

Reactivity of primate sera to foamy virus Gag and Bet proteins

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In order to establish criteria for the serodiagnosis of foamy virus infections we investigated the extent to which sera from infected individuals of human and primate origin react with structural and non-structural virus proteins in immunoblot assays. Using lysates from infected cells as the source of virus antigen, antibodies were preferentially detected against the Gag proteins and the non-structural Bet protein. Both the Gag precursor molecules of 70 and 74K apparent M_r and the cytoplasmic 60K M_r Bet protein were found to be phosphorylated, the latter being synthesized in large amounts in infected cells. Rabbit antiserum raised against recombinant human foamy virus (HFV) Gag major capsid protein cross-reacted with foamy viruses of chimpanzee, gorilla, orang-utan, rhesus monkey and

African green monkey origin. This was reflected by a broad cross-reactivity of the respective monkey sera to the Gag proteins of the various foamy virus isolates. Cross-reactivity of antisera against the Bet protein was restricted to viruses from man and the great apes. Recombinant Gag and Bet proteins expressed in prokaryotes or in insect cells were readily recognized by foamy virus-positive primate sera. Screening serum samples from chimpanzees with HFV Gag and Bet proteins expressed by recombinant baculoviruses revealed that 18 out of 35 (52%) were positive for Gag antibodies. Of these, 13 (72%) showed antibodies against the Bet protein, indicating that Bet antigen is of value in serological screening for foamy virus infections.

Introduction

The foamy viruses are a poorly characterized subgroup of the *Retroviridae* (Aguzzi, 1993; Loh, 1993; Flügel, 1993; Rethwilm, 1994). Foamy viruses are frequently found in wild and captive monkeys, great apes, felines, bovines and occasionally other mammalian species (Aguzzi, 1993; Loh, 1993). As revealed by immunofluorescence assays, the serological prevalence in monkeys has been reported to be high, reaching 70% or more (Hooks & Gibbs, 1975; Hooks & Detrick-Hooks, 1981). Foamy viruses have been isolated from healthy animals, as well as from animals with various diseases, but have never been convincingly associated with a distinct pathological condition (Aguzzi, 1993; Loh, 1993;

M. O. McClure *et al.*, unpublished results). Although highly cytopathic in tissue culture, foamy viruses have been regarded as benign passengers in their natural hosts (Coffin, 1990).

In 1971 a human foamy virus (HFV₇₁) was isolated from a Kenyan patient suffering from nasopharyngeal carcinoma (Achong *et al.*, 1971). Although further foamy virus isolates from human tissue have been reported (Young *et al.*, 1973; Stancek *et al.*, 1975; Cameron *et al.*, 1978; Werner & Gelderblom, 1979), HFV₇₁ is still the only human isolate available for research.

Serological surveys for foamy virus antibodies in the human population have led to controversial results. While Achong & Epstein (1978), Muller *et al.* (1980) and Loh *et al.* (1980) reported HFV seropositivity in East African and Pacific island populations, respectively, Brown *et al.* (1978) could not find HFV antibodies in sera originating from East Africa and the U.S.A. More recently, Mahnke *et al.* (1992) reported antibodies to HFV Gag and Env proteins in 1 to 2% of European sera

The nucleotide sequence data reported here have been submitted to the EMBL database and assigned accession numbers X79000 (pA65) and X79001 (p20A).

and in 6 to 7% of sera originating from tropical Africa, indicating a possible worldwide prevalence of human foamy virus infections. The latter study was carried out by using HFV Gag and Env antigen expressed in prokaryotes (Bartholomä *et al.*, 1992; Mahnke *et al.*, 1990) in ELISA and immunoblot assays, with antigen from virus-infected cells as a confirmatory assay in selected cases. However, the immunoblot assays presented by Mahnke *et al.* (1992) showed a plethora of weak bands, appearing to make the discrimination between specific and non-specific reactions a matter of chance. This study has therefore been criticized for methodological inconsistencies (Neumann-Haefelin *et al.*, 1993).

Since recent immunological studies suggested an aetiological role for foamy virus infection in motor neuron disease (amyotrophic lateral sclerosis; Westarp *et al.*, 1992) and in Graves' disease (Wick *et al.*, 1993; R. Emanoil-Ravier, personal communication) we attempted to define the normal pattern of foamy virus proteins recognized by positive sera in immunoblot assays, in order to establish criteria for foamy virus serodiagnosis.

Methods

Cells and viruses. Baby hamster kidney cells (BHK-21) and primary diploid human embryonic lung fibroblasts (HEL) were obtained from the ATCC and from Dr F. Harms (Würzburg, Germany), respectively. Cells were grown in MEM supplemented with 5% fetal calf serum and antibiotics. HFV was a gift of Dr M. A. Epstein (Bristol, U.K.), the simian foamy viruses (SFV) types 1, 2, 3, 5, 6, 7 and 8 were obtained from the ATCC. The isolation of SFVpp from an orang-utan called Dodo (London Zoo, U.K.) will be reported elsewhere (M. O. McClure *et al.*, unpublished results). For the isolation of SFVgg, peripheral lymphocytes were prepared from heparinized blood from a gorilla called Rann (a gift of Dr H. M. McClure, Yerkes Primate Research Center, Lawrenceville, Ga., U.S.A.) and cocultivated with the human T lymphoid cell line Sup T₁. The culture supernatant was monitored for reverse transcriptase activity (Daniel *et al.*, 1985) on a weekly basis and gave a positive reading on day 28 of culture. Examination of the cell pellet by electron microscopy revealed virus particles with foamy virus morphology (data not shown). The virus was subsequently grown on Vero cells and virus aliquots were stored in liquid nitrogen.

Sera. The HFV-reactive human serum C2 and the orang-utan sera have been described previously (Netzer *et al.*, 1990; Neumann-Haefelin *et al.*, 1993; M. O. McClure *et al.*, unpublished results). Chimpanzee sera were obtained from Dr H. Berthold (Freiburg, Germany), Dr G. Wick (Innsbruck, Austria) and Dr J. Lewis (London, U.K.). Gorilla sera were a gift of Dr H. M. McClure. Rhesus monkey sera were a gift of Dr C. Stahl-Hennig and Dr S. Stille-Siegenger (Deutsches Primatenzentrum, Göttingen, Germany). African green monkey sera were derived from monkeys of the colony at the Institut für Hygiene und Mikrobiologie in Freiburg, Germany. The rabbit antisera generated against recombinant HFV Gag, Bel-1 and Bel-2 proteins have been described previously (Aguzzi *et al.*, 1993; Baunach *et al.*, 1993).

Cloning of cDNA. Total RNA was prepared from BHK-21 cells, lytically infected with HFV for 3 days by the method of Chirgwin *et al.*

(1979). Poly(A)-positive RNA was selected on an oligo(dT) column and 5 µg were used to synthesize cDNA by the method of Gubler & Hoffman (1983) using a commercial kit (Promega). The cDNA was blunt-ended with T4 DNA polymerase and inserted into a *Sma*I-cut and dephosphorylated pUC19 vector. After transformation of competent DH5α MAX Efficiency cells (Gibco BRL), bacterial colonies were screened by hybridization to a ³²P-labelled *Eco*RI-*Bst*EII fragment from the 3' HFV genome as described by Grunstein & Hogness (1975). Colonies (220) displaying a strong signal were further characterized by restriction enzyme digestion for large inserts. In addition, the plasmid clones were analysed by *Eco*RI-*Bgl*II digestion to detect the splicing event leading to the *bet* mRNA. Out of 38 cDNA clones that extended 5' to the *Bel-1* coding region, 21 showed a 0.79 kb *Eco*RI-*Bgl*II fragment (instead of the 1.09 kb *Eco*RI-*Bgl*II fragment in the unspliced version) characteristic of the *bet* splicing event. The nucleotide sequences of the viral inserts of the clones pA65 and pB52 were determined by dideoxynucleotide chain termination sequencing (Sanger *et al.*, 1977). The A20 clone was isolated by screening a cDNA library synthesized from poly(A)-positive mRNA from SFV-1-infected cells with a probe from the 3' region of the SFV-1 genome as described previously (Mergia, 1994). The nucleotide sequence of the viral insert was determined after subcloning into the pUC118 vector as reported (Mergia *et al.*, 1991).

Expression of Gag and Bet in eukaryotic expression vectors. The pA65 cDNA clone has a 12 bp in-frame deletion. After repair of this deletion by exchange of a 0.66 kb *Bgl*II-*Afl*II fragment derived from the infectious molecular clone pHSRV (Rethwilm *et al.*, 1990), the *bet* coding sequences of pA65 (HFV) and p20A (SFV-1) were inserted into simian virus 40 (SV40) promoter-directed expression vectors (Rethwilm *et al.*, 1991; Mergia *et al.*, 1991). This gave rise to pSbet (HFV) and pSV20A (SFV-1).

The complete HFV *gag* sequence, as a 2.54 kb *Msp*II fragment from pHSRV (Fig. 1) and the complete HFV *bet* coding sequence from the repaired clone pA65 were inserted into the *Sma*I site of the baculovirus transfer vector pAcCL29-1 (Livingston & Jones, 1989) by blunt-end ligation. Following cotransfection with AcRP23-*lacZ* virus DNA (Possee & Howard, 1987) in *Spodoptera frugiperda* (Sf9) cells, recombinant baculoviruses were isolated and plaque-purified as described previously (Matsuura *et al.*, 1987).

Expression of Gag and Bet in prokaryotic expression vectors. The HFV *bet* coding sequence, lacking the amino-terminal 15 codons, was excised as an *Eco*RI-*Afl*II fragment from pA65 and inserted into the *Sma*I and *Sma*I site of the bacterial overexpression vectors pROS (Ellinger *et al.*, 1989) and pGEX-2T (Smith & Johnson, 1988), respectively, after treating the ends with the Klenow fragment of DNA polymerase I. In a similar fashion, the SFV-1 *bet* coding region, lacking the amino-terminal 24 codons, was excised as a *Bgl*II-*Hinc*II fragment from pSV20A and inserted into the *Eco*RV and *Sma*I site of pROS and pGEX-3X, respectively. The correct insertion of the *bet* sequences in frame with the *lacZ* (β -galactosidase; β GAL) and glutathione *S*-transferase (GST) genes of pROS and pGEX, respectively, was verified by nucleotide sequencing. The expression of HFV *gag* sequences in the pROS vector has been described previously (Aguzzi *et al.*, 1993). The expression strategy of the recombinant proteins is shown in Fig. 1.

Preparation of virus antigen and immunoblot assays. For the preparation of antigen, all virus isolates were cultivated on primary HEL cells. Infected cells were harvested when approximately 30% of the culture showed typical giant cell c.p.e. After washing in PBS the cells were lysed in detergent buffer [20 mM-Tris-HCl pH 7.4, 0.3 M-NaCl, 1% sodium desoxycholate, 1% Triton X-100, 0.1% SDS, 0.02% sodium azide, 1 mM-PMSF, 20 µM-4-amidino-PMSF, 1 µM-pepstatin A and 10 µM-protease inhibitor E64 (Sigma)]. Following measurement of the protein content with a commercial assay (Sigma),

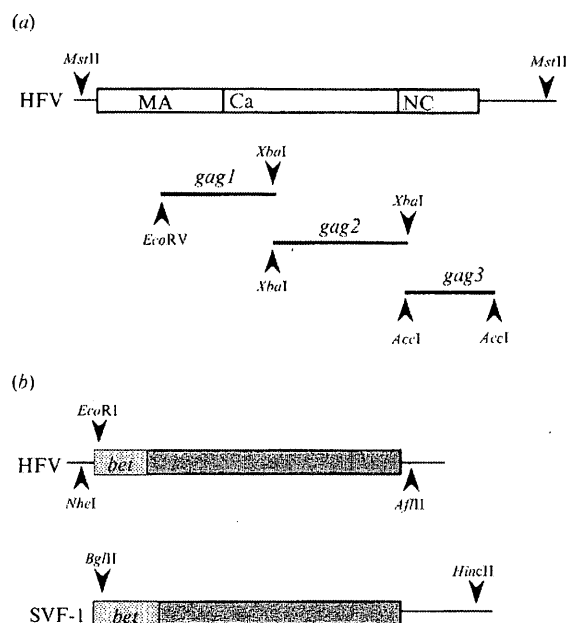


Fig. 1. Fragments of the HFV and SFV-1 genome expressed in bacteria and by recombinant baculovirus. (a) Expression of the HFV gag gene fragments 1 to 3 in the pROS vector has been described recently (Aguzzi *et al.*, 1993). In addition, the complete HFV gag gene was inserted into a recombinant baculovirus as a MspI fragment. (b) The HFV bet coding sequence was cloned into baculovirus as a NheI-AflII fragment and into the pROS and pGEX-2T prokaryotic expression vectors as an EcoRI-AflII fragment. The SFV-1 bet coding sequence was inserted into pROS and pGEX-3X vectors as a BglII-HincII fragment. The positions of the matrix protein (MA), major capsid protein (CA) and nucleocapsid protein (NC) are indicated.

equal amounts of protein were separated by 12.5% SDS-PAGE and semi-dry blotted onto a nitrocellulose membrane (Schleicher & Schüll). The membranes were blocked in Tris-buffered saline (20 mM-Tris-HCl pH 7.6, 137 mM-NaCl; TBS) containing 3% non-fat dried milk (TBS-M). Membranes were incubated with primate sera diluted 1:200 or with rabbit sera diluted 1:500, in TBS-M for 60 min. Following several washes in TBS containing 0.5% Tween 20, the membranes were incubated with the appropriate peroxidase-coupled secondary antibody (Dako) and washed again. The immunostain was developed using the ECL chemiluminescence detection system (Amersham).

BHK-21 cells were transfected with bet expression vector DNA and lysates of transfected cells were harvested for immunoblot analysis as described recently (Baunach *et al.*, 1993). Gag and Bet proteins expressed in bacteria and insect cells were purified by preparative SDS-PAGE to greater than 90% purity as described previously (Netzer *et al.*, 1993). In immunoblot assays 100 ng of purified protein per lane was used.

Radioimmunoprecipitation assays (RIPA). HFV-infected BHK-21 cells were metabolically labelled with either 100 μ Ci/ml [35 S]methionine (Amersham) or 170 μ Ci/ml [32 P]orthophosphate (Amersham) for 4 h. After washing, the cells were lysed in ice-cold detergent buffer. The lysates were cleared by incubation with an excess of normal rabbit serum for 2 h at 4 °C, followed by incubation with staphylococcal protein A-Sepharose (PAS; Pharmacia) for 1 h and centrifugation at 10000 g for 2 min. The clarified supernatant was then immunoprecipitated with Gag and Bet protein-specific rabbit antisera for 2 h at

4 °C. The immune complexes were collected with PAS, washed six times with detergent buffer, resolved by 12.5% SDS-PAGE, and exposed to X-ray film.

Results

Characterization of HFV and SFV-1 Bet and Gag proteins

In a previous study, a foamy virus-encoded intracellular protein with an M_r of 60K was found to be recognized by foamy virus-positive primate sera (Netzer *et al.*, 1990). It has been speculated that this protein might represent the Bet protein (Baunach *et al.*, 1993). To elucidate the nature of the protein and to clarify whether it is identical to the Bet protein, we synthesized cDNA from the mRNA of cells that were lytically infected with HFV. More than 50% of the cDNA clones that were not prematurely terminated showed the bet splicing event. Two different types of bet cDNA clones were identified (pB52 and pA65) and one representative of each was chosen for nucleotide sequencing. Both clones start in the vicinity of the HFV internal start site of transcription (Löchelt *et al.*, 1993). Whereas pB52 was derived from an mRNA singly spliced from the bet-1 open reading frame (ORF) into the bet-2 ORF, the clone pA65 represented a double-spliced mRNA from which an additional intron was spliced out. This splicing event has been described recently to be characteristic for transcripts starting at the foamy virus internal promoter (Löchelt *et al.*, 1994). In both clones the first ATG codon was from the bet-1 ORF. In a similar way to the HFV bet cDNAs clone, phage lambda clone 20A starts in the vicinity of the SFV-1 internal promoter (Mergia, 1994) and shows an identical splicing pattern to the HFV bet clone pA65, using splice sites conserved in all foamy virus genomes sequenced so far (Flügel *et al.*, 1987; Mergia *et al.*, 1991; Kupiec *et al.*, 1991; Renne *et al.*, 1992; Herchenröder *et al.*, 1994). The SFV-1 bet sequence starts with the ORF-1 ATG and is spliced into the ORF-2 frame after 93 codons of ORF-1.

The HFV bet cDNA from clone pA65 and the SFV-1 bet cDNA from 20A were inserted into eukaryotic expression vectors under control of the SV40 enhancer-promoter sequences. Comparison of lysates from BHK-21 cells, either transfected with the HFV Bet-encoding plasmid pSbet or infected with HFV, in an immunoblot assay with Bet-1- and Bet-2-specific rabbit antisera and with a HFV-positive human serum, revealed the 60K protein reactive with the sera in lysates from infected cells to be of the same M_r as the protein encoded by pSbet, indicating that it is Bet (Fig. 1a). Immunoblot analysis of lysates from cells, transfected with the SFV-1 Bet-expressing plasmid pSV20A or infected with SFV-1, with SFV-1-positive rhesus monkey serum gave an analogous result (data not shown).

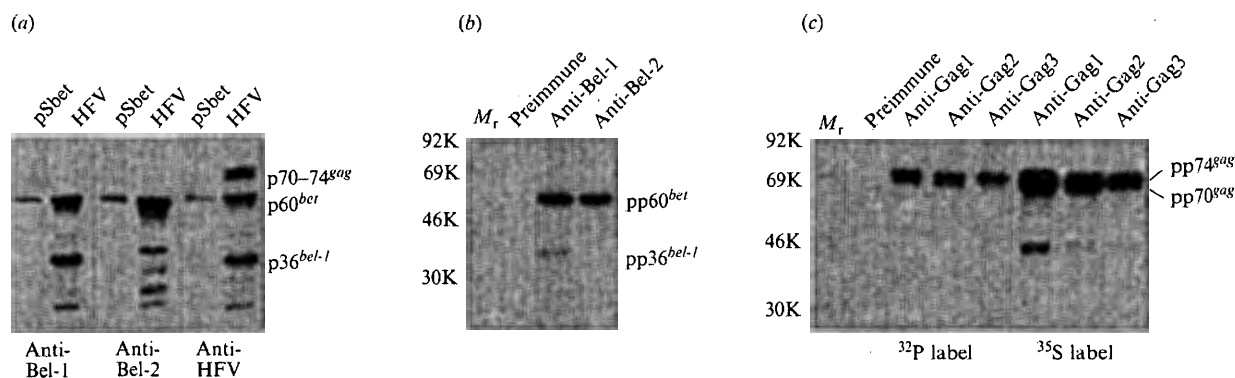


Fig. 2. (a) Identification of the HFV Bet protein. Lysates from BHK-21 cells either transfected with an expression plasmid (pSbet) for the A65 cDNA clone or infected with HFV were reacted with rabbit sera directed against the HFV *bel-1* and *bel-2* ORFs or with human anti-HFV-positive serum in an immunoblot assay. (b) Phosphorylation of Bel-1 and Bet. HFV-infected BHK-21 cells were labelled with [32 P]orthophosphate. The proteins were then precipitated with either Bel-1- or Bel-2-specific rabbit antisera, or preimmune rabbit serum, and resolved by SDS-PAGE. Both antisera precipitated the 60K Bet protein. In addition, Bel-1 antiserum also precipitated the 36K Bel-1 protein. Lane M contains M_r markers. (c) Phosphorylation of the Gag precursor 70/74K proteins. HFV-infected BHK-21 cells were metabolically labelled with [32 P]orthophosphate or [35 S]methionine, the virus Gag proteins were precipitated with rabbit antisera generated against different protein domains from the HFV *gag* ORF (Fig. 1) and resolved by SDS-PAGE.

The HFV Bel-1 protein has been shown to be phosphorylated (Venkatesh *et al.*, 1993). Since the Bet protein of HFV contains 88 amino acids of the *bel-1* exon at its amino terminus, we asked whether Bet might also be a phosphoprotein. As shown in Fig. 2(b), phosphate-labelled Bet can be precipitated from HFV-infected cells with either Bel-1- or Bel-2-specific antisera. However, whether Bet phosphorylation is due solely to its Bel-1 component cannot be deduced from this experiment, since the *bel-2* ORF also possesses potential phosphorylation sites.

The HFV *gag* ORF is expressed as precursor molecules of 70K and 74K (Aguzzi *et al.*, 1993). To investigate whether this M_r difference might be due to phosphorylation, we labelled HFV-infected BHK-21 cells with [32 P]orthophosphate or [35 S]methionine and precipitated the Gag proteins with rabbit antisera generated against different domains of the Gag protein. As shown in Fig. 2(c), both Gag precursor molecules were found to be phosphorylated to approximately the same extent. Interestingly, precipitation of both the 32 P- and 35 S-labelled 70K molecule was always found to be less effective with the Gag3 antiserum, which was generated against the carboxy-terminal *gag* ORF (Fig. 2c).

Cross-reactivity of primate sera to different foamy virus isolates

There have been conflicting reports concerning the prevalence of foamy viruses in the human population (see above). We therefore set out to determine which virus proteins are readily recognized by foamy virus-positive sera in an immunoblot assay. Since human sera

that are positive for foamy viruses are scarce (we know of only two, both resulting from accidental infection; Neuman-Haefelin *et al.*, 1983, 1993) we used the sera of great apes and monkeys. Previous studies on the serological prevalence of foamy viruses among humans rely on virus antigen from one single isolate obtained from human tissue more than 20 years ago (Achong *et al.*, 1971) and, moreover, some retroviruses are known to show considerable variation in their antigenic proteins (Vaishnav & Wong-Staal, 1991). Thus, the extent to which foamy virus-positive primate sera reacted with viruses originating from different species would determine the potential of a given virus antigen to identify infections with distantly related viruses in a serological assay. To analyse this, HEL cells were infected with HFV (isolated from a human source), SFV-1 (macaque monkey), SFV-2 (macaque monkey), SFV-3 (African green monkey), SFV-5 (galago), SFV-6 (chimpanzee), SFV-7 (chimpanzee), SFV-8 (spider monkey), SFVpp (orang-utan) and SFVgg (gorilla). Lysates derived from productively infected cells were then subjected to immunoblot analysis with foamy virus-positive sera from human, chimpanzee, orang-utan, gorilla, rhesus monkey and African green monkey, as well as with rabbit sera generated against recombinant HFV Gag, Bel-1 and Bel-2 protein. Representative immunoblots are shown in Fig. 3.

The proteins of the various viruses predominantly recognized by the homologous sera were the two Gag precursor molecules and the Bet protein. The human serum detected the chimpanzee virus (SFV-6 and SFV-7) Gag and Bet proteins equally as well as the respective HFV proteins, demonstrating the close relationship of the

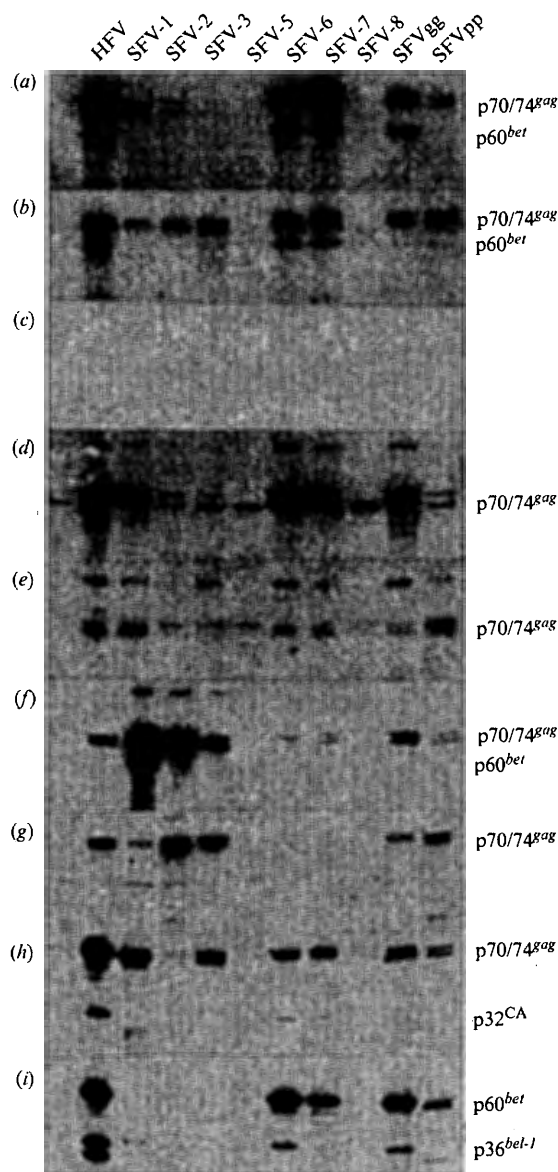


Fig. 3. Immunoblots demonstrating the serological cross-reactivity against Gag and Bet proteins of primate foamy viruses. Lysates from HEL cells infected with the HFV isolate and SFV from macaque monkeys (types 1 and 2), African green monkey (type 3), prosimian galago (type 5), chimpanzee (types 6 and 7), New World spider monkey (type 8), gorilla (SFVgg) and orang-utan (SFVpp) were reacted with foamy virus-positive sera from human (a), chimpanzee (b), gorilla (d), orang-utan (e), rhesus monkey (f) and African green monkey (g), as well as with antisera against recombinant HFV CA (h) and Bel-1 protein (i). In (c) the lysates were reacted with a foamy virus-negative chimpanzee serum.

human and chimpanzee viruses (Nemo *et al.*, 1978; Herchenröder *et al.*, 1994). SFVgg proteins were less well detected. Only a weak cross-reactivity was observed with SFVpp and SFV-1 to SFV-3 Gag, and the Bet proteins

of these viruses were not detected by this serum (Fig. 3a). The chimpanzee sera (Fig. 3b) recognized Gag and Bet proteins of HFV, SFV-6 and SFV-7 and showed a broader cross-reactivity than the human serum to the Gag proteins of most virus isolates. Viral proteins in SFV-5- and SFV-8-infected cells were not recognized by any of the sera used. However, this being due to a lack of virus antigen seems unlikely since the cell lysates were prepared when foamy virus c.p.e. was prominent. SFV-5 and SFV-8 were isolated from a prosimian and a New World monkey, respectively (Hooks & Gibbs, 1975). Therefore, a lack of cross-reactivity of our sera to these presumably distantly related viruses may explain the result. The gorilla serum (Fig. 3d) recognized the Gag precursors of HFV, the chimpanzee viruses and SFV-1. Only weak reactivity with SFV-2, SFV-3 and SFVpp Gag proteins was found, whereas the Bet protein was not detected with any lysate. In addition, this serum recognized proteins of around 130K. The orang-utan serum (Fig. 3e) also reacted well with a protein of approximately 130K. However, of the other virus proteins, only the Gag precursors of SFVpp and, to a weaker extent, of HFV, SFV-1, SFV-6, SFV-7 and SFVgg were detected. The rhesus monkey serum (Fig. 3f) reacted well with the Bet protein of SFV-1 and only barely with the SFV-2 Bet. A strong reaction was also observed with the Gag proteins of SFV-1, SFV-2 and SFV-3, whereas the respective proteins of HFV, SFV-6, SFV-7, SFVgg and SFVpp were less well detected. The serum from an African green monkey (Fig. 3g) showed reaction to the Gag precursors of HFV, SFV-2, SFV-3, SFVgg and SFVpp, whereas the respective proteins of SFV-1 and the chimpanzee viruses were only barely detected. The Bet protein was not recognized by this serum.

A rabbit hyper-immune serum directed against the CA domain of HFV (Gag2 in Fig. 1) reacted with the Gag proteins of viruses isolated from the human (HFV), apes (SFV-6, SFV-7, SFVgg and SFVpp) and with SFV-1 and SFV-3 (Fig. 3h). A rabbit serum directed against recombinant HFV Bel-1 detected the Bet proteins of all great apes (Fig. 3i). HFV, SFV-6, SFV-7 and SFVgg Bet proteins were of an apparent M_r of 60K, whereas the SFVpp Bet protein was approximately 4K smaller. In addition, this serum recognizes the Bel-1 protein of the great ape foamy viruses and to a lesser extent the respective protein of SFV-1 and SFV-2. The SFVpp protein was found to be of higher electrophoretic mobility than the Bel-1 proteins of the other higher primates, corresponding to a 4K lower M_r . This indicated that the smaller Bet protein of this virus is caused by a smaller *bet-1* exon. The use of a Bel-2 ORF-specific rabbit antiserum revealed a similar Bet staining pattern to the Bel-1 antiserum (data not shown).

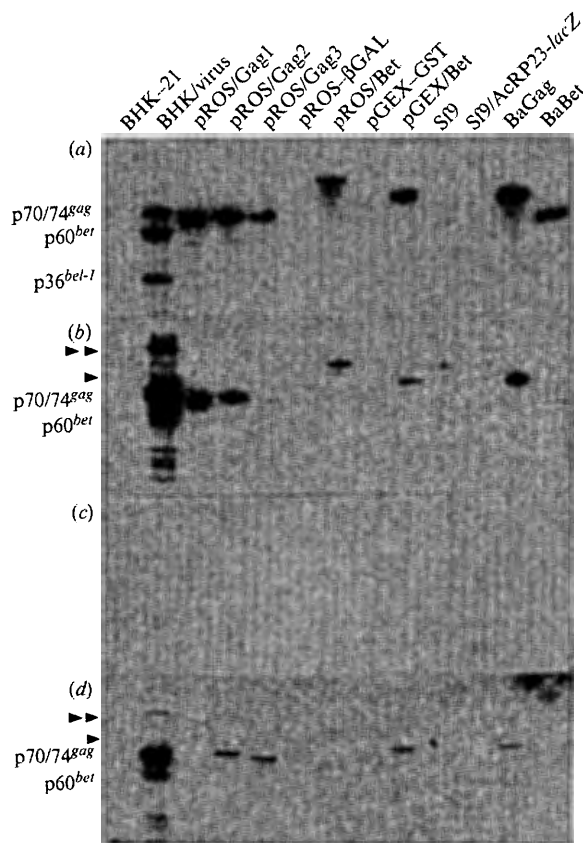


Fig. 4. Immunoblot of the purified recombinant HFV and SFV-1 proteins. Human (a), chimpanzee (b) and rhesus monkey (d) foamy virus-positive sera and chimpanzee foamy virus-negative serum (c) were used. In (a) to (c), lysate from HFV-infected BHK-21 cells (BHK/virus) served as the positive control and β Gal and GST proteins fused to HFV Bet were used. In (d), a lysate from SFV-1-infected BHK-21 cells was used as control and SFV-1 Bet was the fusion partner of β Gal and GST. Sf9 and Sf9/AcrP23-lacZ, lysates from uninfected and AcrP23-lacZ-infected Sf9 cells; BaGag and BaBet, purified recombinant protein from Sf9 cells infected with Gag_{HFV}- and Bet_{HFV}-expressing baculovirus, respectively. The arrows indicate proteins in the range of 130K and 80K M_r in virus-infected cells recognized by some of the sera.

Reactivity of foamy virus-positive primate sera to Gag and Bet proteins expressed in insect cells and in bacteria

We examined the extent to which recombinant Gag and Bet proteins of HFV and SFV-1 could be used as a reliable source of antigen in serological assays. The recombinant proteins were used in an immunoblot assay with primate sera previously identified as containing foamy virus antibodies by detection with virus antigen. As shown in the representative immunoblots of Fig. 4, foamy virus-positive human, chimpanzee and rhesus monkey sera recognized the HFV Gag protein expressed either in bacteria or in insect cells. Human and

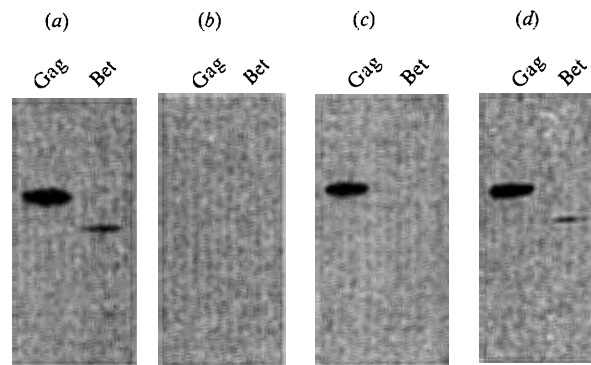


Fig. 5. Immunoblot of HFV Gag and Bet proteins, which were purified from insect cells infected with recombinant baculovirus with a human HFV-positive serum (a), foamy virus-negative chimpanzee serum (b), chimpanzee Gag-reactive serum (c) and chimpanzee serum reactive with Gag and Bet protein (d).

chimpanzee sera reacted better with the HFV Gag portions corresponding to MA (Gag1) and CA (Gag2) protein, but the rhesus monkey sera showed no reaction with Gag1 and reacted equally well with Gag2 and Gag3 antigen. The Bet protein displayed a more restricted reactivity. Bet antibody-positive human and chimpanzee sera detected the HFV Bet protein regardless of the vector system used (Fig. 4) but did not react with SFV-1 Bet (data not shown). Conversely Bet-positive rhesus monkey sera detected the SFV-1 Bet but did not react with HFV Bet (Fig. 4d). In addition, some sera reacted specifically with virus proteins with an M_r that was different to the 70/74K Gag precursor and the 60K Bet proteins in lysates of infected cells (Fig. 4b, d). These were in the range of 130K and 80K.

Finally, we used the HFV Gag and Bet proteins purified from recombinant baculovirus-infected insect cells to screen a panel of chimpanzee sera by an immunoblot assay, taking advantage of the cross-reactivity previously observed in assays with virus antigen from infected cell lysates. Representative results are presented in Fig. 5. Of the 35 sera investigated, 18 (52%) reacted positive with Gag antigen. Of these, 13 (72%) also had antibodies to the Bet protein. Reactivity against the Bet protein was always weaker compared to Gag reactivity, despite the fact that equal amounts of the respective proteins were loaded onto the gels. Notably, no unspecific bands were observed in this assay with any of the sera tested and discrimination between positive and negative sera was easy to accomplish.

Discussion

Similar to the lentiviruses and the human T cell leukaemia virus-bovine leukaemia virus group of retroviruses, the foamy virus subgroup is characterized by the presence of

accessory genes of suspected or proven regulatory function, in addition to the structural *gag*, *pol* and *env* genes (Mergia & Luciw, 1991; Cullen, 1991; Rethwilm, 1994). Thus, the respective accessory proteins may give rise to a host immune response if exposed to the host immune system. Two non-structural accessory proteins have clearly been identified in the foamy virus system (Rethwilm, 1994). The nuclear 36K Bel-1 trans-activator protein is a prerequisite for viral replication, while the cytoplasmic 60K Bet protein has been reported to be dispensable for *in vitro* replication of the virus (Baunach *et al.*, 1993; Yu & Linial, 1993). The use of hyper-immune sera raised against recombinant Bet protein has indicated that the Bet protein might be a preferentially expressed virus protein (Löchelt *et al.*, 1991; Baunach *et al.*, 1993). The cDNA cloning experiments on HFV mRNA described in this study provide strong evidence supporting this view. Restriction enzyme analysis revealed more than 50% of the cDNAs that were not terminated early to be *bet*-specific. Both proteins Bel-1 and Bet were found to be phosphorylated. The functional consequences, if any, of this post-translational modification are not known for either protein.

The Gag precursor proteins of 70/74K were also found to be phosphorylated. It had been suggested that phosphorylation might account for the difference in M_r of these two proteins (Batholomä *et al.*, 1992; Flügel, 1993). The data presented here indicate that this is probably not the case. Instead, the precipitation experiments with an antiserum generated against the carboxy-terminus or the HFV Gag protein suggest that the 70K molecule may either be a cleavage product of p74^{prec} or that the protein was not synthesized to full length. Phosphorylation has also been demonstrated for the human immunodeficiency virus (HIV) and avian retrovirus Gag proteins (Leis *et al.*, 1989; Burnette *et al.*, 1993). However, any biological function of retrovirus Gag phosphorylation remains to be elucidated.

The HFV Gag precursor has been reported to give rise to p32^{CA} and p27^{MA} capsid proteins (Aguzzi *et al.*, 1993; Bartholomä *et al.*, 1992). In addition, a p15 capsid protein with RNA-binding properties, which may represent the foamy virus NC protein, has been reported for SFV-1 (Benzair *et al.*, 1986). When analysing infected cell extracts with foamy virus-positive primate sera or specific antisera raised in rabbits, we noticed that processing of the foamy virus Gag precursor seemed to be slow. For example, p32^{CA} was only occasionally observed by staining with a specific antiserum. Furthermore, when virus harvested from cell-free supernatant was purified by sucrose gradient centrifugation and analysed in immunoblot assays, we regularly observed the 70/74K Gag precursors, whereas the cleavage products were only rarely detected and Bet was

absent (data not shown). We do not yet know to what extent the slow processing of the foamy virus protease is responsible for the stability of the Gag precursor proteins. Certainly culture conditions, type of host cells, and timepoint of harvest of cell lysates and cell-free virus may influence the relative abundance of the Gag cleavage products.

Antibodies to the structural virus proteins Gag, Pol and Env prevail in retrovirus-infected primate hosts (Essex *et al.*, 1985; Schochetman *et al.*, 1989). In a previous study utilizing RIPA, a variety of immunodominant foamy virus proteins has been characterized (Netzer *et al.*, 1993). These include a 130K glycoprotein, most probably representing the *env* precursor (Giron *et al.*, 1993; Aguzzi *et al.*, 1993) and the 70/74K and 60K proteins, now identified as the Gag precursor molecules and the Bet protein, respectively. In the immunoblot assays used in our study, antibodies directed against virus proteins other than Gag and Bet were only occasionally observed. However, we cannot yet tell to what extent these antibodies were directed against foamy virus Env or Pol proteins, since both ORFs give rise to precursors and cleaved proteins of almost similar size (Netzer *et al.*, 1990, 1993; Aguzzi *et al.*, 1993; Giron *et al.*, 1993). The use of recombinant Pol and Env proteins as antigen in immunoblot assays might help to elucidate this point. With respect to antibodies directed against the Env proteins we noticed a marked difference in detectability depending on the assay system used. The same sera that previously recognized Env proteins in RIPA (Netzer *et al.*, 1990) did not detect these proteins in an immunoblot assay. Similar observations have been made by others for foamy viruses (Giron *et al.*, 1993) and HIV (Essex *et al.*, 1985; Gaines *et al.*, 1987).

In the previous study the detection of the 70/74K proteins has been regarded as particularly suitable for diagnostic purposes in RIPA (Netzer *et al.*, 1990). We therefore investigated the extent to which primate sera reacted with the Gag proteins of various foamy virus isolates. Our results demonstrate a broad cross-reactivity among foamy viruses and sera from infected Old World simians. This cross-reactivity was more pronounced with more closely related monkey species. However, rhesus and African green monkey sera detected the Gag proteins from HFV and virus isolates from apes and vice versa. In contrast, the detection of the Bet protein was found to be more restricted. While HFV Bel-1- and Bel-2-specific rabbit antisera detected the Bel-1 and Bet proteins of virus isolates from all great apes, the results from the human and chimpanzee sera indicate an even closer relationship of the viruses isolated from these two species.

There are limited nucleotide sequence data on primate foamy virus genomes available, which support our view

(Flügel *et al.*, 1987, 1990; Maurer *et al.*, 1988; Mergia *et al.*, 1990; Kupiec *et al.*, 1991; Renne *et al.*, 1992; Herchenröder *et al.*, 1994). Pol and Env proteins were found to be highly conserved among all foamy virus isolates. Unlike the immunodeficiency viruses (Vaishnav & Wong-Staal, 1991), the foamy virus Gag sequences are less well conserved compared to Env sequences. However, closer inspection identified small clusters of highly conserved peptides distributed throughout the Gag protein (Schliephake & Rethwilm, 1994; data not shown). Antibodies to these clustered sequences may account for the broad cross-reactivity against Gag proteins. With respect to the Bet protein, significant amino acid similarity, leading to cross reactivity in serological assays, was only found between HFV and the chimpanzee virus (Herchenröder *et al.*, 1994).

When recombinant HFV Gag proteins expressed in bacteria were used as antigens in immunoblot assays, different portions of the Gag protein were recognized to different extents by foamy virus-positive sera. The recombinant protein corresponding to HFV CA showed the broadest antigenicity (Fig. 4). Similarly expressed Bet protein was also found to be a suitable antigen source in our detection system. However, since neither the immunodominant epitopes of the foamy virus Gag protein, nor the influence on antigen recognition caused by phosphorylation of Gag and Bet are known, we used eukaryotically expressed HFV Gag and Bet proteins to screen chimpanzee sera for foamy virus antibodies. Compared to bacterial expression, the baculovirus system has the additional advantage of reducing the possibility of false positive reactions, often a problem with fusion proteins used in bacterial expression systems, although we did not observe such a reaction with any of the sera tested.

Of the chimpanzee sera investigated, 52% had antibodies to foamy virus Gag protein and 72% of these had antibodies to Bet. While the percentage of captive chimpanzees identified by a serological assay to be infected with foamy virus is within the previously reported range (Hooks & Gibbs, 1975; Hooks & Detrick-Hooks, 1981), the finding that a high percentage of Gag antibody-containing sera also reacted with the non-structural Bet protein indicates that the Bet protein is not only synthesized in large amounts during virus replication in tissue culture, but may also be a preferentially expressed and immunogenic viral protein in the infected host. This observation is reminiscent of findings with HIV for which antibodies to the non-structural Nef protein were detected in up to 50% of infected individuals (Allan *et al.*, 1985).

The results reported here and previously (Netzer *et al.*, 1990) indicate that Gag-specific antibody response is a hallmark of foamy virus infection. In addition, it has

been shown recently that Gag-specific immune response is responsible for the typical nuclear fluorescence that is observed when sera from infected individuals are reacted with foamy virus-infected cells in an immunofluorescence assay and which distinguishes the foamy viruses from all other known retroviruses (Teich, 1984; Neumann-Haefelin *et al.*, 1993; Loh, 1993; Schliephake & Rethwilm, 1994). The high percentage of Bet seropositivity among foamy virus-infected chimpanzees now points to a second protein which we suggest will be of value in screening human sera for foamy virus footprints.

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