not have been presented to the rabbits in immunologically optimal conditions.

Our experiments provide conclusive evidence that galactocerebrosides not possibly contaminated with other CNS components can evoke myelination-inhibiting antibodies in rabbits. To date, evocation of demyelinating and myelination-inhibiting antibodies by either natural (*13, 20*) or synthetic cerebrosides has been accomplished only in this species. The minor differences in the molecular structure of cerebrosides from central and peripheral nervous system myelin (*21*) would appear to make the specificity of antibodies to myelin for central or peripheral nervous system cultures (*1, 3, 22*) difficult to explain if cerebroside is the only factor involved.

SARKA HRUBY
ELLSWORTH C. ALVORD, JR.
Laboratory of Neuropathology, Department of Pathology, University of Washington Medical School, Seattle 98195
FREDRICK J. SEIL
Department of Neurology, Veterans Administration Hospital, Palo Alto, California 94304

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Slow Persistent Infection Caused by Visna Virus: Role of Host Restriction

Abstract. *Proviral DNA has been demonstrated by in situ hybridization in foci of cells of a lamb infected with the RNA slow virus visna. A few of these cells also contain the major virion structural antigen p30. This restriction in virus gene expression in the infected animal provides a mechanism for persistence of virus in this chronic infection.*

Visna, an inflammatory and demyelinating disease of the central nervous system (CNS) of sheep, is caused by a virus closely related to RNA type C viruses both in structure (*1*) and in the initial aspects of replication in vitro; in permissively infected cells the genetic information in the RNA genome of the virus is completely transferred to a DNA intermediate or provirus (*2*). Visna is also a prototype of slow and persistent infections (*3*) in which the virus elicits an immune and inflammatory response from its host which fails to eliminate the infectious agent. One of the central issues in understanding chronic virus infections is how a virus like visna is able to persist for periods as long as 8 years in an animal with demonstrable neutralizing antibody (*4, 5*).

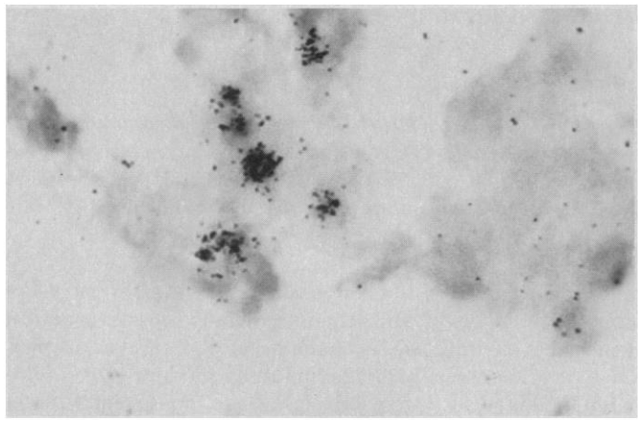
We have proposed (*1, 2*) one mechanism for persistence analogous to the lysogenic relationship between viruses and their bacterial hosts; in the infected animal provirus formation occurs as it does in vitro, but later steps in the life cycle of the virus are believed to be repressed in most cells. Virus genetic information is therefore conserved, and virus survives to perpetuate infection because the infected cell is neither detected nor destroyed by the immune surveillance system of the host animal. The important consequences of this argument now amenable to experimental test are: (i) infected cells should harbor viral DNA; but, (ii) because of host restriction, only a small proportion of infected cells should also synthesize viral proteins or progeny at detectable levels.

We have examined these predictions as follows. Viral DNA can be detected and quantitated in cells in tissue sections by hybridization in situ. A restriction in virus gene expression can then be assessed by determining, by immunofluorescence, the proportion of cells that also contain p30, the major structural polypeptide and gene product of the virus. These studies were conducted in an animal model (*6*) congruent in many respects with the natural slow infection, and the postulated temperate relationship of virus and host cell in vivo. Visna virus causes a persistent infection of American lambs in which virus particles are not observed in tissues, and in which virus cannot generally be recovered from tissue homogenates. However, explant cultures uniformly produce virus after a few days in culture, consistent with the notion that virus growth is blocked in vivo, and that this restriction can be relieved in some manner by the conditions of cultivation in vitro.

The nature of the virus host cell relationship in the animal was investigated in sheep choroid plexus (SCP) tissue. We chose this tissue because, in our studies of virus isolation from various tissues, we found that choroid plexus was invariably infected and contained the largest percentage of cells that could be induced to yield virus on explantation (*7*). In this investigation we examined choroid plexus from one fetus killed 2 weeks after virus inoculation. No virus particles were detected by electron microscopy in a sample of the tissue, and infectious virus was not recovered from cell-free homogenates prepared from another portion of the tissue. Virus, however, was isolated from every explant culture established from this particular choroid plexus tissue. Sections were cut from the remaining tissue and fixed for assay of viral DNA and p30.

To detect viral DNA by in situ hybridization (*8, 9*) RNA was removed by digestion with ribonuclease, and the DNA in the tissue section was denatured and hybridized to a virus specific probe (*10*) labeled to high specific activity with [^{3}H]deoxythymidine triphosphate (dTTP). At the end of the reaction, unreacted probe was removed by extensive washing and digestion with single strand specific S1 nuclease, and the tissues were prepared for radioautography. After exposure of 9 to 14 weeks slides were developed and stained.

Most of the cells in the sections from infected tissues had no more than the background of 1 to 2 grains per nucleus seen in uninfected tissues, but in other areas significant hybridization is evident. In these foci we observed discrete collections of grains over the nuclei of cells (Fig. 1). In two independent experiments we found that the number of cells with a significant number of grains over the nucleus constituted 18 percent of the total



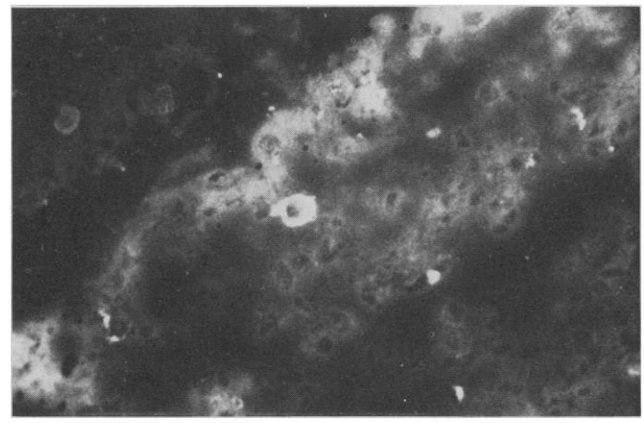


Fig. 1 (left). In situ hybridization of visna complementary DNA to SCP tissues: Visna ^{3}H-labeled cDNA was hybridized to SCP tissue from an infected animal (*9*). After radioautographic exposure of 14 weeks, the slides were developed and stained (original magnification × 1000). Fig. 2 (right). Indirect fluorescent antibody assay for visna virus p30 in tissues. Sections adjacent to those in Fig. 1 were fixed in acetone, reacted with guinea pig monospecific antiserum to visna p30 and then with fluorescein-conjugated antibody globulin to guinea pig serum. The preparation was examined with a Zeiss fluorescence microscope with a BG12 exciter filter and OG4 barrier (original magnification × 400).

population. These data are in good agreement with the proportion of cells expected to carry the viral genome, based on the fraction of cloned cells from SCP tissues that can be induced to synthesize virus in vitro (Table 1).

The nuclear localization of visna proviral DNA is characteristic both of infected tissues and SCP cells infected in vitro, and a similar number of grains occurs in both circumstances. The average of 20 to 40 grains per nucleus over the background, therefore, corresponds to the 100 to 200 copies of viral DNA per cell, measured by hybridization in solution (*11*). The amplification of viral genetic information evidently characteristic of visna virus infection of SCP cells both in the animal and in vitro is fortunate experimentally, as the success of the in situ hybridization technique depends on gene reiteration at this level (*8*). The specificity of the in situ hybridization is substantiated by (i) the expected localization of grains in nuclei, (ii) the lack of hybridization of probe to uninfected tissues, (iii) the absence of hybridization if the cell DNA was not denatured, (iv) the absence of hybridization as a result of prior treatment with deoxyribonuclease (*12*), and (v) the fact that DNA from SCP cells infected (in vitro) effectively reduced hybridization, whereas DNA from uninfected cells did not competitively interfere with hybridization of the labeled probe (*13*).

The principal structural polypeptide (p30) of the virus could only be detected by immunofluorescence (*14*) in a few cells in tissue sections adjacent to those used for in situ hybridization. Cytoplasmic fluorescence (Fig. 2) was observed in cells in areas corresponding to foci that had viral DNA, but, in contrast to SCP cells permissively replicating virus in vitro, where more than 80 percent of cells display similar cytoplasmic fluorescence (*15*), only 0.025 percent of the cells stained positively (Table 1). Thus only about 1/1000 of the cells that contained viral DNA were synthesizing detectable amounts of the major viral gene product in vivo. Presumably the cells that synthesize p30 also give rise to the low levels of infectious virus in tissues, but this has yet to be directly demonstrated.

These experiments provide evidence that there is a block in virus replication beyond the stage of provirus formation in SCP tissue infected with visna virus. The initial events in the viral growth cycle leading to formation of provirus establishes a stable association between viral genetic information and the host cell, but later steps in replication involving transcription or translation of viral RNA are blocked. Virus proteins and progeny are therefore not synthesized, and the infected cell is not detected or destroyed by immune mechanisms. Spontaneous activation occurs in a small proportion of cells in vivo and in a much larger proportion under conditions of growth in vitro. In the animal these minimum levels of viral replication are associated with the slow spread of virus and attendant inflammation and tissue destruction.

Table 1. Restriction of visna virus gene expression in vivo. Visna virus DNA was detected in SCP tissues by in situ hybridization (*9*). The average number of positive cells (those with 15 grains per nucleus over background) was determined by examination (at 1000 ×) of radioautographs of 16 tissue sections representing two independent hybridization experiments. The average total number of cells in tissue sections (40,000) was estimated from counts of nuclei within the area of a reticle 5 by 5 mm (at 400 ×) in 25 random microscopic fields, and the ratio of the area of the reticle to the area of the whole tissue section. The latter was obtained by comparison of the weights of tracings of the tissue sections with the weight of a tracing of known area. The average number of cells with virion p30 was assessed by immunofluoresence (*14*) (Fig. 2, legend 2) in tissue sections immediately adjacent to those used for hybridization. The proportion of cells with p30 is expressed as the average number of cells displaying bright fluorescence per tissue section divided by the average number of cells per tissue section. A minimal estimate of the proportion of cells with a complete viral genome was obtained by cloning SCP cells from a trypsinized portion of tissue not used for hybridization and immunofluorescence. Of 22 clones established from the SCP tissue, three gave rise to virus after 1 to 3 weeks of cultivation in vitro.

Item	Percent	No./total
	In cells	
Viral DNA	18	7,200/40,000
p30	0.025	10/40,000
	By clones	
Virus production	14	3/22

ASHLEY T. HAASE
LINDA STOWRING
Infectious Disease Section, Veterans Administration Hospital, and *Departments of Medicine* and *Microbiology, University of California, San Francisco 94121*
OPENDRA NARAYAN
Department of Neurology and Animal Medicine, Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205
DIANNE GRIFFIN
Department of Medicine and Neurology, Johns Hopkins University
DONALD PRICE
Department of Neurology and Pathology, Johns Hopkins University

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9. For hybridization in situ to detect DNA, tissue sections cut with a cryostat from frozen tissue were fixed in a mixture of ethanol and acetic acid, and subsequently treated with 0.2*N* HCl. RNA was removed enzymatically with pancreatic ribonuclease [100 μg per microliter of sodium saline citrate, double strength (2 × SSC), 37°C, 30 minutes], and the DNA was denatured in 95 percent formamide 0.1 × SSC (65°C, 2 hours). The complementary DNA (cDNA) was hybridized under a cover slip at a concentration of 4 ng per 5 μl in 4 × SSC containing 50 percent formamide at 45°C to a C_0t of 2 mole/liter per second, based on the cDNA in solution. The slides were then washed in buffer (30 m*M* sodium acetate, *p*H 4.5, 100 m*M* NaCl, 3 m*M* $ZnCl_2$), treated with 10 units of S1 nuclease (Sigma) per milliliter for 2 hours at 50°C, washed, dried, and coated with Ilford K2 emulsion. After radioautographic exposure, the slides were developed and stained with Giemsa (*8*) (A. Haase and L. Stowring, in preparation).
10. A relatively symmetric transcript complementary to the RNA genome of visna was synthesized in an endogenous reaction containing actinomycin D. Reaction mixtures (1 ml) contained 50 μmole of tris-HCl, *p*H 7.4, 8 μmole of $MgCl_2$, 10 μmole of dithiothreitol, 0.1 mmole of dATP, dGTP, dCTP, and ^{3}H-labeled dTTP (specific activity 59.5 c/mmole, 10^8 dpm per microgram of DNA), 100 μg of actinomycin D, 250 μg of purified visna virus, and 0.02 percent Triton X-100. After incubation at 37°C for 4 hours, the reaction products were purified, treated with 0.6*M* NaOH for 4 hours at 37°C, neutralized and passed over Sephadex G-50 to remove free isotope and unlabeled nucleotides. The probe fractions in the exclusion volume were pooled and precipitated with ethanol. After recovery by centrifugation, the ^{3}H-labeled probe was suspended in a small volume of buffer (10 m*M* tris-HCl, *p*H 7.4, 1 m*M* EDTA) at a concentration of 2 ng/μl. At least 90 percent of the predominantly single-stranded DNA transcript contained sequences complementary to viral RNA, determined by annealing the DNA to a vast excess of viral RNA to a Cr_0t (concentration of ribonucleotide in moles per liter times the time in seconds) of 2; the DNA probe complementary to virion RNA was representative of the entire genome, as it protected 60 to 100 percent of labeled 70*S* RNA from digestion by ribonuclease at mass ratios of cDNA/RNA of less than 10 [A. Haase, A. Garapin, A. Faras, H. Varmus, J. Bishop, *Virology* **57**, 251 (1974)].
11. SCP cells synchronously infected in vitro contain, late in the viral growth cycle, an average of 100 to 200 copies of viral DNA per cell, measured by liquid annealing methods [(*2*) and B. Traynor, A. Haase, M. Brahic, in preparation]. Under the same conditions of infection the viral DNA content of SCP cells infected in vitro measured by in situ hybridization corresponds to 20 to 40 grains per nucleus (L. Stowring and A. Haase, unpublished data).
12. The deoxyribonuclease treatment was at 200 μg/ml in buffer containing 10 m*M* $MgCl_2$, 37°C, 1 hour.
13. In the competition hybridizations, SCP DNA from uninfected cells, or cells infected for 72 hours with about 150 copies of proviral DNA per cell, was extracted, sheared (*2*), and added to the hybridization buffer along with the 4 ng of [^{3}H]cDNA. To accommodate the 300 μg of unlabeled DNA, the volume was increased to 50 μl. See (*9*) for subsequent hybridization procedures.
14. The major polypeptide of visna virus, like other RNA type C viruses, has a molecular weight of about 30,000 and is designated p30 [J. August, D. Bolognesi, E. Fleissner, R. Gilden, R. Nowinski, *Virology* **60**, 595 (1974), for nomenclature]. The p30 was purified to homogeneity by isoelectric focusing; virus preparations were dissociated in Brij detergent and urea and focused successively in broad (*p*H 3 to 10) and narrow (*p*H 5 to 8) gradients. Material with an isoelectric point of 6.9 was freed of ampholines by desalting on Sephadex G-25, and concentrated by lyophilization. The final product was shown by electrophoresis in gels of polyacrylamide to consist of a single species corresponding in migration to p30. This material was used to immunize guinea pigs. The resultant antiserums reacted with visna virus and p30 in immunodiffusion, but not with other viral antigens, sheep cell extracts, or serum proteins. In indirect fluorescent antibody tests it stained SCP cells infected with visna virus in vitro, but not uninfected SCP cells or tissues; and this staining could be blocked specifically by prior adsorption of the serums with p30.
15. A. Haase and L. Stowring, in preparation.

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Insulin, Glucagon, and Glucose Exhibit Synchronous, Sustained Oscillations in Fasting Monkeys

Abstract. *In overnight fasted rhesus monkeys, synchronous, regular oscillations occurred in the plasma concentrations of glucose, insulin, and glucagon. The oscillations displayed a period averaging 9 minutes. The amplitudes for insulin and glucagon were ten and five times greater than for glucose. Insulin cycled in and glucagon out of phase with glucose. In baboons, oscillations of glucose and insulin were smaller than in rhesus monkeys, while in man, regular oscillations were not observed.*

While studying the changes that occur in circulating fuels and hormones during spontaneous feeding in rhesus monkeys (*Macaca mulatta*), we noted synchronous, regular oscillations in the concentrations of glucose, insulin, and glucagon in the plasma. The period of these oscillations was 7 to 12 minutes and remained relatively constant up to 1 hour in individual animals. The insulin and glucose cycles were nearly in phase while the glucagon cycles were out of phase with insulin and glucose. These observations were made in eight conscious monkeys that were restrained in chairs and had been prepared with venous cannulas permanently implanted in the vena cava at the level of the right atrium for blood sampling. The monkeys were fed on a complete liquid diet (Ensure, Ross) delivered by an automated feeder (*1*). The animals were housed in sound-attenuated chambers, their venous lines being led to the outside of the chamber so that samples could be taken without disturbing the monkey. Samples were drawn at 2- or 5-minute intervals for analyses (*2*) of glucose, insulin, and glucagon.

The cycles were studied in detail in six animals that had fasted for 16 hours. Figure 1 shows the data and time series analysis in one animal (No. 5, Table 1). Plasma was sampled every 2 minutes for 30 minutes in the morning before the first expected meal. The data were analyzed by autocorrelation with values being interpolated to 30-second intervals and periods of delay of (up to) 16 minutes (*3*). The period of the cycles for glucose, insulin, and glucagon did not differ by more than 1 minute (except in animal No. 4). The mean period for all animals was 9.3 ± 1.5 minutes (standard deviation, S.D.) (Table 1). The method of cross correlation was used to determine phase shifts. With glucose used as a reference, the maximum correlation with insulin occurred on an average 0.9 ± 0.6 minute later (or 8.4 minutes earlier) and for glucagon 6.8 ± 1.6 minutes later (2.5 minutes earlier) than glucose. Maximum correlation of the molar ratio of insulin to glucagon (I/G) occurred 1.0 ± 0.6 minute later (or 8.3 minutes earlier) than glucose.

The normalized autocorrelation coefficients for the variables are listed in Table 1. Although not every coefficient reached the level of significance in each animal, at least one of the variables displayed significant autocorrelation at the $P \lesssim .05$ level (in animal No. 6, I/G was the only variable to reach significance). The amplitudes of oscillation of the three variables were significantly different (insulin > glucagon > glucose) (Table 1). In these relatively short experiments the amplitude did not vary greatly from cycle to cycle in most animals. However, amplitudes did change in some time series and when this occurred the amplitudes of insulin and glucagon often changed in parallel.

The effect of a phantom meal was examined in three animals by filling the feeder and turning on the light, but not activating the diet delivery pump. Glucose and insulin cycled unchanged during and after this phantom meal. In contrast, when an authentic meal was eaten, the concentrations of glucose and insulin increased within 10 to 12 minutes and the cycles were interrupted. Atropine given intravenously (0.05 mg/kg) at the midpoint of a 60-minute period of observation did not disrupt the cycles of insulin