Experimental Visna in Icelandic Sheep: The Prototype Lentiviral Infection

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A brief review of experimental infection of Icelandic sheep following intracerebral inoculation of neurotropic strains of visna virus is presented. In vivo replication of the virus is restricted, so that some cells carry the deoxyribonucleic acid provirus as an unexpressed genome. This cellular restriction plays a major role in the slow progression of the infection, abetted by neutralizing antibody in serum and spinal fluid. The latent provirus maintains the viral genome in the presence of an active immune response, since immune surveillance cannot recognize cells that are not synthesizing viral antigens. Infected Icelandic sheep experience two types of diseases of the central nervous system: a subclinical subacute encephalitis begins within weeks of infection in most sheep; and at irregular intervals from 0.5–8 years after infection, clinical paresis develops in the majority of Icelandic sheep and is accompanied by discrete focal demyelinating lesions in the spinal cord. The subacute encephalomielitis is probably mediated by an antiviral cellular immune response, whereas the pathogenesis of the focal demyelinating lesions is still obscure. During persistent infection there is some selection for neutralization-resistant antigenic variants of the infecting serotype, and these are isolated at a frequency of \( \sim 15\% \). However, variants do not replace the infecting serotype, and antigenic drift does not appear essential for persistence of visna virus or for the occurrence of demyelinating lesions.

Iceland has played a special role in the history of visna, since the disease was initially recognized in Iceland and is named after the Icelandic word for wasting, which describes one of the presenting signs of the disease. The same virus also causes a chronic interstitial pneumonia known as maedi in Iceland and as progressive pneumonia in the United States.

Originally, visna virus was inadvertently introduced into the Icelandic sheep population, which was apparently free of the infection, as a consequence of the importation of 20 Karakul rams from Germany in 1933. The infection spread through the northern and western regions of Iceland and was recognized as an epizootic in the early 1940s. Bjorn Sigurdsson, an Icelandic physician, showed that the disease was due to a transmissible agent, and he and his collaborators isolated the virus by growing it in cultures of sheep choroid plexus (SCP) cells. A heroic effort by veterinarians and farmers during the period 1944–1965 led to the eradication of visna, so that Icelandic sheep are now free of infection. These events are described in a series of classic publications [1–7].

More recently, we have conducted a collaborative study of the pathogenesis of visna in Icelandic sheep [8–23]. The following account will summarize selected aspects of these studies, with particular emphasis on the pathogenesis of the lesions seen in the CNS and on the phenomenon of antigenic drift.

Biologic Variation in the Virus and in the Host

As with other viruses, genetic determinants in both the virus and its host have a marked influence on visna infection and its pathologic consequences. This influence largely accounts for the differences in experimental ovine infections with visna, maedi, or progressive pneumonia viruses reported by different laboratories. Studies in Iceland have exclusively utilized virus strains that are highly cytopathic in SCP cells; such viruses grow to high titer in cell culture and tend to kill infected cells, unlike field isolates of progressive pneumonia virus [24], which produce
syncytia with release of only low titers of cell-free virus. Furthermore, we have used viral strains that have been selected for their ability to cause severe CNS disease. The second important variable is the high susceptibility of Icelandic sheep. Compared with certain English breeds found in the United States, these animals are more prone to clinical disease and are more permissive hosts for visna virus [11, 14].

**Viral Replication and Immune Response in Icelandic Sheep**

*Viral replication.* Following intracerebral inoculation of a large dose (10⁴ TCID₅₀) of visna virus in Icelandic sheep, some animals were tested repeatedly for virus in blood and spinal fluid, while others were killed at intervals and many tissues examined for virus. Salient observations were as follows.

1. Viremia appeared at two to four weeks and persisted throughout the lifelong infection [8]. Virus was isolated from buffy coat, but never from plasma, even before the appearance of neutralizing antibody in the serum. Such isolations required co-cultivation of leukocytes with permissive SCP cells, and cultures often yielded virus after only two to four weeks of incubation. Maximal frequency of isolations required the use of 10⁶ leukocytes because fewer isolations were made when 10⁵ or 10⁴ leukocytes were tested. Although most sheep were viremic, only 10%–80% of serial specimens from individual sheep were positive. These observations suggest that virus was carried as a latent provirus in a small number of leukocytes. Consistent with this finding, extensive immunofluorescent or electron microscopic examination failed to identify individual virologic cells. Testing of fractionated cells suggested that mononuclear cells, probably both lymphocytes and monocytes, can carry the genome [8, 9, 24].

2. Visna virus can be isolated from the other tissues [8], mainly from lymphoid organs, CNS, and lung, throughout the life of the infected sheep. Titration of tissue homogenates indicated that only minimal amounts of cell-free infectious virus were produced, and many specimens required explantation and co-cultivation for isolation of virus [8, 11, 14].

3. The CSF yielded low titers of cell-free virus, but only for the first three months after infection. After the appearance of neutralizing antibody in the CSF, virus was no longer isolated [19].

**Immune response.** Visna virus is immunogenic, and infected Icelandic sheep consistently develop an immune response. Serum neutralizing antibody appeared two to three months after infection [8]. Immunodiffusion tests showed that all infected Icelandic sheep generate serum antibody against the viral glycoprotein (neutralizing antigen) and that approximately one-half also made detectable antibody against the major core antigen, p30 [16].

The CSF neutralizing antibody appeared after serum antibody in approximately one-half of the infected sheep [19]. Since the blood-brain barrier remained intact, this antibody represented intrathecal synthesis by B lymphocytes that migrated into the CNS. Plasma cells were seen regularly in the inflammatory lesions in brain and spinal cord [13]. Measurement of the total amount of immunoglobulin in CSF indicated that the level of IgM was elevated in many sheep, but IgG1 levels were not [19]. For ∼50% of the animals, electrophoresis of spinal fluid in long-term infected sheep showed oligoclonal immunoglobulin bands [21].

Cell-mediated immunity was assessed by lymphoblast transformation in response to stimulation by virus or virus-infected fibroblasts. Although a cell-mediated immune response was not detected in Icelandic sheep, studies by other investigators [25–27] showed such a response in peripheral blood and CSF cells of other breeds of sheep, peaking at two to eight weeks after infection, and then waning with irregular reappearance.

**In Vivo Restriction of Viral Gene Expression**

*Slowness and persistence.* The observations presented above are consistent with the view that sheep cells restrict the expression of the viral genome in vivo. Direct evidence for this interpretation has been generated by the painstaking studies of Haase and Brahic and colleagues [28, 29], who have applied in situ hybridization to infected sheep tissues. This restriction is the salient molecular aspect of visna infection and undoubtedly accounts for the slow progression of infection. Another factor contributing to slowness is the damping effect of neutralizing antibody in serum and extracellular fluid of the CNS.

Persistence of the viral genome in the face of a potent immune response is explicable by the same restriction phenomenon. Cells that perpetuate the viral genome as a provirus do not synthesize viral antigens, and such latently infected cells can escape immune surveillance. Presumably, an occasional cell
becomes virogenic, contributing to the indolent spread of the infection.

A cell-culture model of visna virus restriction. Many of the molecular aspects of host-imposed restriction of visna virus could best be studied in a cell-culture model, because the controls on retroviral gene expression are not yet well understood [30]. After infection of a number of continuous cell lines, one system was found that appeared to provide a potential model (J. N., unpublished observations).

Following exposure to visna virus, CV-1 cells, a line of African green monkey kidney cells, showed no cytopathic effect and were consistently nonvirogenic when carried for longer than six months (>10 serial transfers), as judged by the failure to isolate virus from the culture medium on numerous attempts (figure 1). Yet whenever an aliquot of this culture, designated CV-1 (VV), was co-cultivated with permissive sheep cells, virus was isolated within three to six weeks. The proportion of cells in the CV-1 (VV) population that were rescue positive was \(<1:10,000\) as judged by cellular titrations. In addition, 72 single-cell clones derived from CV-1 (VV) cultures were all found to be rescue negative. Consistent with this estimate, preliminary examination of the CV-1 (VV) cells, with a cDNA probe used for in situ hybridization, indicated that viral RNA could be detected in one cell out of 1,000–7,000 (J. Harris and A. Haase, personal communication).

In summary, the CV-1 (VV) model appears to resemble in vivo visna virus infection in that only a small proportion of cells is infected, the infected cells carry the complete genome, and genome expression can be induced by co-cultivation with permissive cells. Further molecular characterization of this model is in progress.

Early and Late CNS Lesions in Icelandic Sheep

All Icelandic sheep inoculated intracerebrally with a large dose \((10^6 \text{ TCID}_{50})\) of a neurotropic strain (1514) of visna virus develop lifelong infections, as signaled by the ability to isolate virus from the blood and by persistent serum antibodies. Two types of pathologic changes occur in the CNS of such sheep: an early and persistent inflammatory response, seen in most sheep; and the irregular and unpredictable occurrence of focal demyelinating lesions which, in the spinal cord, produce clinical paresis. For clarity these two lesions will be described separately before the mechanisms are discussed.

Early inflammatory lesions. The majority of Icelandic sheep infected by intracerebral injection of the 1514 strain of visna virus developed inflammatory CNS lesions as described in several publications [8, 12, 13, 15, 17]. These inflammatory changes were usually subclinical. The CNS lesions were distributed mainly around the ventricles, although lesions were seen in white matter and meninges. These lesions consisted of accumulations of lymphocytes, monocytes or macrophages, and plasma cells, distributed as perivascular cuffs or as focal and diffuse infiltrates of the neuropil. There was little evidence of neuronal death or neuropagia, and overt demyelination was also unusual. Occasional areas of severe inflammation contained foci of necrosis or liquefaction, but otherwise there was little tissue destruction.

Examination of the CSF permitted the serial description of the inflammatory process [19]. The course of inflammation clearly differed in individual sheep, but the most common pattern was an early rise in the number of cells 0.5–3 months after infection, with a subsequent waning of pleocytosis, which persisted at slightly elevated levels or dropped to normal limits. The great majority of CSF cells were lymphocytes or macrophages. There was a general correlation between cell count and severity of inflam-
because of marked loss of myelin and rather sharply demarcated borders. Electron microscopy showed classic primary demyelination within these foci (figure 4) with many naked, but otherwise intact, axons. Remyelination by oligodendrocytes or by Schwann cells was also prominent. Within these chronic demyelinated foci, inflammation was minimal and gliosis was marked. In recently demyelinated lesions, debris-laden macrophages and inflammation were more prominent.

**Mechanisms of CNS lesions.** As an experimental model, visna is cumbersome because of the slow and variable time course of disease, the size and expense of the animals, and the absence of inbred histocompatible sheep. Given the lack of evidence, the following discussion is necessarily speculative.

The lesions of visna, particularly the late lesions, are excessive in relation to the minimal numbers of infected cells. Thus, it is necessary to invoke an amplifying step to explain the severity of pathology. An immune mechanism would provide this amplification step.

**Clinical signs and late demyelinating lesions.** Visna was originally described as a demyelinating disease [1, 3], but these observations were ambiguous in the absence of ultrastructural studies [5]. The uncommon and unpredictable occurrence of clinical disease and the difficulties of perfusion impeded recent work, but it has finally been possible to confirm primary demyelination as an important component of clinical disease [17].

Clinical signs were characteristically irregular and unpredictable in Icelandic sheep, with onset at any time from three months to as late as eight years after infection (figure 2). The salient feature was lameness, usually in one or both hind legs, with progression to all four extremities. In individual animals, the course of paresis was either rapid over several weeks to months, or gradual and irregular over several years. Eventually sheep could not feed themselves and were killed.

Sheep killed because of progressive lameness usually showed focal demyelinating lesions in the spinal cord in addition to subacute encephalomyelitis. These focal lesions were often dramatic (figure 3).
Inflammatory lesions evolve during the first two to four weeks after infection and seem to stabilize after three months. The evolution of the blast-transformation response [23], which peaks between three to six weeks, is congruent with this tempo; whereas the antibody response in serum and CSF is somewhat slower, reaching a peak at three to six months after infection. Suppression of the immune response [18] markedly reduced the early inflammatory lesions without reducing virus isolations from the CNS (table 1). Conversely, postinfection immunization [20] enhanced lesions, although not markedly. The available evidence suggests that the target antigen is viral and not myelin. There is a definite correlation between severity of subacute encephalitis and the frequency of viral isolation (table 2) [8, 14, 20]. Also, the dose of infecting virus correlated with lesion grade.

Experimental allergic encephalitis (EAE) could be induced in sheep immunized with sheep brain in complete Freund’s adjuvant, and sensitized animals raised antibodies against sheep basic protein and galactocerebroside [31]. However, asymptomatic visna-infected sheep failed to raise serum antibodies against either of these myelin antigens up to nine months after infection [31]. Finally, the ability of visna-maedi virus to induce progressive pneumonia, the pulmonary counterpart of subacute encephalitis, is most readily explained by an antiviral immune mechanism.

The focal demyelinating (or late) lesions of visna, which resemble the plaques of multiple sclerosis, are still poorly understood. We do not know if these are more severe manifestations of the same pathologic process responsible for subacute encephalitis. Also, the trigger that initiates the irregular occurrence of demyelination at long intervals after infection remains obscure. Demyelination could be mediated as a bystander effect, secondary to a delayed-type hypersensitivity response.

Figure 4. Demyelination and remyelination in the spinal cord of a sheep with clinical visna. An axon without myelin is seen at lower left, and a partially remyelinated axon at upper right, contrasting with a relatively normal myelin figure at right of field. Magnification: ×24,000.
Table 1. Influence of immunsuppression or of post-infection immunization on severity of early visna lesions in Icelandic sheep.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of blood lymphocytes/μl</th>
<th>No. of CSF cells/μl</th>
<th>CNS lesion grade*</th>
<th>CNS virus isolations (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressed</td>
<td>25</td>
<td>17</td>
<td>0.0</td>
<td>59</td>
</tr>
<tr>
<td>Control</td>
<td>5,000</td>
<td>246</td>
<td>3.0</td>
<td>69</td>
</tr>
<tr>
<td>Immunized</td>
<td>ND</td>
<td>ND</td>
<td>3.5</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>0.5</td>
<td></td>
<td>19</td>
</tr>
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</table>

NOTE. There were eight sheep in each group. Immunosuppression was induced by treating the sheep with horse anti-sheep thymocyte serum or normal horse serum and infecting them with 10⁶ TCID₅₀ of strain 1514 visna virus [18]. They were killed one month later, when all determinations were made. Postinfection immunization was carried out by infecting sheep with 10⁷ TCID₅₀ of strain 1514 visna virus and immunizing them with purified 1514 virions in complete Freund's adjuvant at three and five weeks postinfection [20]. They were killed at nine weeks. Data are median values. ND = not done.

* Inflammatory lesions were graded on a scale of 0–6 (increasing severity) for each animal.
† Percentage of virus isolations was based on four to five specimens tested for each animal.

Antigenic Variation During Long-Term Infection of Icelandic Sheep

Gudnadóttir [6] first reported that a viral isolate from a long-term infected Icelandic sheep could escape neutralization by the animal's own sera, even though the same sera neutralized the infecting strain. Narayan and colleagues [32–34] further studied this phenomenon in Hampshire and Border Leicester sheep. They found that when isolates from the sheep's sera were tested against the animal's own sera (autologous neutralization), variants were isolated from two of seven animals. It was also shown that variants could be selected in culture by passaging visna virus in the presence of neutralizing antisera [32, 35, 36]. Molecular comparison of both the proteins and the genomes of variant viruses [37, 38] indicated that the variants represented point mutations in the env gene encoding the envelope glycoprotein, the protein responsible for neutralization.

Antigenic drift in long-term infected Icelandic sheep. The occurrence of antigenic variation in some visna-infected sheep is well established. However, several questions remained unanswered. What is the frequency with which variants occur? Do variants play a role in viral persistence? Do variants play a role in lesion development? A detailed study of antigenic variants in our long-term infected Icelandic sheep was therefore undertaken [33], and some of the salient observations will be summarized.

A group of 20 Icelandic sheep were infected with strain 1514 and observed for more than seven years, during which 209 isolates were obtained from blood, CSF, and CNS. A stratified sample consisting of 76 of these isolates was selected to represent differing intervals after infection. These isolates were tested by neutralization against three reference sera, and the results are set forth in table 3. Among

Table 2. Correlation between severity of early visna lesions and the frequency of virus isolations.

<table>
<thead>
<tr>
<th>CNS lesion grade*</th>
<th>No. of sheep</th>
<th>No. of CSF cell counts &gt;100/total no. of cell counts obtained (%)†</th>
<th>No. of CNS viral isolations/total no. of specimens tested (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1</td>
<td>17</td>
<td>3/15 (20)</td>
<td>17/80 (21)</td>
</tr>
<tr>
<td>2+</td>
<td>35</td>
<td>18/32 (56)</td>
<td>88/148 (59)</td>
</tr>
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</table>

NOTE. Data are from [8, 20].

* Lesions were graded on a scale of 0–6 (increasing severity).
† Cell counts were measured in number of cells/μl.
‡ Data for viral isolations are based on four to five specimens tested for each animal.

Table 3. Frequency of variant viruses among isolates from blood and CSF or CNS of Icelandic sheep with long-term visna virus infections.

<table>
<thead>
<tr>
<th>No. of months after infection</th>
<th>No. of variants/total no. of isolates tested with indicated system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood CSF/CNS Blood CSF/CNS</td>
</tr>
<tr>
<td>1–12</td>
<td>3/21 0/4 0/12</td>
</tr>
<tr>
<td>13–24</td>
<td>2/10 0/4 0/2</td>
</tr>
<tr>
<td>25–36</td>
<td>0/12 . . . 0/2</td>
</tr>
<tr>
<td>37–48</td>
<td>0/6 0/1 . . .</td>
</tr>
<tr>
<td>49–60</td>
<td>2/6 . . . 1/1†</td>
</tr>
<tr>
<td>61–72</td>
<td>1/3 . . . 1/2†</td>
</tr>
<tr>
<td>73–84</td>
<td>1/1 3/8 1/3†</td>
</tr>
<tr>
<td>Total</td>
<td>9/59 3/17 3/24 3/11</td>
</tr>
</tbody>
</table>

NOTE. Two different systems were used. Reference method: isolates were typed with selected sheep sera to determine whether they were "1514-like" by comparison with strain 1514 used for infection or "not 1514-like." Autologous tests: isolates were tested against serial serum samples from the same animal and compared with infecting strain 1514. Data are from [23].

* Of the 35 samples tested against autologous sera, 23 were from five sheep with clinical visna; none of these 23 were variants.
† These six variants are from a single sheep, no. 1557 (see figure 5).
Figure 5. Autologous neutralization tests on two Icelandic sheep infected with strain 1514. Sera were tested against the infecting strain (anti-1514) and against isolates from blood (B) or CNS (C). Arrows and symbols indicate the time at which each isolate was obtained. Sheep no. 1553 shows no drift, and all isolates were neutralized, whereas sheep no. 1557 shows drift since the last six isolates were not neutralized. Data are from [23].

The 76 isolates, 12 (16%) were typed as variants. These variants were dispersed over time and among sheep. In only one instance (sheep no. 1557) did variant viruses replace the infecting strain; in the remaining 19 animals, the frequency of variants did not increase with time after infection.

Among the 20 sheep, 35 isolates from seven animals were selected (with emphasis on those with clinical visna) and were each tested against serial sera from the same sheep (autologous neutralization). The findings are summarized in table 3. Only isolates from sheep no. 1557 evidenced over drift, whereas none of the 27 isolates from the other six animals demonstrated this phenomenon. Also, none of the CNS isolates from sheep with clinical visna were typed as antigenic variants. Sheep no. 1557 showed antigenic drift and no. 1553 did not (figure 5).

Biologic significance of antigenic variation. Our observations and those of Narayan and colleagues [32–34] are quite consistent. Antigenic variants of visna virus are constantly generated by spontaneous mutations in the env gene of the virus. This phenomenon is a manifestation of the plasticity of the genomes of RNA viruses [39]. Persistent infection in the sheep must select somewhat for these variants, since they were found at a frequency >10%, which is much higher than their frequency in the initial inoculum (presumably <1 per 1,000 virions, since reference antisera readily neutralize 1,000 TCID<sub>50</sub> of the 1514 strain).

In the long-term infected sheep, variants persist together with the parental virus and do not gradually replace the parental serotype. If variants were responsible for continued spread of infection, they should increase in relative frequency, but this increase was not seen. Therefore, antigenic variation does not appear to be essential for virus persistence. Isolates from the CNS of animals with clinical visna are usually not variants, i.e., are neutralizable by autologous antisera. Within the limits of these observations, there is no evidence that variants are involved in the occurrence of focal demyelinating lesions.

Persistent Infections and Slow Viruses

Sigurdsson [2] originally defined a slow infection as having a long incubation period, attacking a single organ system, and being inevitably fatal. These views should be modified in light of 30 years of research. We prefer to distinguish between the infectious process and consequent disease. Infections persist by utilizing a variety of strategies to evade the defense mechanisms of the host. A persistent infection may be subclinical or may initiate disease weeks, months, or years later. The disease process is usually chronic but may or may not be fatal and may or may not be limited to a single organ. Thus, Sigurdsson’s early views must be expanded to include a number of different patterns of persistent infection and chronic disease.

References


