The Emergence of Antigenic Variants is a Rare Event in Long-term Visna Virus Infection in vivo

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SUMMARY

Six sheep persistently infected with visna virus were studied for 4 1/2 to 5 3/4 years until they became ill. Virus was isolated at intervals from peripheral blood leukocytes and cerebrospinal fluid (CSF), and at the time of sacrifice from various parts of the brain and the lungs. Both brain and lungs showed lesions typical of advanced visna/maedi. All the sheep formed antibodies in sera and CSF. Virus isolates from each sheep were tested in neutralization tests against sera and CSF collected from the same animal. In one sheep all isolates were found to be identical to the inoculated virus by this test. In each of the other sheep an antigenic variant emerged from 1 to 3 years after inoculation and remained in circulation even after the formation of autologous antibodies. In one case a variant was isolated from the lungs, whereas in all cases the virus isolated from the brain was identical to the inoculated virus. The results show that antigenic variants are rare in visna and do not seem to have a role in the pathogenesis of the disease.

INTRODUCTION

Several studies have recently been done on the pathogenesis of visna in experimentally infected sheep, but most have concentrated on the early events of the infection, occurring during the first months after inoculation (Pétursson et al., 1976; Georgsson et al., 1977; Nathanson et al., 1976). However, visna virus is known to persist in infected sheep for 8 years or even longer (Thormar & Pálsson, 1967; Gudnadóttir, 1974) and to cause lesions in the central nervous system (CNS) or the lungs which may or may not lead to clinical signs during the lifetime of the animals. It has been suggested that a continuous antigenic drift of visna virus may explain its ability to replicate and cause damage in the infected host in the face of high titres of neutralizing antibodies (Gudnadóttir, 1974; Narayan et al., 1978).

In this paper we report observations of Icelandic and Dorset sheep inoculated with visna virus, and showing persistent viral infection in their CNS and lungs when sacrificed up to 6 years later. We confirm earlier findings that antigenic variants develop in sheep during the course of the infection. However, our study found such variants to be rare and did not support the notion that they are important in the pathogenesis of the disease.

METHODS

Animal experiments. Five 1-year-old female Icelandic sheep and a castrated male lamb of Dorset breed were inoculated intracerebrally with 10⁶.0 to 10⁶.8 TCID₅₀ of visna virus strain K796 or with 10⁵.6 TCID₅₀ of strain K485 (sheep no. 14). Samples of clotted and heparinized blood were collected from the jugular vein, at first bi-weekly and then monthly or bi-monthly. Peripheral blood leukocytes (PBL) were separated from the heparinized blood by incubation with a solution of dextran T250 (Pharmacia), followed by washing in Eagle’s basal medium (BME) (Thormar et al., 1980). Suspensions of at least 10⁷ PBL were inoculated onto monolayers of sheep choroid plexus (SCP) cells in 30 ml Falcon flasks for isolation of virus. Cerebrospinal fluids (CSF) were collected by lumbar puncture 3 or 4 times a year. The fluids were centrifuged and the cells, suspended in 0.5 ml CSF, were inoculated onto SCP cultures for virus isolation. The clarified fluids were frozen at −86°C for later determination of anti-
body. The brain was removed at the time of sacrifice and explant tissue cultures were made from various separate areas, i.e. the right cerebral hemisphere, midbrain, cerebellum and medulla. Lung tissue was similarly explanted. Explant cultures were incubated with growth medium (BME with 15% lamb serum) until widespread cellular outgrowth had formed (Brown & Thormar, 1975); they were then changed to maintenance medium (BME with 2% lamb serum). All explant and inoculated cultures were kept for at least 2 months and examined for cytopathic effect. Blind passages of fluid were made every 2 to 3 weeks.

The left half of each brain, the spinal cord and a piece of lung tissue were fixed in 10% buffered formalin. Paraffin-embedded brains were cut by coronal sections at five levels and stained with haematoxylin-eosin (HE), Kluver-Barrera, Loyez, Holtzer and combined Bodian-PAS techniques. Sections of lung tissue were stained with HE.

**Virus neutralization.** Neutralization tests were done as described earlier (Thormar, 1963) using 100 TCID$_{50}$ of virus mixed with doubling dilutions of serum or CSF in maintenance medium. The mixtures were incubated at 22°C for 20 h and then inoculated onto monolayers of SCP cells in 96-well Linbro microtitre tissue culture plates. One-tenth ml of each mixture was inoculated into each of two wells. Cytopathic effect was read at 7 and 14 days. Titres were expressed as the highest dilution showing complete neutralization of virus. All sera were heated at 56°C for 30 min before use.

**Hyperimmunization of rabbits.** Visna virus was purified by the method of Lin & Thormar (1971). A 50 to 100 µg amount of virus in phosphate-buffered saline pH 7.2 was mixed with an equal volume of complete Freund's adjuvant and injected intradermally into the hind footpads of New Zealand white male rabbits. A booster was given 6 to 10 weeks later by intramuscular injection into the hind legs. The rabbits received three or four boosters and were bled 10 days after the last injection.

**RESULTS**

**Clinical signs and histological lesions in infected sheep**

Of the six sheep included in this study, one (no. 13) was found dead without previously showing noticeable signs of illness. The other five sheep became ill from 4 1/2 to 5 3/4 years following inoculation. They developed weakness of the legs which progressed into a severe paralysis in a few days to weeks. All showed loss of weight for some time before they became paralysed, but otherwise no signs of a slowly progressing visna infection were noticed.

In neuropathological examination changes in the myelin of the brain was the most striking feature. In the cerebral hemisphere large areas of centrum semiovale and the white matter of gyri showed diffuse pallor of myelin, but with no sharp borders between the intact myelin and those undergoing demyelination. The small, sharp-bordered demyelinating foci were found in capsula interna and in striopallidal bundles, giving the picture of 'moth-eaten' foci. Demyelination was more intensive in centrum semiovale and in white matter of frontal cortex gyri, while corpus callosum and white matter of parietal and temporal cortex were less damaged. There was a marked fibrillary gliosis in the areas of demyelination. Similar gliosis was seen in subependymal areas adjacent to the fourth and lateral ventricles and around the central canal in the spinal cord. Neuronal changes consisted of dispersed loss of neurons in cerebral cortex with numerous pictures of neuronophagia and satellitosis as well as microfoci of necrosis in hippocampus and in the depth of cerebral sulci. No inflammatory infiltrations were observed in any of the brain, cerebellum or spinal cord.

In the lungs a chronic, interstitial inflammation was found. The alveolar walls were thickened, and numerous lymphocytes and mononuclear cells were seen within the septae or forming peribronchial cuffs. In some areas an almost total consolidation of tissue was seen, while in other areas, in the lungs of the same sheep, the alveolar walls were broken, forming spikes and large bullae that gave a picture of compensatory emphysema.

**Virus isolations**

Table 1 summarizes the results of attempts to isolate virus from the six sheep during the persistent infection and at the time of sacrifice. Virus was isolated from the brains of all four sheep from which explant cultures were successfully grown. The virus was found to be widespread in the brain, since it was in most cases isolated from several areas, such as the cerebral hemisphere, cerebellum, medulla and choroid plexus. Virus isolation was made from the lungs of two out of four sheep. In all cases, virus cytopathic effect appeared in the primary cultures from 12 to 18 days after explantation. Virus isolations were made from 10% or less of spinal fluids tested and from 5% (sheep no. 6) to 20% (sheep no. 13) of the PBL samples.
Antigenic variants in visna

![Graphs](image)

**Fig. 1.** Sheep no. 3. Titre against: (a) the inoculating virus strain K796; (b) virus isolated from PBL (●), CSF (○) and brain (B) at the indicated times; (c) virus isolated from PBL (●) and lung (L). O, Sera; ●, CSF; †, time of death.

**Fig. 2.** Sheep no. 1. Titre against: (a) the inoculating virus strain K796; (b) virus isolated from PBL (●) and CSF (○) during the infection and at the time of death (†). O, Sera; ●, CSF.

Table 1. **Virus isolations from infected sheep**

<table>
<thead>
<tr>
<th>Sheep no.</th>
<th>Time of sacrifice (years)</th>
<th>PBL</th>
<th>CSF</th>
<th>Brain</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 1/4</td>
<td>6/46*</td>
<td>2/17*</td>
<td>ND†</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>5 3/4</td>
<td>7/46</td>
<td>2/18</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>5 3/4</td>
<td>2/43</td>
<td>0/14</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>5 1/2</td>
<td>10/46</td>
<td>2/20</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>4 1/2</td>
<td>6/36</td>
<td>0/17</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D12</td>
<td>4 1/2</td>
<td>2/37</td>
<td>0/4</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* No. positive/no. tested.
† ND, Not done.

**Neutralizing antibodies in sera and CSF**

Virus isolates from PBL, CSF, brain and lungs were passaged 2 or 3 times in SCP cultures and tested in neutralization tests against sheep serum and CSF samples. The virus strains used for inoculation of the sheep were also included in the tests. The results are shown in Fig. 1 to 4. First, all the sheep formed neutralizing serum antibodies against the inoculated virus within 1 to 4 months post-inoculation. The antibodies appeared a little later in the CSF but reached significant titres in most of the animals and remained elevated for the duration of the persistent infection. In one sheep (no. 3) the CSF titres were equal to or greater than the serum titres (Fig. 1 a, b). When PBL virus isolates from each sheep were tested against a series of sera from the same sheep collected at intervals throughout the duration of the infection various patterns were
observed. In one sheep (no. 1) all five PBL virus strains isolated either within 2 years post-inoculation or as late as 5 1/2 years post-inoculation were neutralized by the sera in a pattern identical to that of the inoculated virus (Fig. 2). In another sheep (no. 14) there was no significant formation of neutralizing antibodies until about 3 1/2 years post-inoculation. There was then a similar increase in titre against virus isolates from PBL, brain and lungs as against the inoculated virus strain K485 (data not shown). In sheep no. 6 (Fig. 3) the two PBL isolates were identical, although separated in time by more than 3 1/2 years, but were slightly different from the inoculated virus. Virus isolated from various parts of the brain at the time of sacrifice showed a pattern identical to that of the inoculum. Similar results were obtained for Dorset sheep no. D12 (data not shown). Two PBL virus strains isolated from sheep no. 13 within 2 1/2 years after inoculation were identical to the inoculum (Fig. 4a, b). However, four strains isolated at 3 years post-inoculation or later were different from the inoculum and the early PBL isolates, although they were all identical to one another (Fig. 4c). Finally, four PBL strains isolated from sheep no. 3 at 1 to 2 years and 4 to 5 years post-inoculation were identical to the inoculum and to virus isolated from the brain of the sheep (Fig. 1a, b). On the other hand, two PBL isolates obtained at 2 1/2 and 4 years post-inoculation and virus isolated from the sheep's lungs were identical but different from the inoculum and the other PBL and brain isolates. In this sheep, therefore, two serotypes of visna virus co-existed for 3 1/2 years. One was located in the lungs and the other one in the brain at the time of death. A series of spinal fluids were tested in neutralization tests against virus strains isolated from CSF of the same sheep. In all cases the CSF isolates were identical to the virus strain used for inoculation (Fig. 1 to 4). Therefore, virus was apparently circulating in the CSF as well as in the blood for years after the formation of autologous neutralizing antibodies.
Antigenic variants in visna

Table 2. Cross-reaction between inoculating virus strain and brain and lung isolates

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Anti-K796</th>
<th>Anti-no. 3B</th>
<th>Anti-no. 3L</th>
</tr>
</thead>
<tbody>
<tr>
<td>K796*</td>
<td>512</td>
<td>512</td>
<td>256</td>
</tr>
<tr>
<td>No. 3B†</td>
<td>1024</td>
<td>1024</td>
<td>128</td>
</tr>
<tr>
<td>No. 3L‡</td>
<td>64</td>
<td>16</td>
<td>128</td>
</tr>
</tbody>
</table>

*Strain used for inoculation.
† Brain isolate.
‡ Lung isolate.

Cross-reactions between virus isolates

Cross-neutralization tests with virus isolates from sheep no. 3 using hyperimmune sera raised in rabbits showed that the brain isolate and the inoculated virus strain were identical. On the other hand, hyperimmune sera against these strains had 8- to 64-fold lower titres against the lung isolate than against the autologous virus (Table 2). This confirms the difference found in tests with sheep sera (Fig. 3).

DISCUSSION

Our study agrees with previously published results (Narayan et al., 1978) which showed that antigenic variants of visna virus emerge in sheep during the slow infection, probably by mutation. However, we did not observe a continuous progression of antigenic variants in any of our sheep. On the contrary, the appearance of antigenic variants was rare in the persistently infected sheep and occurred only once in any one of them during the almost 6 years of infection. The discrepancy in our observations and those of Narayan et al. (1978) may be due to differences in virus strains used or to the fact that these investigators used 10- to 100-fold larger inocula in some of their sheep, resulting in a greatly increased number of virus isolations from the PBL.

Our results are similar to those of Pétursson and co-workers (Pétursson et al., 1981; Lutley et al., 1983) who found antigenic variants to be rare in sheep that had been persistently infected with visna for several years. They also agree with earlier studies (Thormar & Helgadóttir, 1965) which found that a few antigenic variants occurred among virus strains isolated from the lungs of natural cases of maedi, but most of the strains were antigenically closely related to one another, and even to a visna virus strain isolated from the brain of a natural case of visna a decade earlier and passaged many times both in sheep and in tissue cultures. Similarly, Icelandic visna and maedi virus strains were neutralized by sera from Dutch sheep naturally affected with Zwoegerziekte (Thorshar, 1966), a lung disease caused by visna/maedi virus (De Boer, 1975). Since the Icelandic and Dutch strains of the virus had been separated geographically for at least three to four decades and probably much longer, this demonstrates the antigenic stability of the virus in nature. This stability is remarkable in view of the fact that the virus persists in sheep for several years and most of this time in the presence of high concentrations of circulating antibodies. However, our present study does not rule out minor antigenic differences among the visna virus isolates.

At the time of paralysis and death, virus identical to the inoculated strain was found to be widespread in the brains of sheep showing lesions typical of advanced visna. Similarly, virus isolated from lungs with typical maedi lesions was identical to a variant which was present in the sheep’s blood 4 years earlier. Therefore, this study does not support the idea that a continuous antigenic drift of visna virus has an important role in the pathogenesis of the slow disease.

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REFERENCES


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