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Isolation of a New Foamy Retrovirus from Orangutans

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We have isolated a new foamy virus from blood samples taken from two apparently healthy orangutans (*Pongo pygmaeus*). The older orangutan has since died with encephalopathy after a brief acute illness, while the younger one, his grandson, remains well. These animals and 12 other orangutans had specific antibodies to foamy virus as measured by immunofluorescence. The new foamy virus and the antisera showed strong and specific neutralization, with only weak cross-reaction with other simian foamy virus strains. Southern blotting with *gag* and *env* probes of human foamy virus and PCR amplification showed that the new foamy virus, designated SFV-11, is related to, yet distinct from, previously characterized strains from humans, chimpanzees, and monkeys.

Spumaviruses, or foamy viruses, constitute one of three retrovirus subfamilies, the other two being the lentiviruses (e.g., human immunodeficiency virus) and the oncoviruses (e.g., human T-cell leukemia virus [HTLV]) (37). Foamy viruses cause persistent infections in nonhuman primates, cattle, cats, hamsters, and, possibly, humans (12). Serological surveys indicate that the prevalence of foamy virus in human populations is low, although it may be higher in certain geographical areas (1, 19, 23). The first human isolate, derived from an African patient with nasopharyngeal carcinoma (2), has been cloned and is well characterized (9, 21, 22, 29). The second was isolated from the brain of a British patient suffering from a neurological disease of unknown etiology who died with subacute encephalopathy after dialysis for chronic kidney failure (6). In the absence of a definitive diagnosis, the encephalopathy was attributed to aluminum intoxication, a not uncommon side effect of hemodialysis in 1978, but this was not confirmed postmortem (3). Foamy viruses have also been isolated from patients with de Quervain subacute thyroiditis (34, 38) and Graves' disease (17). The association of these viruses with human disease, however, remains controversial (36). The pathogenic potential of foamy viruses in animals is also unclear. Infected primates have not been reported with associated illness, although there is a report of immunosuppression in rabbits inoculated with a simian foamy virus (SFV), SFV-7 (11). It seems that foamy viruses, which are highly cytopathic in cell culture, establish an asymptomatic infection in the host and persist for long periods in the presence of neutralizing antibody.

SFVs have been isolated from a number of monkeys and great apes, including chimpanzees, but have not been isolated previously from orangutans. In this study, we report the isolation and preliminary characterization of a new foamy virus from peripheral blood samples taken from two orangutans (*Pongo pygmaeus*). Other captive orangutans tested were seropositive for this foamy virus, designated SFV-11.

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MATERIALS AND METHODS

Cells and cell lines. The following human T-cell lines were used: C8166, a subclone of CR63/$CR_1$1-4 cells (7, 31); the H9 clone of Hut 78 cells (27); and Sup-T1 cells (33). The other human cells used were Raji cells (B cells) (8), rhabdomyosarcoma cells (RD/TE671) (35), and the embryonic lung cell lines HEL (HEL 299) (26) and MRC-5 (14). Vero (African green monkey kidney) and BHK-21 (baby hamster kidney) clone 13 cells were also used.

Nonadherent cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS); adherent cells were maintained in Dulbecco's modified Eagle's medium with 5% FCS (10% FCS for HEL and MRC-5 cells).

Peripheral blood mononuclear cells (PBMCs) from orangutans were derived from a 3-ml fresh blood sample collected in preservative-free heparin and fractionated by density gradient centrifugation (2,000 × *g* for 40 min at 4°C) on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Cells, initially stimulated with phytohemagglutinin (1 μg/ml) were maintained in RPMI 1640 medium containing 20% FCS and recombinant interleukin-2 (100 U/ml; Biogen).

Viruses. Viruses (human foamy virus [HFV] and SFV-1, SFV-2, SFV-3, SFV-4, SFV-6, and SFV-11) were propagated on either BHK-21 cells or HEL cells and harvested either from culture supernatants or from twice-freeze-thawed cells. Virus stocks were filtered (Nalgene; 0.45-μm pore diameter) and stored in liquid nitrogen.

Virus isolation. Virus isolation was carried out by cocultivation of the PBMCs (2×10^5 in a volume of 0.5 ml) with an equal number and volume of indicator cells (human cells and Vero cells). Cultures were checked daily for multinucleated giant cell formation (syncytia) or other cytopathic effects (CPEs). Once infection was established in Sup-T1, HEL, and BHK-21 cell lines, the virus titer in the supernatant did not exceed 7×10^3. This was determined by CPE in the cultures after serial 10-fold dilutions of the infected cell supernatant on uninfected cells. Detection of virus in the cultures was con-

firmed both by electron microscopy (EM) and by detection of reverse transcriptase (RT) activity in the culture supernatant collected at twice-weekly intervals after cocultivation with the indicator cells.

Neutralization assay. Serum neutralizing activity was titrated either as described in the table legends or as follows. Infected cell supernatant containing 10^3 infectious units of foamy virus was incubated for 1 h at 37°C with serial dilutions of orangutan sera before addition of 10^5 Sup-T1 cells. The appearance of CPE was scored up to 10 days postinfection. Neutralization titers are expressed as the reciprocal of the serum dilution responsible for >90% reduction in the CPE.

Sera tested for neutralizing activity against the orangutan foamy virus included homologous antisera taken from the index animal, Dodo, and from his grandson, Kadim, as well as stored sera taken from a variety of orangutans who had been housed at the London Zoo in the past. Three serum samples came from the Zurich Zoo, taken from orangutans who had never been kept at the London Zoo but who had had contact with other London-bred orangutans.

Specific neutralizing antisera raised in horses or rabbits or taken from primates naturally infected with other known foamy viruses, both HFV and SFV, were also assayed for their capacity to neutralize the orangutan virus.

Immunofluorescence microscopy. Dilutions of orangutan antisera were incubated for 30 min at room temperature with 5×10^5 Sup-T1 cells or Sup-T1 cells productively infected with SFV-11. After two washes in phosphate-buffered saline (PBS) containing 0.02% sodium azide and 1% FCS, the cells were further incubated for 30 min in fluorescein isothiocyanate conjugated to goat anti-human immunoglobulin G, which cross-reacts with orangutan immunoglobulin G. Cells were then examined for surface fluorescein isothiocyanate staining by fluorescence microscopy.

EM. (i) Thin-section EM. Infected cells were centrifuged for 10 min at $1,000 \times g$ and the pellet was fixed in 2.5% glutaraldehyde in PBS for 60 min. After being washed in PBS, the cell pellet was postfixed in 1% osmium tetroxide for 60 min, washed in distilled water, dehydrated in ethanol, and embedded in Agar 100 resin (Agar Aids, Stansted, United Kingdom). Sections were stained with uranyl acetate and lead citrate and examined at 60 kV.

(ii) Negative staining EM. Pelleted infected cells were suspended in distilled water. A carbon-coated grid was placed on a drop of cell suspension for 2 min, washed on a drop of distilled water, and stained for 10 s on a drop of either 3% phosphotungstic acid (pH 6.5) or 4% ammonium molybdenate (pH 6.0). The grid was air dried and examined at 80 kV.

(iii) Immune EM. Infected cells were pelleted at $1,000 \times g$ for 10 min, and the pellet was resuspended vigorously in distilled water. The cells were repelleted, and the supernatant was centrifuged for 30 min at $100,000 \times g$. The pellet was resuspended in PBS, and 0.2 ml of this viral suspension was incubated for 60 min at 37°C with an equal volume of homologous orangutan antiserum diluted 1:50 in PBS. After centrifugation at $20,000 \times g$ for 30 min, the pellet was resuspended in distilled water, negatively stained with phosphotungstic acid, and examined for agglutination of virus particles.

RT assay. Supernatant taken from cell cultures was centrifuged for 1 h at $40,000 \times g$, and the pellet was resuspended in 100 μl of 50 mM Tris buffer (pH 7.8) containing NaCl (100 mM), dithiothreitol (1 mM), Triton X-100 (0.5%), and glycerol (20%). RT activity was assayed in duplicate by the method of Liu et al. (18), with poly(rA) · oligo$(dT)_{12-18}$ as a template primer and poly(dA) · oligo$(dT)_{12-18}$ as a control for cellular DNA polymerase activity. The results shown are means of duplicate samples in counts per minute minus the poly(dA) background (routinely <1,000 cpm).

Southern blot. Total DNA was extracted from Sup-T1 cells infected with the orangutan virus SFV-11, rhabdomyosarcoma cells infected with HFV, and uninfected cells. The DNA was digested under standard conditions with *Eco*RI, *Hin*dIII, *Bam*HI, and *Bgl*II, and the fragments were separated on a 0.8% agarose gel. After transfer to GeneScreen membranes, the blots were probed with two probes derived from the HFV genomic sequence: a *gag* probe (*Eco*RV-*Hpa*I fragment, positions 1480 to 3394) and an HFV *env* probe (*Eco*RI-*Eco*RI, positions 6037 to 9527) (9, 21) under conditions of low stringency (30% formamide at 52°C). The blots were then washed at low and high stringency as detailed in the legend for Fig. 2.

PCR. DNA was prepared from cultured cells or homogenized tissue samples by standard methods and amplified with primers directed to areas of the HFV long terminal repeat that are well conserved in SFV-1 and SFV-3. Virus isolate samples were amplified for 30 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 60 s) by using PBF1 and PBF2 (CACTACTCGCTGCGTC GAGAGTGT, positions 786 to 809; GGAATTTTGTATAT TGATTATCC, positions 1115 to 1093), and the products were analyzed directly on agarose gels.

DNA from tissue samples was amplified for 40 cycles under the same conditions, and 2 μl of product was transferred to a fresh reaction mixture and amplified with PBF2 and PBF3 (AAAGGATTTGTATATTAGCCTTGCT, positions 1006 to 1031) for a further 30 cycles before analysis of the final products on agarose gels.

Sequencing and alignment. PCR-amplified products were diluted and subcloned into a plasmid vector according to the manufacturer's instructions (TA cloning kit; Invitrogen). The nucleotide sequence was determined by the dideoxynucleotide chain termination method, and alignments were performed with the NALIGN and CLUSTAL programs (Intelligenetics).

RESULTS

We screened, for evidence of retroviral infection, heparinized blood samples taken from primates at London Zoo when the animals were admitted to the zoo hospital for routine health assessment or veterinary care. In short-term cultures of phytohemagglutinin-stimulated PBMCs fractionated on a Ficoll-Paque density gradient, giant cell formation was observed in the orangutan PBMCs. This CPE was transmissible by cocultivation of the PBMCs with a variety of cell lines, including African green monkey kidney cells (Vero), Syrian hamster BHK cells, human T cells (Sup-T1, H9, and C8166), B cells (Raji), and embryonic lung fibroblasts (HEL and MRC-5). The end point titers ($10^{3.5}$) and RT activity (about 10,000 cpm/ml) of cell-free virus were highest in Sup-T1, HEL, MRC-5, and BHK cells. The progress of the CPE over time on experimentally infected cells was dependent on the viral inoculum. For example, 10^3 infectious units produced syncytia in Sup-T1 cells within 3 days and completely destroyed the culture within 8 to 10 days. Thin-section (Fig. 1A) and negative stain (Fig. 1B) EM of infected cells revealed the presence of virions with typical foamy virus morphology. These virions, 100 to 150 nm in diameter, consisted of a central core and a fringed envelope with projections 15 nm in length and 15 nm apart (Fig. 1C). Cores were observed singly or in groups in the cytoplasm (Fig. 1A, insert). Budding occurred at the cell surface, often from microvilli, with entire cores acquiring the fringed envelope as budding took place (Fig. 1C). Although

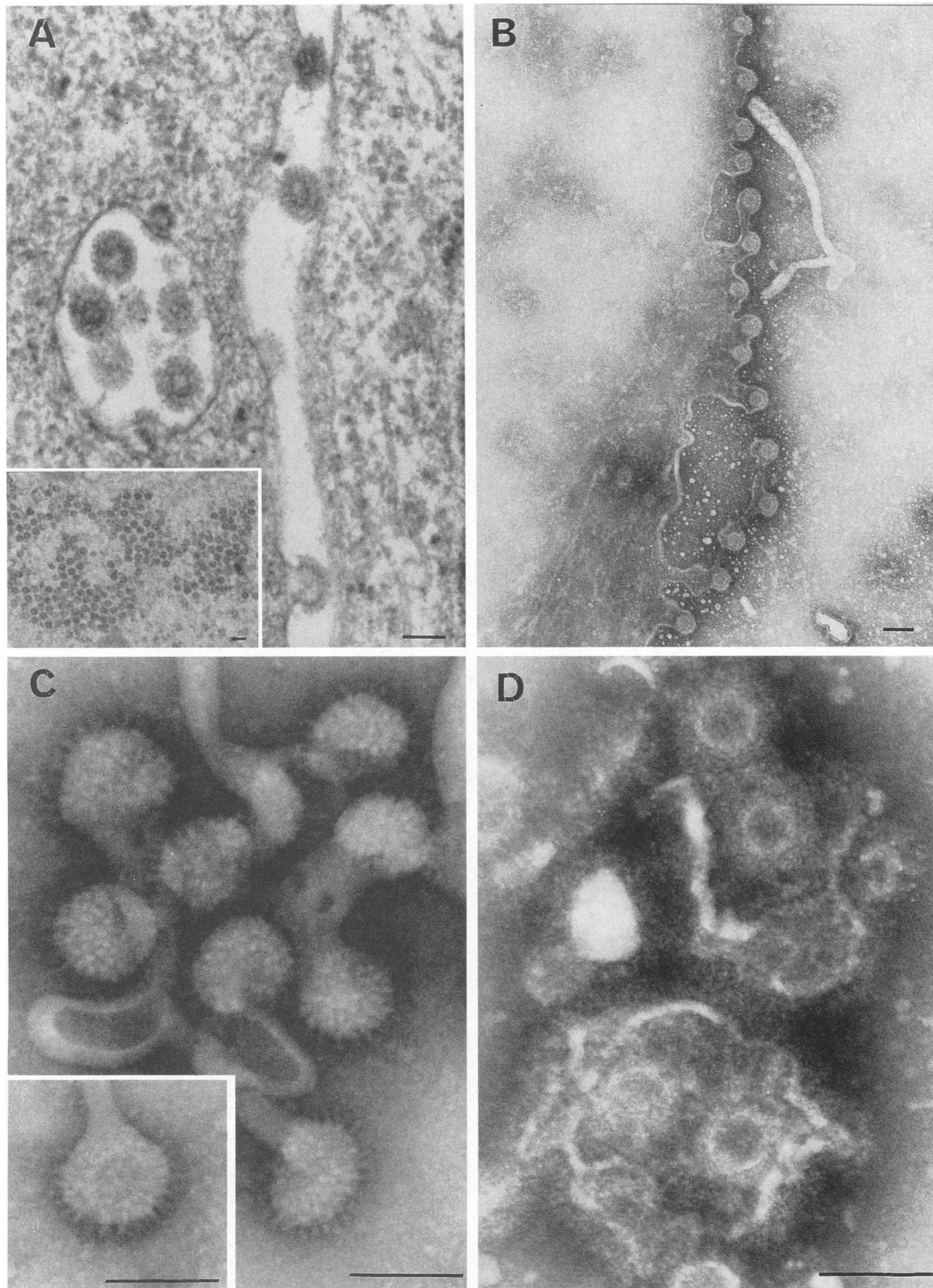

FIG. 1. Electron micrographs of SFV-11. Each bar represents 100 nm. (A) Thin section of infected Sup-T1 cells. Virus particles are seen to bud from intracellular as well as cell surface membranes. The budding particles have acquired the characteristic fringed envelope of foamy viruses. Virus particles are also present in large numbers in the cytoplasm (inset). (B) Negatively stained virus particles budding from the cell surface. Note that the cores are already condensed before budding is complete. (C) Negatively stained virions budding from microvilli. The envelope spikes are clearly visible, as is the inner core (inset), revealed by penetrating stain. (D) Immune EM of SFV-11 particles. Negatively stained particles, partially purified by differential centrifugation, were incubated with serum taken from the infected orangutan, Dodo, and are seen to clump. The coating of both envelope and cores is visualized.

TABLE 1. Captive orangutans have neutralizing antibody to foamy virus infection

Orangutan	Sex/yr of birth[a]	Antigen detection by immunofluorescence microscopy[b]	Neutralization titer[c]
Dodo[d]	M/1964		
1982		++	6,144
1987		+++	6,144
1988		+++	6,144
1991		+++	6,144
Bula[e]	F/1961	+	6,144
Azimat[d]	F/1968	+	3,072
Jantan[e]	M/1974	+	>6,144
Twiggy[d]	F/1962	++	3,072
Maria[d]	F/1967	++	1,536
Katie[d]	F/1964	+	3,072
Njonja[d]	F/1965	+/−	96
Amal[e]	M/1973	−	192
Martha[d]	F/1965	+	1,536
Blossom[d]	F/1964	++	3,072
Kadim[e]	M/1983	+++	6,144
Inianak[f]	F/1982	+++	ND
Jane[f]	F/1993	+++	ND
Pongo[f]	F/1961	+++	ND

[a] M, male; F, female.
[b] Orangutan sera were incubated with Sup-T1 cells infected with SFV-11 from Dodo, and cellular fluorescence was scored by eye as follows: +++, >50% of the cells are positive; ++, 5 to 10% of the cells are positive; +, <5% of the cells are positive; −, cells are negative.
[c] Inhibition of foamy virus CPE after a 7- to 10-day period is recorded as positive neutralization. Numbers shown are reciprocal serum dilutions at which CPE is inhibited 100%. ND, not determined.
[d] Animal caught in the wild for which the date of birth is approximate.
[e] Animal born at the London Zoo.
[f] Animal born at the Zurich Zoo.

TABLE 2. Foamy virus neutralization by homologous and orangutan sera

Virus	Neutralization by[a]:					
	SFV-1	SFV-2	SFV-3	SFV-4	SFV-6	SFV-11
SFV-1	864					32
SFV-2		7,776				96
SFV-3			288			32
SFV-4				864		32
SFV-6					7,776	32
SFV-11	32	32	32	32	32	23,328

[a] One hundred tissue culture infective doses of virus in 50 μl was mixed with an equal volume of threefold serum dilutions up to 1:23,328. After a 1-h incubation, this mixture was added to BHK cells (2,000 cells seeded in 96 wells the previous day) and cultured for 7 to 10 days. Cultures were passaged every 2 to 3 days by trypsinization and transfer of 1/10 of the cells to fresh wells. Values indicate the reciprocal of the highest dilution of antiserum able to inhibit CPE formation by 90%.

the majority of particles contained only one core, multicored virions, up to 300 nm in diameter, were frequently seen (Fig. 1D).

A second virus isolated from the grandson, Kadim, of the index animal, Dodo, had similar characteristics. Table 1 shows that serum samples from both animals reacted with both isolates in immunofluorescence microscopy assays specific for infected cells, as, indeed, did the serum samples from three orangutans housed at Zurich Zoo who had previous contact with the London animals. Serum samples from Dodo and Kadim as well as stored serum samples from orangutans previously housed at London Zoo were examined with immunofluorescence microscopy and neutralizing antibody assays. Most of these animals had high titers of neutralizing antibodies against the orangutan foamy virus (Table 1).

Table 2 shows the results of cross-neutralization assays between the new virus, SFV-11, and five previously characterized SFV strains. The five antiserum samples against SFVs (serotypes 1 to 4 and 6) neutralized the orangutan virus at a much lower titer (1/32) than did the homologous orangutan sera, and likewise, the orangutan virus poorly neutralized the other SFV serotypes. These findings indicate that the foamy virus isolated from the two orangutans belongs to a different neutralization group from previous isolates established from other species and for which virus and neutralizing antisera were available. The virus was designated SFV-11.

In order to confirm that these two virus isolates were indeed members of the foamy retrovirus subfamily and to explore the relationship between them and the prototype HFV isolate, we hybridized probes derived from the *gag* and *env* genes of HFV to restricted DNA extracted from cells infected with the two orangutan viruses under conditions of high and low stringency. The restriction patterns of the two orangutan viruses were identical (not shown) but were different from that of HFV. Figure 2 shows that the HFV *env* probe cross-hybridized with the orangutan virus DNA under conditions of high stringency, whereas the HFV *gag* probe did not. These results indicate that the *env* genes of SFV-11 and HFV are more closely related than their *gag* genes. This finding is consistent with the higher degree of conservation previously reported for the *env* genes of HFV, SFV-1 from a rhesus macaque (16), and SFV-3, the foamy virus isolated from an African green monkey (28). The diversity between the *gag* genes of the two orangutan viruses and HFV is further underlined by the lack of serological cross-reactivity of *gag*-derived bands on Western immunoblots (data not shown).

To carry out PCR, oligonucleotide primers were selected from regions of the HFV long terminal repeat that are well conserved in other foamy viruses for which the genomic sequences are known, namely, SFV-1 (16) and SFV-3 (28). An amplified product of approximately 310 bp was obtained from BHK cells infected with the orangutan virus (Fig. 3A). When similar experiments were performed with postmortem tissue samples, seminested amplification was necessary to obtain PCR products of approximately 108 bp (Fig. 3B). We found viral DNA in spinal cord, lymph node, spleen, and skeletal muscle tissue (Fig. 3), indicating the presence of viral genomes in those organs. Sequences obtained from spleen, muscle, and spinal cord tissue were identical to that of cultured SFV-11, while that amplified from lymph node tissue showed minor variation (Fig. 4). Furthermore, sequences obtained from SFV-11 indicate that this virus is clearly related to, but distinct from, previously sequenced foamy viruses (Table 3 and reference 4).

Two years after isolation of the virus, Dodo became acutely ill, becoming lethargic and ataxic and demonstrating progressive difficulty in mounting his climbing frame. Ultimate tetraparesis, which developed in the space of 1 week, necessitated that the animal be humanely killed. A blood sample taken postmortem indicated that the level of neutralizing antibody had not changed over time (Table 1). Postmortem examination revealed lung lesions and generalized encephalopathy. The lung changes consisted of giant cell formation similar to a primary retroviral pneumonia found in macaques infected with simian immunodeficiency virus (20). The encephalopathy was histologically characterized by a widespread inflammatory process, astrocyte infiltration in the forebrain involving the optic

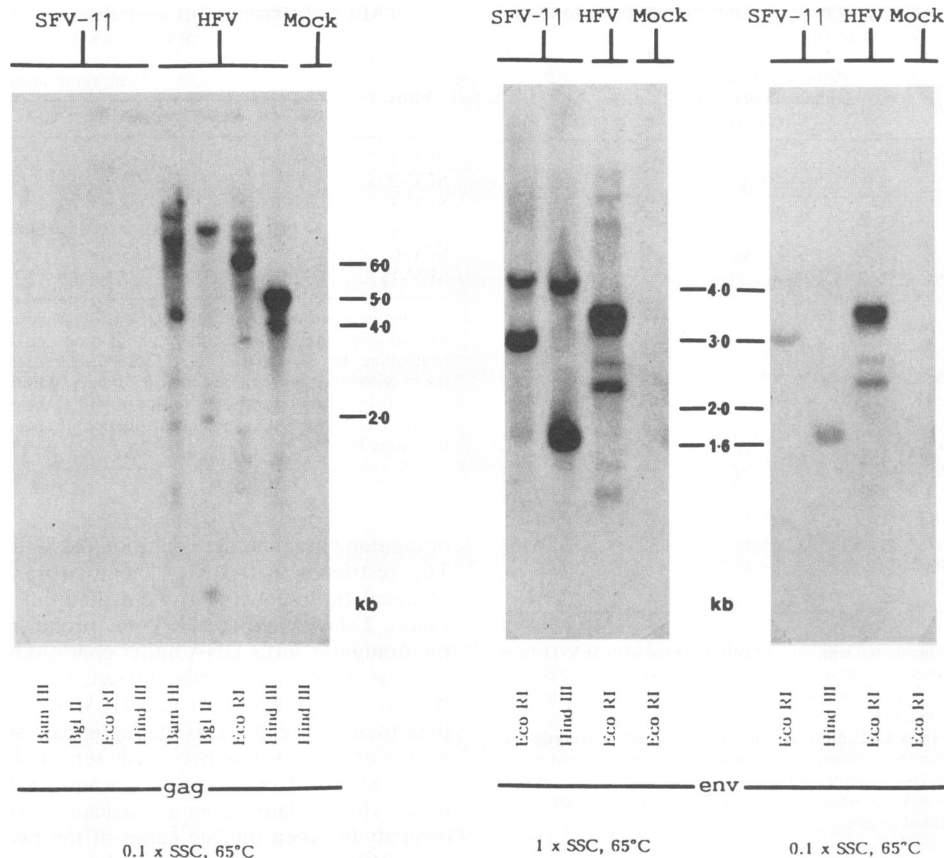

FIG. 2. Southern blot of SFV-11 and HFV DNA. Lanes marked SFV-11, HFV, and mock contained DNA from cells infected with the first orangutan isolate, DNA from cells infected with HFV, and DNA from uninfected Sup-T1 cells, respectively. Lanes marked gag and env were probed with probes derived from the *gag* and *env* genes of HFV under the indicated stringency conditions. No specific signal was obtained with the *gag* probe on SFV-11 after washing with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C (not shown). The *Eco*RI restriction pattern for SFV-11 is different from that of HFV. Similarly, the *Hin*dIII digestion pattern of SFV-11 differs from that expected for HFV on the basis of the published sequence (9, 21).

tract, and loss of neurons in the cerebellum, all of which were compatible with the diagnosis of a subacute encephalitis.

DISCUSSION

As part of a serological survey of captive primates for infection with known viruses, we obtained a heparinized blood sample from a 28-year-old orangutan. Unusually, in our experience, giant cell formation in the PBMC culture was apparent immediately after fractionation from whole blood. Virus showing classical foamy virus morphology was readily isolated by cocultivation with human B- and T-cell lines, particularly Sup-T1 cells. Although leukocytes from the buffy coat have been a consistent source of productive foamy virus culture (15, 24), it is worth recalling, in view of the subsequent pathology, that foamy viruses exhibit an unambiguous tropism for brain cells in vitro, were first isolated from the brain suspensions of chimpanzees previously experimentally inoculated with kuru (30), and have been subsequently isolated from the brains of macaques (25) and spider monkeys (13). The orangutan in question had carried high levels of neutralizing antibody to his own virus at least since 1982 with no apparent ill effects until 1990. The virus resisted neutralization by sera against other known foamy virus serotypes. Sera from Dodo only weakly neutralized other primate foamy viruses, SFV-1, -2, -3, -4, and -6 and HFV.

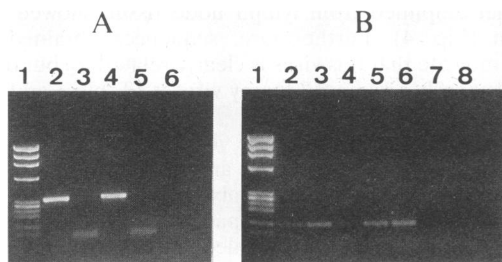

FIG. 3. Ethidium bromide-stained agarose gel showing PCR amplification products from isolated SFV-11 (A) and nested PCR products from tissue samples (B). (A) Lanes: 1, ΦX174 RF/*Hae*III molecular weight marker; 2, SFV-11-infected BHK cells; 3, uninfected BHK cells; 4, HFV-infected BHK cells; 5, uninfected BHK cells; 6, water control. (B) Lanes: 1, ϕX174 RF/*Hae*III molecular weight marker; 2, spleen; 3, lymph node; 4, kidney; 5, skeletal muscle; 6, spinal cord; 7 and 8, water controls.

Orangutans have a life span of approximately 50 years in captivity or 35 years in the wild. At the time of screening, Dodo, the animal from whom the first isolate was obtained, was considered to be in prime condition, being admitted to the zoo hospital only to have his toenails clipped. However, his condition changed dramatically about 2 years later without apparent precipitating cause. His acute illness was character-

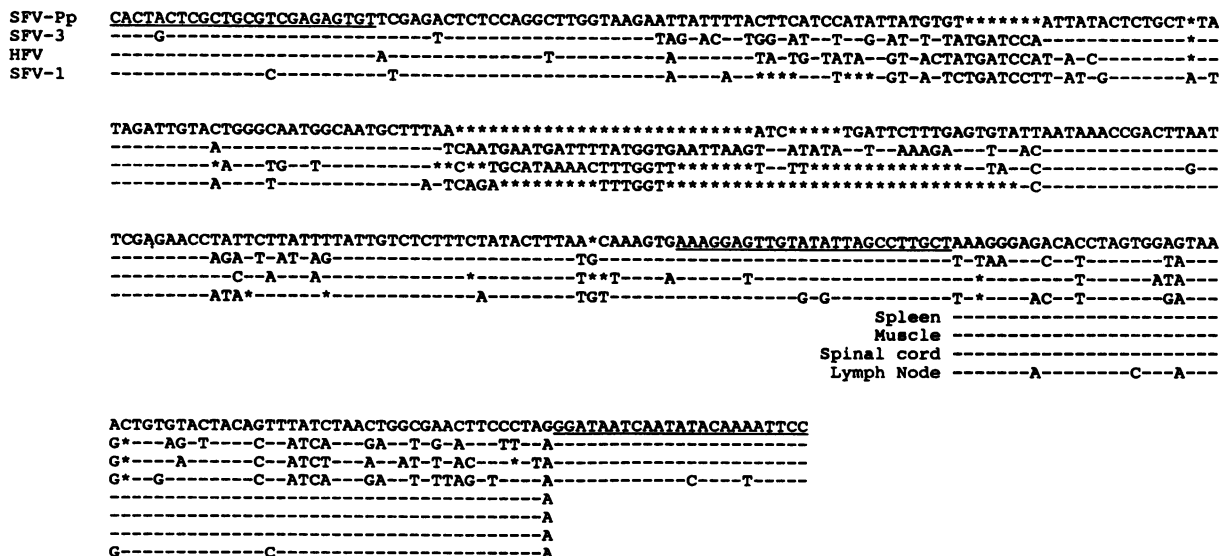

FIG. 4. Alignment of nucleotide sequences obtained from SFV-11 isolate (SFV-Pp) and tissue-derived PCR products with previously published sequences. Underlined sequences denote primers used for amplification.

ized by an ataxia and muscle weakness, manifested by marked difficulties in climbing, which worsened rapidly over a period of 1 week, with accompanying loss of appetite and body weight, lethargy, and apparent depression. Because of his deteriorating condition (ultimately tetraparesis), he was humanely killed. Postmortem examination revealed that, apart from a marked scoliosis (possibly of long duration and without relevance to the terminal illness), pathological changes were confined to the brain and spinal cord. Although these pathological and clinical changes might be observed in a wide variety of central nervous system infections, they were reminiscent of the abnormalities found in transgenic mice carrying the HFV genome (5).

We have at present no evidence to prove a causal link between the foamy virus infection of this animal and its neurological disease. In light of the fact that one of the two known HFVs originated from a patient who also died with encephalopathy (6) after an undefined neurological disease (3), it is, however, interesting to speculate that foamy virus infection of some primates might on occasion lead to overt disease, perhaps in older animals. Infection with foamy viruses may be comparable to infection of humans with another retrovirus, HTLV-I, and the associated neurological disorder, tropical spastic paraparesis, because only a small percentage of HTLV-I-infected individuals develop tropical spastic paraparesis (32).

TABLE 3. Long terminal repeat R/U5 homology between SFV-11, HFV, SFV-1, and SFV-3

Virus	% of nucleotide identity with[a]:		
	HFV	SFV-1	SFV-3
SFV-11[b]	78.8	77.8	82.3
HFV		83.3	79.5
SFV-1			86.0

[a] The percentage of nucleotide identity with previously sequenced foamy viruses (HFV, SFV-1, and SFV-3) was determined by using the NALIGN program (Intelligenetics).

[b] The SFV-11 sequence was obtained by PCR amplification of virus cultured in BHK cells as described in Materials and Methods.

In the past, foamy viruses isolated from chimpanzees, SFV-6, SFV-7, and the most recent, SFVcpz (10), have been catalogued as simian viruses. Although it is inaccurate, since the orangutan, like the chimpanzee, belongs to the great ape family, rather than the simian family (monkeys), we have adhered to this practice for the sake of uniformity. Ten different SFV serotypes are documented. Although we traced as many of these as we could, we were unable to obtain all 10 viruses; yet, it seems appropriate to name this virus SFV-11. Our limited analysis of the phylogenetic relationship of primate foamy viruses (4) confirms that SFV-11 is not a close relative of any known SFV for which sequence data are available. However, we feel that a definitive nomenclature for foamy viruses will have to be addressed once more sequence information is available.

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