American Journal of Pathology, Vol. 142, No. 4, April 1993

Human Foamy Virus Proteins Accumulate in Neurons and Induce Multinucleated Giant Cells in the Brain of Transgenic Mice

Adriano Aguzzi,* Erwin F. Wagner,* Kai-Olaf Netzer,† Katrin Bothe,† Ingrid Anhauser,* and Axel Rethwilm†

From the Institute of Molecular Pathology, Dr.-Bohr-Gasse 7, A-1030 Vienna, Austria, and the Institute of Virology and Immunobiology, University of Würzburg, Versbacherstrasse 7, D-W-8700 Würzburg, Germany†*

Human foamy virus (HFV) is a retrovirus encoding structural genes and, like human immunodeficiency virus and human T cell leukemia virus I, several ancillary reading frames collectively termed the* bel *genes. We have previously shown that HFV transgenic mice develop an encephalopathy with neuronal loss in hippocampus and cerebral cortex. We have now raised and characterized rabbit antisera to various recombinant portions of* gag, pol, env, *and* bel-1, *the viral transactivator. Immunoreactivity for* gag *and* bel-1 *was observed in nuclei and processes of hippocampal and cortical neurons before the onset of morphological lesions and correlated with the appearance of HFV mRNA. Astrocyte-derived multinucleated giant cells containing HFV proteins were present in the brain of transgenic mice coexpressing full-length HFV genes but not in mice expressing truncated* gag *and* env, *suggesting that these genes contain a fusogenic domain. Expression of full-length structural genes decreased the life expectancy of transgenic mice, implying an adjuvant role for these proteins in HFV-induced brain damage. (Am J Pathol 1993, 142:1061–1072)

Human foamy virus (HFV) was originally isolated 20 years ago from an African patient suffering from nasopharyngeal carcinoma.[1] Ever since the first HFV isolate was cloned and sequenced[2,3] characterization of HFV has been rapidly progressing.[4–6] The genomic region between *env* and the 3′ long terminal repeat bears particular interest since it encodes several ancillary reading frames, including a retroviral *trans*-activator termed *bel-1*.[7–9]

According to several serological surveys, HFV has attained a high prevalence in significant geographical areas, such as East Africa and the Pacific Islands,[10–12] and evidence for a role of HFV in specific pathologies is accumulating. For example, HFV has been isolated from patients with a variety of diseases, including, notably, encephalopathy (reviewed in ref. 13). Nasopharyngeal carcinoma in Kenya and Tanzania is associated with an extraordinarily high prevalence of antibodies to HFV, and most intriguingly, seropositivity for HFV was recently reported to be very frequent in East African patients with acquired immune deficiency syndrome and its related complex.[12]

Despite the increasing number of epidemiological clues pointing to pathogenic properties of HFV for humans and its prominent cytopathic effects in various types of cultured cells,[1,14] the pathogenic properties of HFV have thus far remained ill defined, and its potential threat to humans still awaits ultimate proof. In the present situation, characterized by an incomplete availability of epidemiological and molecular data, *in vivo* model systems are proving invaluable to the study of the biology and pathogenic effects of HFV. We chose to study the biology of HFV in transgenic mice, since such a system enables extremely powerful yet flexible approaches to the study of genetically induced pathologies (for a review, see ref. 15). In fact, the first unequivocal demonstration that HFV can induce lesions in mammalian hosts was provided by our experiments with transgenic mice expressing various parts of the HFV genome.[13,16]

Mice expressing truncated amino terminal fragments of *gag* and *env* (382 and 191 amino acids, respectively) and the intact *bel* region of HFV under transcriptional control of the homologous long terminal repeat develop a rapidly progressive neurodegenerative disease and a myopathy with clinical

Supported by grants of the Deutsche Forschungsgemeinschaft (SFB 165) (to AR) and a grant of the Sassella Foundation (to AA).

Accepted for publication October 13, 1992.

Address reprint requests to Dr. Adriano Aguzzi, Institute of Neuropathology, Sternwartstrasse 2, CH-8091 Zürich, Switzerland.

symptoms as early as 7 weeks after birth. *In situ* hybridization experiments have shown that single neurons in affected areas express very high levels of transgenic mRNA, while neighboring cells with identical morphological characteristics do not express detectable levels of the transgene. However, although expression in adult mice is restricted to myogenic and central neuroepithelial tissues, a transient burst of expression occurs in several tissues during embryonic development without causing deleterious effects.[17] These remarkably complex patterns of expression in HFV transgenic mice suggest that regulation of HFV gene expression is controlled *in vivo* by an interplay of virally encoded genes and by host factors, some of which are likely to be expressed only in specific tissues. In particular, the irregular distribution of cells expressing the transgene within affected tissues is rarely observed in transgenic mice and has led us to propose that *bel-1*, a transactivating factor, may deregulate HFV transcription by triggering an autocatalytic cascade of transactivation in single permissive cells and eventually lead to their degeneration.[17]

In order to gain more insights into the regulation of HFV gene expression *in vivo*, we set out to determine which of the several reading frames encoded by HFV are translated into proteins in the diseased tissues of transgenic mice. To this end, we have cloned DNA fragments corresponding to parts of the structural and regulatory reading frames of HFV into bacterial expression vectors. We have then expressed recombinant protein fragments as bacterial β-galactosidase fusion proteins in an *Escherichia coli* expression system and used these recombinant proteins to raise rabbit antisera. In the present work we show that such antisera react with the appropriate proteins in radioimmunoprecipitation experiments and recognize denatured epitopes in paraffin-embedded tissues when used for immunohistochemical stains. Taking advantage of these reagents, we have proceeded to describe the pattern of immunoreactivity in the brain of HFV transgenic mice shortly before and after onset of the neurodegenerative disease.

Materials and Methods

Construction of Prokaryotic Expression Plasmids

HFV gene fragments corresponding to defined regions of *gag*, *pol*, *env*, and *bel-1* were derived from the coding regions of pHSRV[4] (as shown in Figure 1) and molecularly cloned into the bacterial expression vector pROS.[18] The correct in frame insertion of the fragments at the carboxy terminus of the β-galactosidase coding sequence was verified by DNA sequencing of the molecular clones (A. Rethwilm, unpublished observations). Fusion proteins were purified from induced BMH 71–18 bacteria[18] by preparation of inclusion bodies and preparative

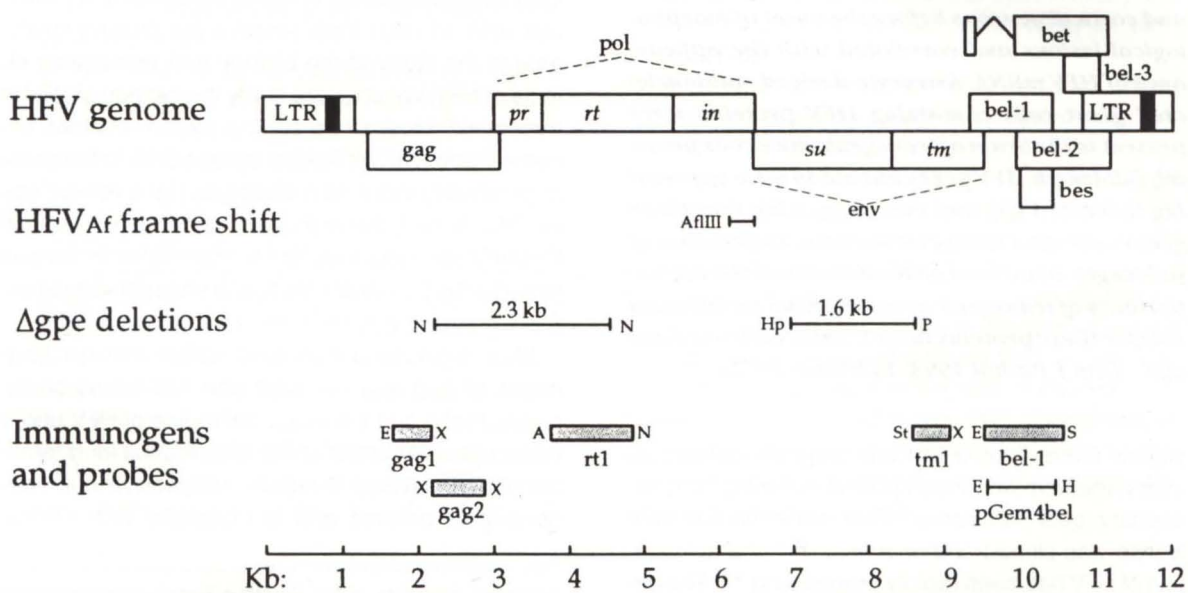

Figure 1. *Genomic structure of HFV and position of the deletions introduced in the pΔgpe construct. The shaded boxes in the lower part of the figure indicate the portions of HFV reading frames cloned into* E. coli *expression vectors. The bar indicates the cDNA fragment cloned into pGem4bel, an* in vitro *transcription vector utilized to generate the ribonucleic acid probes for* in situ *hybridization. Abbreviations for the gene products of HFV are: gag, group-specific antigen; pr, protease; rt, reverse transcriptase; in, integrase (endonuclease); su, surface protein; tm, transmembrane protein. Restriction sites are abbreviated as follows: A,* Acc*I; E,* Eco*RI; H,* Hind*III; Hp,* Hpa*I; M,* Msp*I; N,* Nco*I; P,* Pvu*II; S,* Sph*I; St,* Stu*I; X,* Xba*I.*

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described.[19] Proteins used to immunize rabbits were more than 90% pure as deduced from Coomassie blue stained SDS-PAGE gels.

Detection of Viral Proteins

Metabolic labeling of HFV-infected baby hamster kidney cells (BHK-21) and radioimmunoprecipitation (RIPA) were essentially performed as described recently.[6] For detection of HFV proteins by Western blotting (WB), HFV-infected BHK-21 cells were lysed in detergent buffer [20 mM Tris-HCl, pH 7.4; 0.3 M NaCl; 1% sodium deoxycholate; 1% Triton X-100; 0.1% SDS; 1 mM phenylmethyl sulfonyl fluoride], separated in SDS containing 10% polyacrylamide gels (SDS-PAGE) and blotted onto nitrocellulose membranes (Schleicher & Schuell). Strips were cut from the blotted membranes, blocked overnight in Tris-buffered saline [20 mM Tris-HCl, pH 7.6; 137 mM NaCl] containing 5% bovine serum albumin (TBS/bovine serum albumin), washed in TBS containing 0.5% Tween-20, and reacted with the first antibody. Antibody dilutions were 1:50 in the case of the rabbit anti-*pol* serum and normal rabbit serum; 1:100 for the human anti-HFV serum; and 1:500 for the rabbit anti-*gag* and *bel-1* sera. Antisera were allowed to react in TBS/bovine serum albumin for 30 to 60 minutes. Strips were then washed again and reacted with peroxidase-conjugated secondary antibody (Dako) for 60 min. After washing, the immunostain was developed using a chemiluminescence detection system (Amersham).

Purification of Recombinant HFV Proteins and Generation of Rabbit Antisera

Lyophilized fusion proteins were reconstituted with water at a concentration of 1 mg/ml and emulsified with 3 volumes of complete or incomplete Freund's adjuvants (Gibco). New Zealand albino rabbits were primed with 4 ml of complete Freund's adjuvant/protein emulsion injected subcutaneously at multiple sites and boosted after 6 weeks with 4 ml of incomplete Freund's adjuvant/protein emulsion intramuscularly. Blood (~40 ml) was collected prior to the priming injection and 2 weeks after each boost. Serum was isolated by centrifugation after allowing overnight clotting at 4 C. Immunoglobulins were partially purified by a two-step ammonium sulfate precipitation method.[19] After dropwise addition of 0.5 volumes of a saturated ammonium sulfate solution at 4 C, the precipitate was removed by centrifugation. Additional ammonium sulfate solution was added to the decanted supernatant to a final 50% saturation. Immunoglobulins were allowed to precipitate overnight at 4 C, collected by centrifugation, resuspended in 5 ml of phosphate-buffered saline, and dialyzed against 3 × 2 liters of phosphate-buffered saline. Aliquots of antiserum were stored at −20 C after addition of 0.05% sodium azide.

Processing of Mouse Tissues for Histological Analyses

Transgenic and control mice were sacrificed by intracardial perfusion with buffered formaldehyde (4% w/v) in deep anesthesia. Tissues were postfixed overnight in the same solution, transferred to 70% ethanol, and embedded in paraffin under RNAse-free conditions in a VIP automatic infiltrator (Ames) through an ascending series of ethanols and three clearing baths of xylene. Samples were then vacuum-infiltrated with low-melting-point paraffin (Vogel) at 50 C overnight. Tissue sections of 3 μm nominal thickness were cut, dried overnight on TESPA-treated slides[20] at 56 C, deparaffinized in xylene, and either processed for *in situ* hybridization, for immunohistochemistry, or stained with hematoxylin and eosin. Slides were finally dehydrated and mounted in a xylene-compatible medium (Entellan Neu, Merck).

In Situ *Hybridization*

The transcription vector pGem4bel, which contains a *Eco*RI-*Hind*III fragment derived from the *bel* region of HFV, was used to prepare sense and antisense complementary RNA probes by *in vitro* transcription in the presence of ^{35}S-rUTP. The resulting probe was previously shown to recognize all HFV transcripts identified thus far.[17] Additional riboprobes were prepared from the pKS*bel*-1 vector. pKS*bel*-1 was derived by cloning a 1.1-kb *Ssp*I fragment from the *bel* region into the *Sma*I site of the pBluescript-KS$^+$ *in vitro* transcription vector (Stratagene) and was shown to yield superimposable results in *in situ* hybridizations (A. Aguzzi and A. Rethwilm, unpublished observations). The radiolabeled transcripts were partially degraded by controlled hydrolysis in 100 mM bicarbonate buffer (pH 9.4, 65 C, 60 minutes) to an average length of 50–100 bp and resuspended in 50% freshly deionized formamide. The extent of hydrolysis was monitored on a denaturing agarose gel (2% agarose, 10% formaldehyde). *In situ* hybridizations were performed

essentially as described.[21] Our hybridization mixture contained the radiolabeled probe at a concentration of 200–300 × 10^3 dpm/μl in 4× standard saline citrate, 50% formamide, 10% dextrane sulfate, 1× Denhard's solution, 100 ng/μl transfer RNA, 1 mM unlabeled S-rUTP, and short unrelated unlabeled S-rUTP containing transcripts in approximately 10× molar excess of the probe. Sense-transcribed RNA probes were used as negative controls. Posthybridization processing included RNAse A digestion (20 μg/ml, 30 minutes at 37 C), and several low- and high-stringency washes over a period of 30 hours. Slides were then dried, dipped in NTB-2 nuclear track emulsion (Kodak), and exposed at 4 C. The exposure times ranged between 1 and 4 days.

Immunohistochemical Methods

Deparaffinized, rehydrated tissue sections of HFV transgenic and control mice were incubated with antisera 122, 123, 124, 125, 127, 161, and 162 diluted in phosphate-buffered saline to the following strengths: 1:20, 1:100, 1:500 (Table 1). Dilutions of 1:250 of antisera 161 and 162 were applied on selected sections. Predigestion with various concentrations of pronase E (Sigma) did not improve the sensitivities of the immunostains. Detection was accomplished essentially as described[22] using the avidin-biotin complex–peroxidase method and diaminobenzidine as a chromogenic substrate. Sections were either lightly counterstained with diluted hematoxylin or mounted without counterstaining. Inspection and microphotography were carried out on a Zeiss Axiophot microscope equipped with differential interference contrast optics.

Table 1. *Synopsis of Antisera to HFV Gene Products and Their Immunohistochemical Properties*

Antiserum no.	Immunogen	Dilution	Signal pHFV$_{Af}$	Signal pΔgpe	Nonspecific stain
122	*gag1*	1:20	+++	+++	++
		1:100	+++	+++	–
		1:500	+	+	–
123	*rt1*	1:20	–	–	++
		1:100	–	–	–
		1:500	–	–	–
124	*tm1*	1:20	+	–	++
		1:100	+	–	–
		1:500	–	–	–
127	*gag2*	1:20	+++	–	++
		1:100	+++	–	+
		1:500	+	–	–
161	*bel-1*	1:20	+++	+++	++
		1:100	+++	+++	+
		1:500	+	+	–
162	*bel-1*	1:20	+++	+++	++
		1:100	+++	+++	+
		1:500	+	+	–

Results

Characterization of Viral Proteins by Rabbit Antisera

Rabbit antisera were raised to various portions of the HFV reading frames, as detailed in Figure 1, and their specificity was tested by WB and by RIPA. The human serum containing antibodies to HFV[6] recognized viral proteins in the range of 27 to 74 kd in WB (Figure 2A, lane 1).

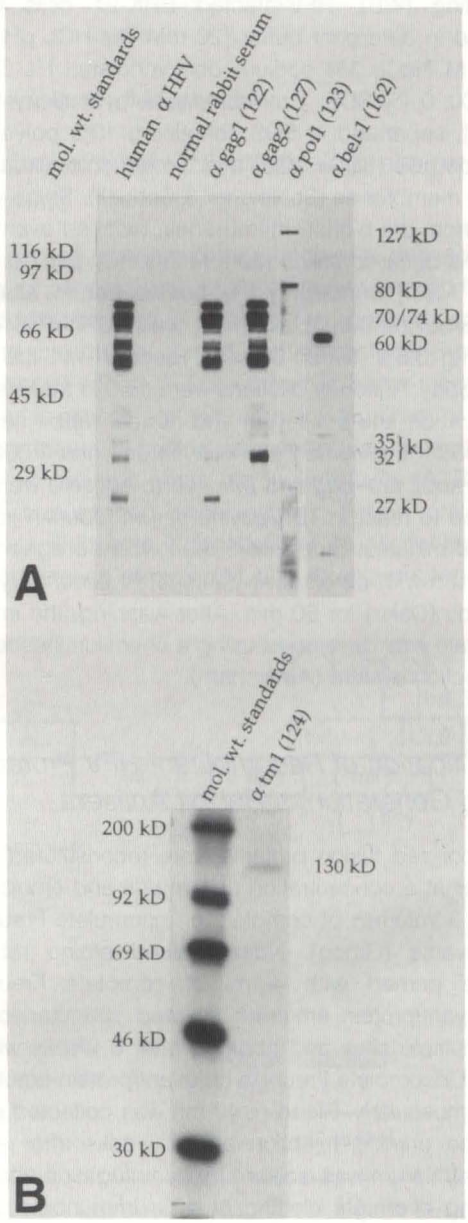

Figure 2. *Western blot* (A) *and radioimmunoprecipitation* (B) *with antisera to HFV.* A, *human positive serum C2*[6] (lane 1); *normal rabbit serum* (lane 2); *antiserum 122 anti* gag1 (lane 3); *antiserum 127 anti* gag2 (lane 4); *antiserum 123 anti* rt1 (lane 5); *antiserum 161 anti* bel-1 (lane 6). B *precipitation of the 130 kD* env *precursor molecule by antiserum 124 (anti tm).*

Viral proteins of 70 and 74 kD have been described to be diagnostically useful in discriminating between foamy virus positive and negative sera.[6] Both the 70 and 74 kd proteins were recognized by our anti-*gag* antibodies generated against the amino terminus and the domain of the major capsid protein of HFV *gag* (Figure 2A, lanes 3 and 4). We therefore conclude that the 70/74 kd double band represents the *gag* precursor pr70/74^{gag} of HFV. Retroviral *gag* precursor proteins are cleaved by the viral protease into the amino terminal matrix protein (MA), the major capsid protein (CA), and the carboxy terminal nucleocapsid protein (NC).[23]

The detection of p27^{gag} and p32^{gag} by the *gag* antisera 1 and 2 identifies these proteins as the MA and CA proteins, respectively. Both proteins are readily recognized by the human anti-HFV-positive serum (Figure 2A, lane 1). The human serum and the *gag* antisera recognize several viral proteins in the molecular weight range of 50 to 60 kd. These viral proteins are likely to represent intermediate cleavage products of the *gag* precursor, in analogy to the *gag* proteins of human immunodeficiency virus.[24,25]

When the *pol* antiserum was applied in a WB assay, two proteins of 127 and 80 kd were identified (Figure 2A, lane 5). These represent the *pol* precursor molecule (127 kd) from which the protease, reverse transcriptase (80 kd), and the integrase of HFV are cleaved.[26]

The *bel-1* antiserum detected two proteins of approximately 60 and 35 kd in HFV-infected cells (Figure 2A, lane 6). *Bel-1*-related proteins displaying these molecular weights had been previously identified as representing the *bel-1* transactivator proteins and *bet*, a fusion protein of unknown function.[27] Identical results were obtained with rabbit anti-*bel-1* sera (data not shown).

To demonstrate the reactivity of the *env* antibody we used the RIPA methodology (Figure 2B) because we were not able to stain the viral glycoproteins with *env*-specific serum or serum of infected humans and monkeys in WB (data not shown). Viral glycoproteins of 47, 130, and 160 kd have been described previously and were suggested to represent the transmembrane protein, the surface protein, and the *env* polyprotein precursor, respectively.[6] However, the former study was performed without the use of specific antisera raised against defined portions of the *env* polypeptide. Precipitation of a 130 kd molecule by our *env* antiserum generated against the transmembrane portion of the *env* protein (Figure 2B) indicates that gp130 represents the *env* precursor protein. The lack of precipitation of the mature 47 kd transmembrane protein by the *env* antiserum is likely due to minute amounts of the cleaved *env* proteins in infected cells.

Characterization of Antisera to HFV Proteins by Immunohistochemistry

The salient features of the transgenic mouse families analyzed in this report have been described in detail elsewhere.[13,16] The pHFV$_{Af}$ transgene encodes the entire HFV genome, but its ability to generate infectious retroviral particles is disabled by a frameshift mutation in the *pol* reading frame. Two additional large deletions in the *gag-pol* and *env* regions were introduced in the pΔgpe construct (Figure 1). Consequently, mice harboring the pHFV$_{Af}$ transgene are able to express all full-length genes of HFV, with the exception of the retroviral endonuclease (integrase), whereas pΔgpe transgenic mice can express only truncated *gag* and *env* fragments of 382 and 191 amino acids, respectively, in addition to the genes contained in the *bel* region. Immunohistochemical studies and *in situ* hybridizations were performed on tissues from 46 transgenic mice (founders and their heterozygous and homozygous offspring) originating from seven independently microinjected zygotes (three pHFV$_{Af}$ and four pΔgpe). Partially purified rabbit antisera were tested on sections of transgenic brain tissues known to yield a signal when hybridized to a HFV riboprobe (as a positive control) and to brain sections of nontransgenic C57Bl/6 mice (as a negative control). Various dilutions of the antisera were applied as summarized in Table 1.

While a positive signal was never detected in wild-type mouse tissues, abundant immunoreactive material for antisera 122 (*gag1*), 161, and 162 (*bel-1*) was demonstrated on sections of pΔgpe transgenic mice. The brain of pHFV$_{Af}$ mice displayed additional immunoreactivity for antisera 127 (*gag2*) and 124 (*tm1*). Optimal signal-to-noise ratios were obtained with dilutions of the purified, 5× concentrated antisera ranging between 1:100 and 1:500 (Table 1). In contrast, antiserum 123 raised against the reverse transcriptase never yielded a positive reaction on paraffin-embedded tissues, despite its reactivity in RIPA.

HFV Immunoreactive Material Localizes to Neurons and Glial Cells in the Brain of Transgenic Mice

In all mice containing the pΔgpe construct the main cell type with accumulation of HFV proteins was the

neurons in the CA3 sublayer of the hippocampus (Figure 3, A and B) and the pyramidal neurons of the cortex (Figure 3, C and D). No immunoreactivity was detected in the white matter of these animals. However, $pHFV_{Af}$ mice displayed additional immunoreactivity in a fraction of subcortically situated glial cells in both forebrain and cerebellum. This observation parallels our data on the expression of HFV mRNA in these regions.[13] Interestingly, immunoreactivity for *gag, env,* and *bel-1* was not confined to the perikaryon of neuronal cells, but extended into the axonal and dendritic processes of neurons (Figure 4, A and B).

Multifocal, somewhat diffusely staining deposits of immunoreactive material were observed in the cortex and subcortex (Figure 4A). Since no such deposits were observed in the brain of nontransgenic littermates upon immunostain with antisera 161 and 162 (data not shown), and no cortical immunostain was observed in the brain of transgenic mice when preimmune sera from rabbits 161 and 162 were used, we conclude that the deposits depicted in Figure 4A reflect the expression, and possibly the extracellular release, of transgenic *bel-1* protein.

In the brain of both $pHFV_{Af}$ and pΔgpe animals older than 6 weeks, glial fibrillary acidic protein (GFAP) immunostains demonstrated a marked cortical and subcortical astrogliosis that extended to areas devoid of neuronal degeneration. Typical reactive astrocytes with long delicate GFAP-positive processes represented the dominating cell type. Back-to-back figures suggestive of completed cell divisions were frequently detected among astrocytes.

Comparison of HFV in in Situ *Hybridization and Immunohistochemistry*

We have visualized transcription and translation of HFV genes by in situ hybridization and immunohistochemistry on consecutive serial sections of the brain of $pHFV_{Af}$ and pΔgpe mice. Quantitation was

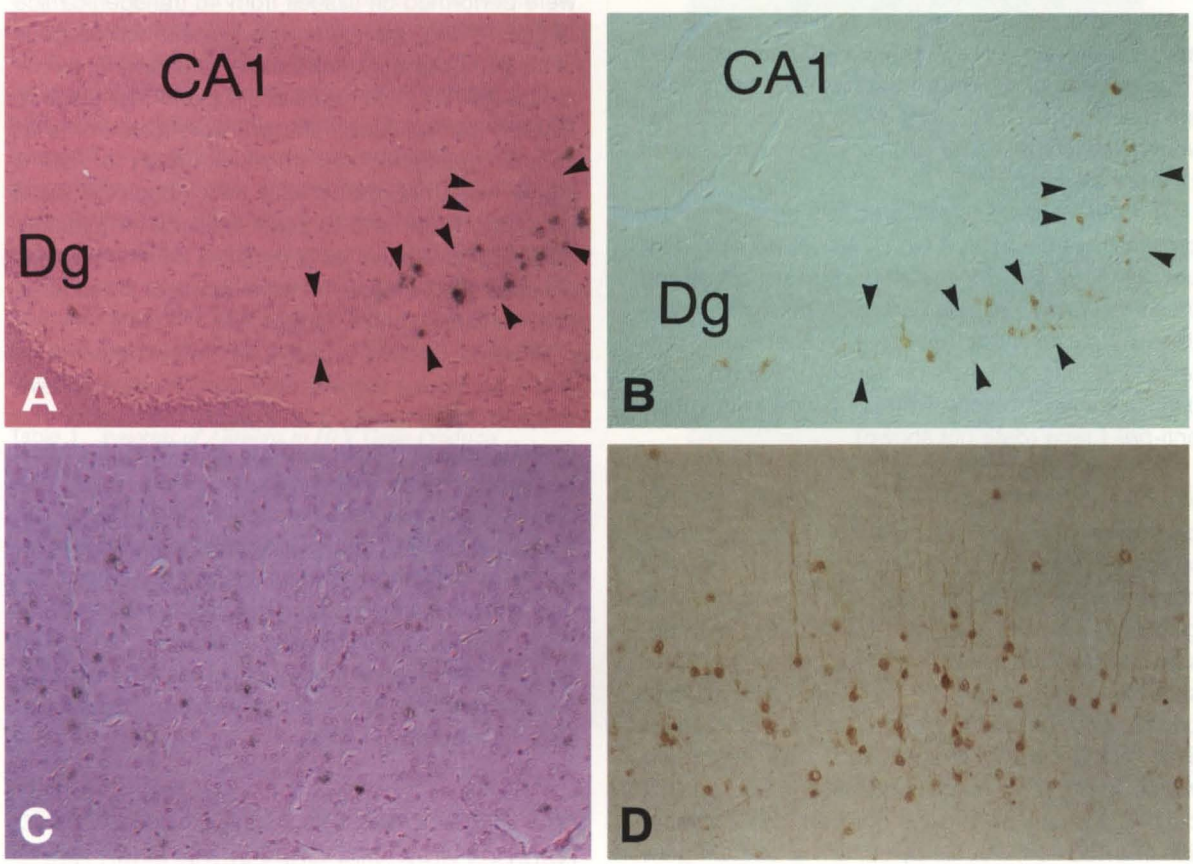

Figure 3. *Consecutive serial sections of the brain of a pΔgpe transgenic mouse at the age of 5 weeks* (A *and* B, *hippocampus;* C *and* D, *parietal cortex). Arrowheads mark the CA3-CA2 region in the hippocampus. Neurological symptoms were not yet apparent at this stage. The micrographs on the left* (A *and* C) *depict* in situ *hybridizations with the pGem4bel RNA probe, whereas those on the right side* (B *and* D) *show immunohistochemical stains with antiserum 122* (gag1). *The similarity in distribution and number of cells containing mRNA and HFV proteins indicates that these two methods detect positive cells in HFV transgenic mice with comparable sensitivity.* ×100.

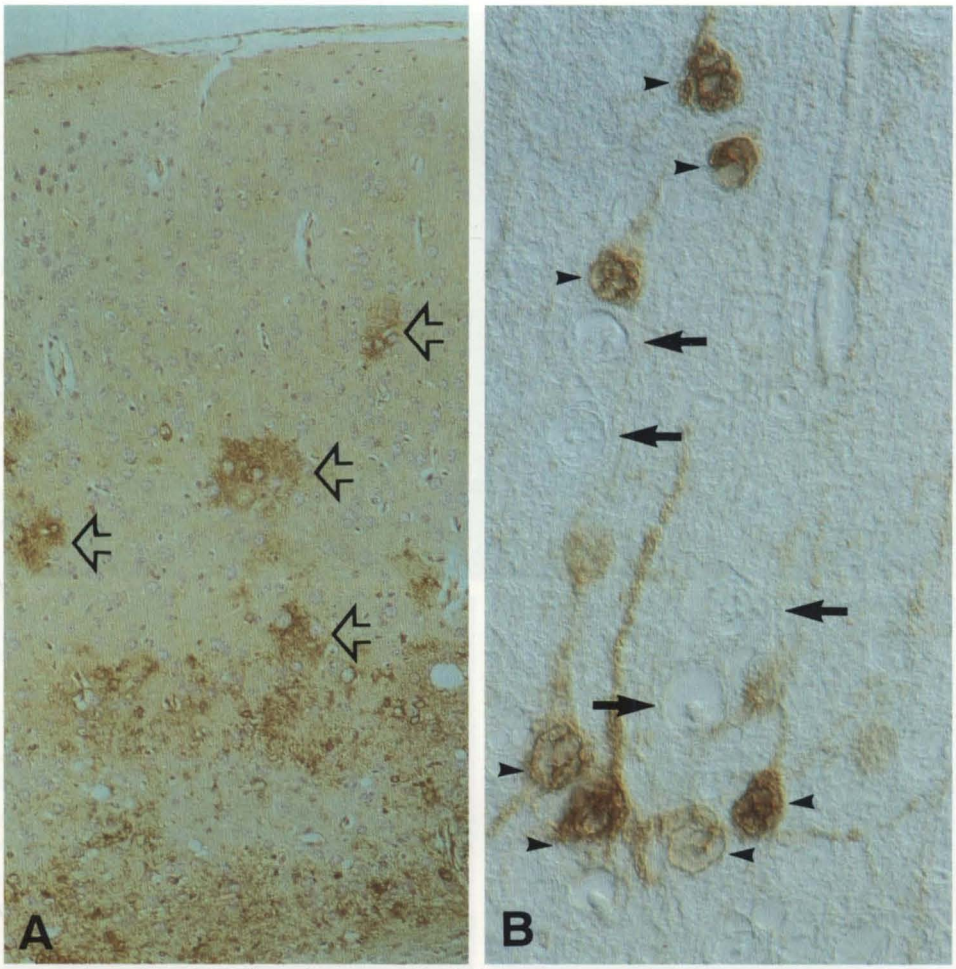

Figure 4. *Immunohistochemical stains for* bel-1 *with antiserum 161.* A, *low-power micrograph demonstrating focal reticular deposits of the* bel-1 *transactivator protein within the molecular layer of the cerebral cortex (open arrows). The patchy, somewhat diffuse pattern of immunoreactivity suggests that deposits of* bel-1 *accumulate at synaptic junctions and/or are released in the extracellular space. ×50.* B *high-power micrograph displaying* bel-1 *immunoreactive material in nucleus, perikaryon, and processes of cortical neurons. ×630. Note the highly irregular distribution of immunoreactivity with strongly immunoreactive (arrowheads) and negative neurons (arrows) in close spatial proximity.*

achieved by counting the total number and the number of positive pyramidal neurons in the hippocampus and in the parietal cortex with both detection methods. Neurons were regarded as positive for HFV mRNA if the number of silver grains situated on the perikaryon exceeded the background counts by a factor of 5 or more on emulsion-coated, hybridized slides. In immunohistochemical stains, only neurons displaying a strong signal (Figures 3 and 4) were considered to be positive for HFV proteins. Identification of HFV gene products with antisera to *gag1* and *bel-1* in all mice, and additionally of *gag2* and *tm1* in $pHFV_{Af}$ mice, correlated closely with the number of cells containing HFV mRNA. In a typical series of hippocampal sections from a 5-week-old pΔgpe transgenic mouse, 21 and 25 positive cells among 501 hippocampal pyramidal neurons were identified by *in situ* hybridization and *gag1* immunohistochemistry, respectively (Figure 3, A and B). In the parietal cerebral cortex of the same animal, 58 and 75 cells scored positively for HFV mRNA and *gag1* immunohistochemistry from a total of 704 neurons (Figure 3, C and D).

Multinucleated Giant Cells and Atypical Astrocytes in the Brain of $pHFV_{Af}$ Transgenic Mice

Two additional cell types were found in the brains of clinically symptomatic $pHFV_{Af}$ transgenic mice, the first consisting of atypical, large multinucleated cells located most often in the hilar and parahippocampal regions (Figure 5A). These cells will henceforth be called multinucleated giant cells (MGC). The shape of these cells was round, and their diameter was often in excess of 50 μm. They contained 5 to 10 nuclei embedded in an abundant cytoplasm. Nuclear morphology was similar to that of neighboring activated astrocytes: the multiple nuclei were

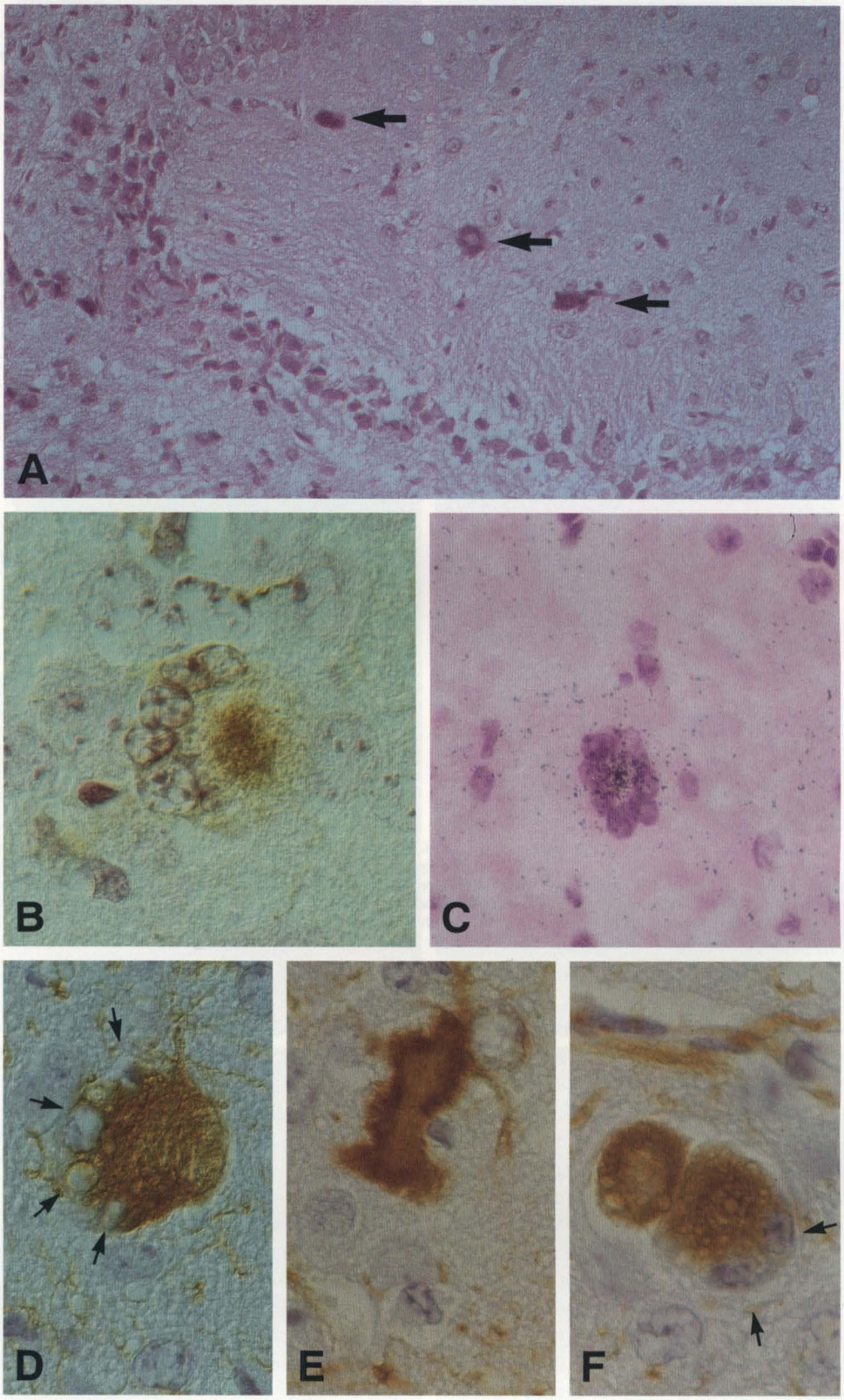

Figure 5. *Multinucleated giant cells in the brain of pHFV$_{Af}$ transgenic mice.* **A**, *micrograph of the hilar region of the hippocampus showing one multinucleated and two bizarrely shaped giant cells. H&E, ×200.* **B**, *gag1 immunostain of a multinucleated giant cell.* **C**, in situ *hybridization with the same probe as in Figure 3, showing transgenic HFV mRNA in the cytoplasm of MGC.* **D–F**, *immunostain for glial fibrillary acidic protein of MGC* (**D**) *and of bizarre, mono-, and binucleated cells (×400). These latter cells are essentially devoid of processes and may represent precursors of MGC. Multiple nuclei are highlighted by arrows.*

substantially isomorphic and contained finely distributed, occasionally peripheral, and somewhat condensed chromatin, and were arranged in proximity to the plasma membrane in a horseshoe-like fashion reminiscent of Langhans giant cells. Mitotic figures or dyskaryosis were never observed in these cells, suggesting that MGC originated through syncytial fusion rather than endomitosis. In contrast to the widespread occurrence of the astrogliosis, MGC were strictly confined to the areas of telencephalon displaying histological lesions; they were not found in the brainstem or in the cerebellum. MGC were also not observed extracranially in mice showing expression of HFV in skeletal and smooth muscle.

Immunostains of MGC with antisera to HFV revealed that they contained *gag*, *env*, and *bel-1*, whereas none of these gene products were detectable in the more typical, reactive astroglial compartment. *gag* proteins tended to accumulate in the central part of the cytoplasm of MGC (Figure 5B), whereas *env* and *bel-1* were more homogeneously distributed. Cytoplasmic HFV mRNA was readily detectable by *in situ* hybridization analysis in MGC (Figure 5C).

Immunostains for GFAP revealed strong immunoreactivity of MGC for this marker protein, implying that they derive from astrocytes. However, the GFAP immunostaining pattern was coarsely vacuolar and differed markedly from that of neighboring reactive astrocytes (Figure 5D). No immunoreactivity was detected in MGC by using antibodies to leukocyte common antigen/CD45 or to synaptophysin (data not shown).

We also identified atypical large cells devoid of processes in brain sections of the same $pHFV_{Af}$ transgenic mice, which stained strongly with the GFAP antiserum (Figure 5, E and F). The appearance of such cells was often globular, but occasionally very irregular shapes were encountered (Figure 5E). The occurrence of intermediate, occasionally binucleated forms (Figure 5F) suggests that these may represent MGC precursors. Although the morphological characteristics of such cells profoundly differed from those of neighboring reactive astrocytes and were more reminiscent of neoplastic astroglia, nuclear atypias and mitotic figures were never detected.

Discussion

The specificity of the antisera described in this manuscript has been verified in a RIPA assay and by comparing the results of immunohistochemical stains to those of *in situ* hybridization experiments. Northern hybridization analyses of pΔgpe transgenic mice had previously demonstrated the presence of two mRNA transcripts of 7.1 and 3.8 kb, and a prominent group of transcripts of 1.8–2.5 kb.[17] These transcript lengths are consistent with an unspliced genomic mRNA enabling translation of a truncated *gag* product, a spliced *env* transcript, and a family of shorter transcripts, presumably resulting in expression of the *bel* genes, in analogy to the splicing pattern of replication-competent HFV.[9] Interestingly, all cells containing HFV mRNA were found to express structural gene products and *bel-1* simultaneously, and no evidence of differential gene expression during the course of the disease was detected. This may suggest that HFV, in contrast to human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV-I), could lack a *rev*-like function controlling the splicing pattern during the retroviral life cycle (for a review, see ref. 28).

HFV Proteins Are Detectable in Nerve Cell Processes

The detection of uniform, strong immunoreactivity for *env* and *gag* in nerve cell processes over several hundreds of microns indicates that transport of these proteins to the neuropil is very efficient. Particularly surprising is the finding that *bel-1* immunoreactivity was also found to be localized predominantly in the cytoplasm and in the processes of affected neurons rather than in their nuclei. Although the details of *bel-1* action are poorly understood, its *trans*-activating function[8] demands its presence in the nucleus. Indeed localization, of *bel-1* is predominantly nuclear in transiently transfected cultured cells,[29] but during the early phases of the retroviral life cycle *bel-1* was identified in the cytoplasm of infected fibroblasts.[27] Together with these data, our observations suggest that subcellular localization of *bel-1* may undergo cell-cycle–dependent shifts. Alternatively, extranuclear immunoreactivity to the *bel*-1 antiserum may be due to the formation of compound proteins that may be partially homologous to *bel-1*, such as *bet, bes,* and the putative 170kD *env-bel-1* fusion protein. In fact, antisera 161 and 162, although raised to recombinant *bel-1,* coprecipitated several additional proteins of various molecular weights in a RIPA assay.

The atypical cellular localization of *bel-1* in transgenic mice may be related to the pathological processes observed: the spotty pattern of HFV immunoreactivity and its localization in the neuropil suggest that *bel-1* may influence neighboring neurons in a cell-autonomous fashion. It was not possible to gather direct evidence for this possibility *in*

vivo, but the antisera described in the present work should help to clarify this issue in organotypic neuronal cultures.[30]

The antiserum raised against the reverse transcriptase domain of the *prot* and *pol* reading frames did not yield positive immunohistochemical reactions, although this reagent was shown to contain specific antibodies in a RIPA assay. It is possible that the epitopes recognized by this serum are not paraffin-stable or, alternatively, the concentration of *pol* gene products *in vivo* may be too low for immunohistochemical detection.

Syncytiogenic Activity of HFV

Induction of syncytia in infected cells is a property shared by disparate enveloped viruses including complex retroviruses (*e.g.*, HIV, SIVmac, and HTLV-I), paramyxoviruses (measles virus and respiratory syncytial virus), and visna virus. To date HIV is by far the best characterized fusogenic retrovirus, and a large body of information has been gathered on mechanisms and the biological significance of its syncytium-forming potential. *In vitro* induction of syncytia in lymphoid cell lines by HIV closely correlates with the spreading of retroviral infection and its cytotoxic potential and has been shown to require the action of viral *env*-derived proteins.[31,32] Specifically, a conserved undecapeptide has been identified to be shared by HIV-1 and HIV-2, paramyxoviruses, Visna, and several simian retroviruses.[27] While syncytiogenic activity of HFV proteins is made likely by their immunohistochemical detection in the cytoplasm of MGC, the demonstration of transgenic HFV mRNA within MGC argues that immunoreactivity results from endogenous synthesis of HFV proteins in these cells rather than from neuronophagia of HFV-expressing cells or from uptake of extracellular protein.

By analogy to HIV and HTLV-I, the HFV *env* gene product is the most likely candidate responsible for this activity. This view is supported by the fact that MGC were only observed in mice transgenic for the $pHFV_{Af}$ construct, which is a full-length viral construct allowing expression of the intact *env* gene, but not in pΔgpe transgenic mice. Furthermore, a potential fusogenic sequence has been mapped to the HFV transmembrane protein.[2]

A critical role in HIV-mediated cell fusion is attributed to host proteins, the presence of which is required in addition to HIV-*env*. Domains have been identified within CD4,[33,34] intercellular adhesion molecule 1,[35] and LFA-1[36], the deletion of which abrogates the fusogenic potential. Although such host factors have not been identified in HFV-induced fusion, our observations suggest that similar interactions may be important. Syncytia in HFV transgenic mice were invariably composed of glial cells expressing GFAP, whereas neuronal fusions were not observed, despite the much higher proportion of neurons expressing HFV. Therefore, one could speculate that presumptive cellular partner molecules may be present *in vivo* only in astrocytes. It will be of interest to search for MGC in the brain of experimental animals infected with HFV, since *in vitro* studies have revealed that HFV preferentially replicates in cell lines of astroglial origin.[4]

Although the prominent fusogenic properties of HFV in cultured cells have been recognized for a long time, and indeed foamy viruses have been referred to in the past as "syncytial viruses,"[10,11,37] formation of MGC *in vivo* was not observed in infected patients or experimental animals. Also, MGC were not observed in transgenic mice expressing genes of HIV[38] and HTLV-I.[39–41] Therefore, this feature makes HFV transgenic mice an attractive *in vivo* model system in which syncytiogenic activity of human retroviruses can be studied.

MGC of monocytic origin are an important diagnostic hallmark of HIV-associated giant cell encephalitis (GCE).[42,43] Although at first glance our findings bear striking similarities to the histopathology of GCE, the resemblance is rather superficial: MGC in GCE essentially originate from hematopoietic cells expressing the CD4 receptor. In contrast, the presence of GFAP and the absence of immunoreactivity for leukocyte common antigen and synaptophysin argue that HFV-associated MGC derive from the astrocytic lineage.

The finding that antibodies inhibiting syncytia formation are of prognostic significance in HIV-seropositive children lends support to the hypothesis that *in vivo* fusogenic activity may play an important role in HIV pathogenicity.[44] In the case of HFV transgenic mice the relevance of MGC to the course of the disease is not yet clear, since we have observed neurodegenerative pathologies, also in the absence of MGC. However, mice expressing full-length structural genes harbor MGC in the brain lesions and have a markedly reduced life expectancy as compared to pΔgpe mice, which do not develop MGC.[16] Therefore, while MGC are not a necessary factor in the pathogenesis of the described neurodegenerative disease, the strong correlation between MGC and particularly severe neuroglial damage suggests that they may contribute to the HFV-induced neurological disease.

Possible Application in Research and Diagnostics

The present study also shows that our antisera recognize epitopes that are not destroyed by formaldehyde fixation and paraffin embedding, and that the sensitivity of the immunohistochemical reaction using a partially purified immunoglobulin fraction is comparable to that of radioactive *in situ* hybridization. These useful properties have enabled us to characterize the pathology developing in HFV transgenic mice more systematically and to advance pathogenetic hypotheses which can now be approached in further molecular and morphological studies. Even more importantly, the above-mentioned characteristics of the antisera described here make them suitable reagents for archival and prospective studies on human material. In view of the lack of knowledge about variability of HFV *in natura*, it is conceivable that antisera to potentially conserved, functionally important domains of HFV proteins may prove more sensitive than *in situ* hybridization with ribonucleic acid probes. For these reasons we believe that these reagents will provide histopathologists and virologists with a further useful tool for evaluating the pathogenic potential of natural HFV infection in humans.

Acknowledgments

We are indebted to Norma Howells for maintaining the rabbit colony, to Manuela Kranz for technical assistance, and to Friedemann Kiefer and Otmar Wiestler for discussions, suggestions, and critical reading of the manuscript.

References

1. Achong BG, Mansell PW, Epstein MA, Clifford P: An unusual virus in cultures from a human nasopharyngeal carcinoma. J Natl Cancer Inst 1971, 46:299–307
2. Flügel RM, Rethwilm A, Maurer B, Darai G: Nucleotide sequence analysis of the *env* gene and its flanking regions of the human spumaretrovirus reveals two novel genes. EMBO J 1987, 6:2077–2084
3. Rethwilm A, Darai G, Rosen A, Maurer B, Flügel RM: Molecular cloning of the genome of human spumaretrovirus. Gene 1987, 59:19–28
4. Rethwilm A, Baunach G, Netzer KO, Maurer B, Borisch B, ter Meulen V: Infectious DNA of the human spumaretrovirus. Nucleic Acids Res 1990, 18:733–738
5. Maurer B, Serfling E, ter Meulen V, Rethwilm A: Transcription factor AP-1 modulates the activity of the human foamy virus long terminal repeat. J Virol 1991, 65: 6353–6357
6. Netzer KO, Rethwilm A, Maurer B, ter Meulen V: Identification of the major immunogenic structural proteins of human foamy virus. J Gen Virol 1990, 71:1237–1241
7. Rethwilm A, Mori K, Maurer B, ter Meulen V: Transacting transcriptional activation of human spumaretrovirus LTR in infected cells. Virology 1990, 175:568–571
8. Rethwilm A, Erlwein O, Baunach G, Maurer B, ter Meulen V: The transcriptional transactivator of human foamy virus maps to the bel 1 genomic region. Proc Natl Acad Sci USA 1991, 88:941–945
9. Muranyi W, Flügel RM: Analysis of splicing patterns of human spumaretrovirus by polymerase chain reaction reveals complex RNA structures. J Virol 1991, 65:727–735
10. Loh PC, Matsuura F, Mizumoto C: Seroepidemiology of human syncytial virus: antibody prevalence in the Pacific. Intervirology 1980, 13:87–90
11. Muller HK, Ball G, Epstein MA, Achong BG, Lenoir G, Levin A: The prevalence of naturally occurring antibodies to human syncytial virus in East African populations. J Gen Virol 1980, 47:399–406
12. Mahnke C, Kashaiya P, Rössler J, Bannert H, Levin A, Blattner WA, Dietrich M, Luande J, Löchelt M, Friedman-Kien A, Komaroff AL, Loh PC, Westarp ME, Flügel RM: Human spumavirus antibodies in sera from African patients. Arch Virol 1992, 123:243–253
13. Aguzzi A, Bothe K, Wagner EF, Rethwilm A, Horak I: Human foamy virus: an underestimated neuropathogen? Brain Pathol 1992, 2:61–69
14. Werner J, Gelderblom HR: Isolation of foamy virus from patients with de Quervain thyroiditis. Lancet 1979, 2:258–259
15. Wagner EF: On transferring genes into stem cells and mice. EMBO J 1990, 9:3025–3032
16. Bothe K, Aguzzi A, Lassmann H, Rethwilm A, Horak I: Progressive encephalopathy and myopathy in transgenic mice expressing human foamy virus genes. Science 1991, 253:555–557
17. Aguzzi A, Bothe K, Horak I, Rethwilm A, Anhauser I, Wagner EF: Developmental modulation of human foamy virus expression. New Biol 1992, 4:225–237
18. Ellinger S, Glockshuber R, Jahn G, Pluckthun A: Cleavage of procaryotically expressed human immunodeficiency virus fusion proteins by factor Xa and application in western blot (immunoblot) assays. J Clin Microbiol 1989, 27:971–976
19. Harlow E, Lane D: Antibodies. Cold Spring Harbor, NY, CSH Press, 1988.
20. Henderson C: Aminoalkylsilane: an inexpensive, simple preparation for slide adhesion. J Histotechnol 1990, 12:123–124
21. Aguzzi A, Wagner EF, Williams RL, Courtneidge SA: Sympathetic hyperplasia and neuroblastomas in transgenic mice expressing polyoma middle T antigen. New Biologist 1990, 2:533–543
22. Aguzzi A, Kleihues P, Heckl K, Wiestler OD: Cell-type specific tumor induction by oncogenes in fetal forebrains transplants. Oncogene 1991, 6:113–118