown in 10% FBS during the first week after trypsinization and maintained in 0.5% FBS with medium changes at 4 to 5 day intervals. Continuous virus production was evident during all of the subcultures. The fluctuations in RT levels were due to accumulation of virions in the supernatant fluids until medium changes when the levels declined precipitously. The low levels of virus production and slight c.p.e. in cultures seen during the first few days after trypsinization were caused by a combination of medium change, low plating efficiency of infected cells (50%) and slow generation of cells with single nuclei. However, the steady increase in virus production through each subculture was probably attributable to increased numbers of cells producing virus and/or greater output by cells in the later subcultures.

To determine the percentage of infected cells in the culture and to compare the number of infected cells with those actually synthesizing virions, cultures were examined at the 4th passage level after infection by infectious centre assay (ICA) and by electron microscopy. In the ICA, an average of 42 plaques developed in indicator cultures inoculated with 50 cells, showing that nearly all the cells possessed the virus genome which was expressed during co-cultivation. In contrast, the electron microscopic examination (described below) showed that only about 10% of the cells had evidence of virus assembly at the height of c.p.e. Thus, although all the cells may have been infected, only a small number had evidence of virus morphogenesis.
Table 1. **Timed GLV production by GSM cultures under different growth conditions**

<table>
<thead>
<tr>
<th>Culture</th>
<th>1-4</th>
<th>4-5</th>
<th>5-6</th>
<th>6-8</th>
</tr>
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<tbody>
<tr>
<td>0.5% FBS</td>
<td>500*</td>
<td>300</td>
<td>450</td>
<td>550</td>
</tr>
<tr>
<td>10% FBS</td>
<td>500</td>
<td>500</td>
<td>450</td>
<td>1200</td>
</tr>
<tr>
<td>Trypsin + 10% FBS</td>
<td>200</td>
<td>300</td>
<td>800</td>
<td>500</td>
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* Numbers represent ct/min of incorporated ³H-TTP in supernatant fluids of the three cultures. Immediately after samples were taken the cultures were washed twice and the various media replaced. Samples were then taken again. These had less than 50 counts.

**Effect of metabolic alteration of infected cells on virus production**

To examine the possibility that virus production was inhibited by limited cellular DNA synthesis, replicate flasks of infected cells in the fifth subculture were subjected to different growth promoting conditions. Cells in one flask were trypsinized and replaced in 10% FBS; in the second flask, medium was changed from maintenance to growth promoting (10% FBS); in the third flask cells were maintained in medium with 0.5% serum.

Supernatant fluids from the cultures were examined 4 days later by RT assay for cumulative virus production. Thereafter, the cultures were washed and supernates examined on three consecutive days for virus production. As seen in Table 1, the amounts of virus produced by the three cultures were equivalent despite a visible burst of mitoses in the two cultures treated with 10% FBS.

The results suggested that factors other than cellular DNA synthesis and cell division were responsible for the control and modulation of virus synthesis in these infected cultures and that this control mechanism was still in effect through multiple subcultures of the cells. It is noteworthy that infected cultures did not have a longer life than inoculated control cultures and the cells did not have any of the morphological or cultural hallmarks of virus transformation.

**Comparison of replication of goat virus and visna virus in goat and sheep cell cultures**

To determine respective host ranges of visna virus and GLV, both viruses were inoculated into both sheep and goat cultures. One flask of each cell type, SCP and GSM, was inoculated with visna virus and another set of flasks with GLV. Both agents were inoculated at an approx. m.o.i. of 1. Unattached virus was removed from the cultures after the 2 h incubation and maintenance medium was replaced. Small volumes of supernatant fluids were withdrawn at daily intervals and examined for infectivity and RT activity.

Fig. 3 shows the results of infection of GSM cells with the two viruses. Visna virus replicated at a faster rate than GLV causing rapid development of c.p.e. and total degeneration of the cells by 7 days post-inoculation. Infectivity of the visna virus samples was determined by titration in both SCP and GSM cultures. These cultures developed c.p.e. at similar endpoint dilutions, indicating that they were equally sensitive to this virus. In contrast, GLV developed at a slower rate, causing a more gradual development of c.p.e. and, as mentioned above, a persistent productive infection of the cultures.

Differences between the two viruses were more evident by their manner of replication in sheep cell cultures. Visna virus caused its usual productive lytic infection of SCP as described previously (Narayan et al. 1978). The goat virus, however, showed no evidence of replication in SCP cells. Supernatant fluids taken from SCP infected with GLV at different intervals after inoculation did not have RT activity and failed to cause c.p.e. after inoculation of GSM cells.
Goat leukoencephalitis virus

Fig. 3. Comparative growth rates of visna virus and GLV in goat synovial membrane cultures. The cultures were inoculated at an m.o.i. of 1 and supernatant fluids examined at intervals for infectivity and RT activity. ———, Visna TCD₅₀; . . . . GLV TCD₅₀; ——, visna RT; ———, GLV RT. The visna virus/GSM culture degenerated by day 8 post-inoculation whereas GLV/GSM became persistently infected.

Despite the failure to replicate in sheep cultures, the goat virus could be rescued from the SCP cells as long as 2 weeks after inoculation by co-cultivation with GSM cells. To test further whether a low level of replication was occurring in the GLV-inoculated SCP cells, four flasks of SCP were inoculated with GLV at an m.o.i. of 0.1 and cells examined on days 2, 5, 10 and 14 by infectious centre assay on permissive GSM cells. The percentage of infected SCP cells was 0.1, 0.4, 0.5 and 0.01, respectively, suggesting that no replication had occurred and that the goat virus may have produced a latent infection in SCP cells. Furthermore, this 'latent' infection was not changed to a 'productive' infection in the SCP cells by inducing cellular DNA synthesis (trypsinization followed by dilution and cultivation in growth medium).

Antigenic relationships between goat virus and visna/progressive pneumonia viruses

Immunodiffusion studies showed that GLV had a protein antigenically related to the p30 of visna and PPV. This antigenic relationship was noted in two reciprocal immunodiffusion tests (Fig. 4 and 5). Lines of homology were produced when antiserum to visna p30 in the centre well was reacted with GLV, PPV and visna viruses, and also when GLV in the centre well was reacted with anti-visna p30 and sera from two goats infected with the goat virus. Pre-infection sera from the latter two animals did not produce lines. Despite the antigenic cross-reactivity of one protein among these viruses, the goat agent is also antigenically distinct because it was not neutralized by highly cross-reactive neutralizing antisera to visna viruses. For example, a hyperimmune goat serum which neutralized visna virus 1514 at a dilution of 1/10000 failed to neutralize GLV at a dilution of 1/10.

Electron microscopy

Most of the GSM cells in the cultures resembled type A synovial lining cells, considered to be phagocytes (Wynne-Roberts et al. 1978). The cell pellets contained much cell debris which consisted principally of disordered membranes. Numerous multinucleated cells were
Fig. 4. Immunodiffusion pattern obtained by reacting antiserum to visna virus p30 in centre well 6, against three NP40-treated virus suspensions: GLV in well 1, visna virus in well 5 and PPV in well 2. Note lines of homology shared by the three viruses. Control wells 3 and 4 received lamb serum and FBS serum, respectively.

Fig. 5. Immunodiffusion pattern obtained with GLV in well 5 against post-GLV infectious sera of goat 1 in well 1, goat 2 in well 3 and pre-inoculation serum from goat 2 in well 4. Anti-visna virus p30 serum is in well 2; the double lines of homology seen in Fig. 4 and 5 may represent either precipitation by two different classes of antibody in the immune sera or specific reactivity of a single class of antibody with the p30 plus a precursor or a breakdown product of the same protein.

seen. The cytoplasm of cells with single nuclei contained large amounts of glycogen and many large lysosomes. Cells which were obviously infected, i.e. with evidence of virus formation, formed approx. 10% of the population in the samples with maximum amounts of virus. Such cells showed an increase in membrane-bound cytoplasmic vacuoles and there were numerous myelin figures. Nuclei were swollen, rounded and showed decreased density; there were large nucleoli.

Virus structures were seen at three sites in the sample (Fig. 6a to d). (1) Extracellular virions were found in clusters lying between cells or along cellular membranes. These particles were roughly spherical and ranged in size from 70 to 110 nm. Most of the particles contained dense nucleoids. (2) Many virus forms were seen budding from cellular membranes. Most budding occurred at the cytoplasmic membrane on the side of the cell which had not been attached to the glass. Buds were extremely pleomorphic and ranged from small, roughly spherical forms 80 to 90 nm in diam. to long tubules of the same diam. and irregular areas of membrane with a thickness of 25 nm. The number of budding particles was variable and dependent on the extent of c.p.e.; it was, however, less than that observed and reported for ovine cell cultures infected with visna virus. Buds were also seen, but less commonly, at intracytoplasmic membranes lining vesicular spaces. The third virus form occurred as spherical or incompletely spherical intracytoplasmic structures with an average diam. of 95 nm. Such structures possessed small radiating projections and occurred in groups of 2 to 50. These variable methods of morphogenesis of GLV are basically similar to those of ovine retroviruses in sheep cell cultures (Coward et al. 1970; Harter & Coward, 1974).
DISCUSSION

This study shows that the aetiological agent of goat leukoencephalitis-arthritis is morphologically a retrovirus, contains a reverse transcriptase, is antigenically related to the retroviruses of sheep but has a host range restricted to goat cells. The pathogenic properties of the goat virus are in keeping with those of the ovine retroviruses. All these agents share some common antigenic determinants and cause persistent infections which
may lead to slowly progressive debilitating diseases of their respective hosts. Until recently, the ovine viruses have been associated only with neurological (visna) and pneumonic (maedi, progressive pneumonia) disease. However, retroviruses have now been recovered from synovial fluid of sheep with arthritis (unpublished data), thus adding a new dimension to the pathogenic potential of these viruses. However, whether the sheep viruses have a multi-system or a specific disease potential is still unclear. Although it is generally recognized that maedi virus can cause visna and vice versa, the pathogenic potential of the ovine arthritis viruses has yet to be evaluated. In contrast, the strain of GLV isolated in this laboratory has multi-system pathogenicity for the goat, causing visna-like lesions in the central nervous system, maedi-like lesions in the lung and arthritis in the joints (Cork & Narayan, 1980). Whether this strain of virus is representative of the agents causing a similar disease complex in other parts of the world (e.g. Stavrou et al. 1969; Weinhold, 1974; O’Sullivan et al. 1978) has yet to be determined.

The tight restriction of replication of GLV in sheep cells is a unique property of this virus. This narrow host range is surprising in view of the wider host range of visna-maedi viruses which can replicate in caprine and bovine cell cultures (Thormar, 1965). The failure of GLV to replicate in SCP, but at the same time persist in the cells, suggests that proviral DNA may have formed a stable association with the cellular DNA and minimal, if any, transcription occurred from the virus templates. The direct inference of this species restriction of virus replication is that virus from infected goats would not be a source of infection for sheep under natural conditions. Preliminary studies of cultivation of tissues from sheep inoculated with GLV showed that unexpressed virus genome was present in cells of these explant cultures since virus was readily rescued by addition of GSM cells (unpublished data). This may be a reason why the goat disease can occur in places like Australia (O’Sullivan et al. 1978) in the absence of its ovine counterpart.

Both GLV and visna virus replicate in a slow manner in their respective host animals, but in cell culture visna virus causes a rapid cytolytic infection. The replication of GLV in cell cultures more accurately reflects the manner of replication of these viruses in vivo, in which slow continuous replication in some cell types along with latent infections of others (e.g. blood leukocytes) may be occurring. The further demonstration that cellular DNA synthesis may have minimal effects on GLV synthesis adds new insights to this retrovirus-cell interaction. These interactions, which seem to mimic the events in the animal, may thus provide valuable information regarding the mechanisms of virus restriction in vivo. For example, the GLV-cell culture system provides an in vitro system in which the molecular events leading to latency as well as slow replication of a non-transforming exogenous retrovirus can be examined.

Further biological significance of the slow replication of GLV in comparison to the rapid growth of visna virus may be inferred from studies of other ovine retroviruses. During the past 2 years more than eight strains of retroviruses have been isolated in this and other laboratories from blood and organs of sheep with visna-like disease, progressive pneumonia and arthritis (O. Narayan, unpublished data; J. R. Gorham, unpublished data). All of these viruses share common antigens with the p30 of visna virus, and replicate in both sheep and goat cultures. However, with the exception of Icelandic visna virus, these ovine viruses replicate slowly and cause persistent infections in sheep cultures in an analogous manner to that of GLV in GSM cultures (unpublished data). Visna virus seems unique among these agents because of its highly cytolytic property in cell culture. This property may not be a natural trait and could have been acquired by mutation and selection during the multiple passaging between sheep brain and sheep cell cultures in Iceland during the past 20 years (Peturssson et al. 1976). It is of interest that all of the antigenic mutants of visna virus retain this lytic property (Narayan et al. 1978). Because of this property,
visna viruses are easier to study in the laboratory. However, virus strains such as GLV and the recently isolated visna–maedi–arthritis (VMA) of sheep may more accurately reflect the biological properties of these viruses in nature.

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