Das zoonotische Potenzial feliner und boviner Foamyviren (*Spumaretrovirinae*)

Etablierung und Anwendung serologischer Nachweisverfahren und Identifikation einer zellulären antiretroviralen Restriktion

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Hiermit erkläre ich diese Dissertation selbständig verfasst zu haben.

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Summary

Foamy- or Spumaretroviruses (FV) belong to the retrovirus subfamily *Spumaretrovirinae*. They infect primates, cats, cattle and horses worldwide and reach partially high prevalences. In humans they were detected only sporadically in persons with intensive contact to primates indicative of a zoonotic infection. At present, little is known about the biology of FVs in their authentic host or after inter-species transmission.

For the development of diagnostic systems for the feline and bovine FV (FFV and BFV) the Gag structural proteins were identified by immunoblotting as the best diagnostic antigens. Subsequently GST-capture ELISAs with the FV antigens Gag, Bet and the ectodomain of Env were established and validated.

These ELISAs were used to detect serological markers of FV infection in field samples from cats and cattle. The studies showed a prevalence of FFV-specific antibodies of 36% in Swiss cats. The ELISAs were adapted to allow distinction of IgG and IgM antibodies in order to determine the stage of an infection. FFV infections were not associated with a disease.

Cattle from Poland was to 42% BFV-positive. In Germany the BFV prevalence was at 7%. Antibodies directed against the antigens of BFV were in addition to serum also detected in raw-milk. This facilitates the monitoring of large dairy herds. To complement the serodiagnostics, BFV infectivity was directly isolated from blood cells and raw-milk but not from saliva of infected cows. Milk could therefore be considered as carrier of infectivity for inter-species transmissions. Large scale studies with human samples are planned.

In parallel, it was investigated whether FVs are targeted by the recently discovered cellular antiretroviral defence mechanism on the basis of APOBEC3. We show that Bet deficient FFV is efficiently suppressed by a newly identified feline APOBEC3 cytidin-deaminase. Viral genome sequencing and biostatistical analysis revealed extensive genome hypermutation. The FFV accessory protein Bet adopts the role of Vif in HIV and counteracts feline APOBEC3. Little is known about the mechanism of APOBEC3 inhibition, it is however certainly different than in HIV infections.

The inhibition of APOBEC3 proteins by FV Bet proteins is species-specific. This specificity is an additional hurdle that FVs crossing the species barrier from non-primate FV to humans have to overcome.

Zusammenfassung

Foamy- oder Spumaretroviren (FV) gehören in die Retrovirus Unterfamilie *Spumaretrovirinae*. Sie sind in Primaten, Katzen, Rindern und Pferden weltweit verbreitet und erreichen teilweise einen hohen Durchseuchungsgrad. Beim Menschen hat man sie bislang nur vereinzelt – indikativ für eine Inter-Spezies-Übertragung ausschließlich bei Personen, die intensiven Kontakt zu Primaten hatten - gefunden. Zur Zeit weiß man nur wenig über die Übertragungs- und Abwehrmechanismen der FV-Infektionen.

Zur Entwicklung eines Diagnosesystems für das feline und bovine FV (FFV und BFV), wurden mittels Immunoblot Experimenten die Gag Strukturproteine als diagnostische Antigene identifiziert. Anschließend wurde ein „GST-capture“ ELISA mit den FV Antigenen Gag, Bet und der Ektodomäne von Env etabliert und validiert.

Die ELISAs wurden für die Durchführung von Studien mit Katzen- und Rinder-Feldseren eingesetzt. Dies ergab bei Schweizer Katzen eine Prävalenz von 36%. Für FFV wurde für die Bestimmung des Stadiums einer Infektion ein modifizierter Assay zur spezifischen Unterscheidung von IgG und IgM Antikörpern entwickelt. FFV Infektionen waren nicht mit einer Erkrankung der Tiere assoziiert.

Rinder aus Polen waren zu 42% FV-positiv. In Deutschland lag die BFV-Prävalenz bei 7%. Antikörper gegen Antigene des bovinen FV wurden neben dem Serum auch in Rohmilch detektiert. Dies vereinfacht die Überwachung großer Herden von Milchkühen. Zusätzlich zur Sero-diagnostik wurde BFV-Infektiösität direkt aus Blutzellen und aus Milch, nicht aber aus Speichel infizierter Rinder isoliert. Milch könnte demnach als Überträger von Infektiösität für Interspezies-Übertragungen in Betracht kommen. Groß angelegte Untersuchungen von humanen Proben sind derzeit in Vorbereitung.

Parallel wurde untersucht, ob auf FV ein zellulärer, antiretroviraler Abwehr-mechanismus auf Basis von APOBEC3 einwirkt, wie er bei HIV-Infektionen entdeckt wurde. Wir zeigen, dass feline FV mit defektem Bet Protein ebenfalls gezielt durch eine hier erstmals identifizierte feline APOBEC3-Cytidin-Desaminase supprimiert werden. Virusgenom-Sequenzierung und biostatistische Auswertung zeigten eine signifikante Genomhypermutation. Das akzessorische Protein Bet übernimmt - wie die vorliegende Studie zeigt - bei FV die Rolle von HIV-1 Vif und wirkt APOBEC3 entgegen. Über den Mechanismus der APOBEC3 Inhibition ist bislang fast nichts bekannt, er unterscheidet sich jedoch von dem bei HIV-1.

Die Inhibition von APOBEC3-Proteinen durch die FV Bet-Proteine ist spezies-spezifisch. Diese Spezifität ist ein weiterer Faktor den FV, die von Nicht-Primaten zum Menschen übertragen werden, überwinden müßten.

Einleitung

Foamyviren

Aus der Familie der Retroviren (*Retroviridae*) haben die Foamyviren (FV), die früher auch als Spuma- oder Spumaretroviren bezeichnet wurden, bislang wenig Beachtung gefunden (123), obwohl sie bereits 1954 in Affennierenzellkulturen beschrieben wurden (43). FV wurden phylogenetisch vor kurzem als eigene Unterfamilie klassifiziert: die *Spumaretrovirinae*. Sie sind bislang deren einzige Mitglieder und waren daher namensgebend. Diese neue Klassifizierung wurde aufgrund wesentlicher Besonderheiten (siehe unten) vorgenommen, die sie klar von allen anderen bekannten Retroviren unterscheiden, die in der Unterfamilie der *Orthoretrovirinae* zusammengefasst sind.

Ein Jahr vor der Entdeckung des ersten humanpathogenen Retrovirus, dem humanen T-Zell-Leukämie-Virus I (HTLV-I), isolierten Achong et al. 1971 das erste humane Foamyvirus-Isolat aus lymphoblastoiden Zellen eines kenianischen Nasopharynx-Karzinom-Patienten. Dieses und weitere Isolate aus Menschen (34, 149, 169, 180) wurden damals als humane Foamyviren (HFV) bezeichnet. Inzwischen ist unter anderem mittels Sequenzvergleichen gezeigt worden, dass es sich in allen daraufhin untersuchten Fällen um Interspezies-Übertragungen von FV aus Nicht-Humanen-Primaten handelte (Affenfoamyviren, simian foamy viruses, SFV) (60). Diese Isolate werden seither nicht mehr als HFV, sondern als Prototyp-Foamyvirus (PFV) bezeichnet.

Affen, Katzen, Rinder und Pferde sind die natürlichen Wirte der Foamyviren (85). In diesen Spezies haben sie eine hohe bis sehr hohe Prävalenz von 10 bis 70 % (31, 131). Die genauen Übertragungswege der FV sind nicht bekannt. Es wird jedoch angenommen, dass sie über oropharyngeale Gewebe ausgeschieden werden und dann über den Speichel durch Beißen und Lecken übertragen werden (27, 68, 73, 175).

Da bisher weder in natürlichen noch in zoonotisch infizierten Wirten eine Erkrankung mit FV assoziiert wurde, gelten sie als apathogen (85). *In vitro* führt eine Infektion mit FV hingegen zu deutlichen cytopathischen Effekten in Form von Zellfusionen benachbarter Zellen und dadurch zur Bildung sogenannter Riesenzellen (Syncytien) sowie zu Vakuolisierung und schließlich dem Zelltod. Das schaumartige Aussehen infizierter Zellkulturen war ausschlaggebend für die Namensgebung der FV (engl.: foam, lat.: spuma für Schaum). FV transgene Mäuse entwickelten schwere neurologische Krankheiten (5).

Aufgrund des asymptomatischen Verlaufs der Infektionen *in vivo* sind FV interessante Kandidaten für die Entwicklung von Vektoren für die Gentherapie (21, 113, 129). Erste Erfolge im Einsatz als Vektoren wurden bei einer Vakzinierungsstudie mit Katzen (140) und einer therapeutischen Studie mit Hunden (76) erzielt. In dieser Doktorarbeit wurden

Langzeitstudien mit neu entwickelten FFV basierten Vektoren durchgeführt (**Construction and Characterization of Efficient, Stable, and Safe Replication-Deficient Foamy Virus Vectors,** S. 62**).**

FV haben ein komplexes Genom und besitzen neben den für alle Retroviren typischen Genen *gag* (Strukturproteine), *pol* (enzymatische Proteine) und *env* (Hüll-Glykoproteine) regulatorische Gene am 3´-Ende. Da sie zwischen dem Env-Gen und dem 3`-LTR liegen, werden sie als *bel* (engl.: Between Env and LTR (Long Terminal Repeat)) Gene bezeichnet. Das virale Transaktivator-Protein Bel1, auch Tas (Trans-Aktivator der Spumaretroviren) genannt, wird sowohl für die Aktivierung des LTR Promotors als auch des internen Promotors, einer Besonderheit der FV (siehe unten), benötigt. Das Produkt des *bel2* Gens wurde bisher nicht identifiziert. Die Funktion von Bet, einem Protein, das durch Spleißen aus Teilen von *bel1* und *bel2* besteht, war lange Zeit unklar. Es wurde eine Rolle bei der Etablierung der viralen Persistenz postuliert (133, 134, 184). In dieser Arbeit wird gezeigt, dass Bet hauptsächlich als Gegenspieler einer zellulären, antiviralen Abwehr von Bedeutung ist.

Zu den Besonderheiten der FV gehört das Vorhandensein eines internen Promotors am 3´-Ende des *env* Gens. Durch den internen Promotor besteht die Möglichkeit einer zeitlich regulierten Transkription (88). Weitere Besonderheiten sind das Fehlen eines Gag-Pol-Fusionsproteins und der Pol-Expression von einem gespleißten Transkript. Im Gegensatz zu den Orthoretroviren fehlen bei den Gag-Proteinen der FV die Major Homology Region (MHR) in der Kapsid-Domäne und die Cystein-Histidin-Boxen in der Nukleokapsid-Domäne. Außerdem ist die proteolytische Gag-Prozessierung unvollständig und/oder zeitlich verzögert. Gag wird nicht in die Domänen Matrix, Kapsid und Nukleokapsid prozessiert. Stattdessem wird nur ein 4 kDa großes Fragment vom C-Terminus proteolytisch abgespalten (85). Des Weiteren läuft die Reverse Transkription zum Teil bereits in der Virus-produzierenden Zelle ab, wodurch ein beträchtlicher Anteil von Viruspartikeln DNA-Genome enthält (185). Diese frühe Reverse Transkription ist essentiell für die Infektivität der FV.

Das zoonotische Potential der Foamyviren

Das Neuauftreten oder Wiederauftreten von Infektionskrankheiten stellt eine permanente Gefahr für die Gesundheit der Weltbevölkerung sowie der Nutz- und Wildtiere dar. Der weltweite Reiseverkehr und Gütertransport haben das Risiko einer schnellen und globalen Ausbreitung von Infektionskrankheiten erhöht. SARS und der Erreger der Vogelgrippe H5N1 waren die jüngsten Bedrohungen mit Pandemie-Potenzial (94).

Da Viren nicht *de novo* entstehen, muß definitionsgemäß ein für eine Spezies sogenanntes „neues“ Virus immer von einer anderen Spezies stammen (164), also über die Spezies-Grenze (interspezies) übertragen werden. Bei manchen Viren gehört ein

Wirtswechsel auch zum regulären Ablauf des Replikationszyklus. In dieser Arbeit werden Interspezies-Übertragungen unabhängig von einer Krankheitsassoziation als zoonotische Übertragung bezeichnet. Während der langen Koevolution von Virus und Wirt bilden sich zahlreiche Wechselwirkungen aus (Koadaptation), durch die erst der komplexe Prozess der Virusreplikation ermöglicht wird. Dies führt zu einer Spezies-Spezifität (Einnieschung), die die Übertragung auf einen neuen Wirt erschwert (166). Dennoch überschreiten Viren und andere Pathogene sporadisch die Wirtsgrenzen zu einem neuen Wirt, einschließlich dem Menschen. Nur selten adaptieren sie sich so an den Menschen, dass eine weitere direkte Mensch-zu-Mensch-Ausbreitung möglich wird.

Viren, die die Wirtsschranken überschreiten, müssen viele Hürden überwinden: 1. Es muss zum Kontakt mit dem neuen Wirt kommen, 2. Die Viren müssen einen permissiven Zelltyp antreffen, in den sie eindringen können, 3. Sie müssen sich selbst vervielfältigen, 4. Sie müssen mögliche Abwehrmechanismen überwinden und 5. Sie müssen wieder aus der Zelle austreten und auf andere übertragen werden. Die nächst höhere Leistung ist der Übertritt auf einen weiteren Organismus.

RNA-Viren haben besonders viele Möglichkeiten entwickelt, sich schnell an neue Bedingungen anzupassen, sie gelten daher als die Viren mit dem größten zoonotischen Potential (110). RNA-Viren besitzen keine oder kaum Korrekturlesemechanismen bei der Replikation ihrer Genome (42). Deshalb entstehen während der Replikation viele Mutationen. Im infizierten Wirt existieren parallel zahlreiche RNA-Viren als Quasi-Spezies mit vielen genetischen Unterschieden. Bei einer Veränderung der Umgebungs-bedingungen kann sich eine Variante mit vorteilhaftem genetischem Programm schnell vervielfältigen (41).

FV haben verglichen mit den anderen Retroviren ein sehr konserviertes Genom (21). Obwohl dies gegen die schnelle Anpassung an einen neuen Wirt spricht, ist wiederholt gezeigt worden, dass von wilden und in Gefangenschaft lebenden Affen FV auf Menschen übertragen worden sind. Erste Untersuchungen deuteten darauf hin, dass das Prototyp-FV zum Teil stark verbreitet war (1, 2, 92, 95, 111). Dies konnte nicht bestätigt werden, und heute geht man von einer sehr geringen Prävalenz aus (6, 28, 112, 143, 144). Die betroffenen Personen sind Affen-Jäger in Afrika, die Affenfleisch als Nahrung zubereiten, Zootierpfleger, die berufsbedingt Kontakt zu Affen haben und Besucher von sog. Affentempeln. Insbesondere bei der Bevölkerung Zentralafrikas ist aufgrund der mangelnden Gesundheitsversorgung mit einer möglicherweise hohen Zahl unidentifizierter SFV-infizierter Personen zu rechnen. Zwei bis vier Prozent der untersuchten Tierpfleger weisen ein klare, gegen PFV/SFV, nicht aber gegen andere Affenretroviren gerichtete, Seroreaktivität auf (26, 61, 135). Es wurde gezeigt, dass die auf den Menschen übertragenen SFV von Schimpansen (*Pan troglodytes*), Pavianen (*Papio sp.*), Gorillas (*Gorilla gorilla*), Mandrillen (*Mandrillus sphinx*), Makaken (*Macaca sp.*) und afrikanischen Grünen Meerkatzen (*Cercopithecus aethiops*) stammten. Die Tatsache, dass von verschiedenen Primatenspezies, die jeweils ihr

eigenes, genetisch und antigenetisch klar unterscheidbares SFV haben, Interspezies-Infektionen des Menschen stattgefunden haben, verdeutlicht das allgemein hohe zoonotische Potential der SFV. Bissverletzungen, Kontakt zu möglicherweise infektiösem Affenspeichel (45) oder zu Körperflüssigkeiten während des Schlachtens und Xenotransplantationen von Pavianorganen waren ursächlich für die SFV-Infektionen (9, 61, 177). Die Infektion verlief auch nach zoonotischer Übertragung im Menschen persistent mit permanent hohem Spiegel PFV-spezifischer Antikörper. Während der Infektion wurden intakte und unvollständige Genome verbreitet. In einigen Fällen wurden infektiöse Viren isoliert (23, 32, 141). Bei keinem der wenigen bekannten Fälle wurde die zoonotische SFV-Infektion mit einer Erkrankung des infizierten Menschen in Zusammenhang gebracht. Weder Familienmitglieder noch Empfänger von Blutprodukten SFV-infizierter Menschen wurden positiv auf SFV getestet (23). Kürzlich wurde jedoch gezeigt, dass SFV durch Bluttransfusion zwischen Rhesusmakaken übertragbar ist (74). Bis heute wurde keine weitere Ausbreitung der auf Menschen übertragenen SFV beobachtet. Aufgrund der geringen Anzahl dokumentierter Fälle kann noch kein abschließendes Urteil über die Gefährdung durch auf den Menschen übertragene SFV gefällt werden. Für eine dauerhafte Etablierung von SFV im Menschen könnte entscheidend sein, dass die meisten der bekannten SFV-Infektionen beim Menschen in Zentralafrika auftraten, einer Region in der auch HIV-Infektionen häufig sind. Wie sich eine Doppelinfektion mit HIV und SFV beim Menschen auswirkt, ist nicht bekannt. Es wäre beispielsweise denkbar, dass sich SFV in den durch eine HIV-Infektion immunsupprimierten Patienten leichter adaptiert und etabliert (168).

Das zoonotische Potential der Nicht-Primaten-FV von Katzen (felines Foamyvirus, FFV), Rindern (bovines Foamyvirus, BFV) und Pferden (equines Foamyvirus, EFV) ist kaum untersucht worden. In den westlichen Industrienationen ist der Kontakt zu Nicht-Humanen-Primaten auf eine sehr geringe Zahl von Personen beschränkt. Im Gegensatz dazu ist in diesen Ländern beinahe die gesamte Bevölkerung den Pathogenen von Rindern ausgesetzt. Ein Teil der Bevölkerung kommt durch Haustierhaltung (Katze, Pferd) in den Kontakt mit FV infizierten Tieren. Die Zoonose-Gefährdung besteht entweder beim Kontakt mit FFV-, BFV- oder EFV- kontaminierten Körperflüssigkeiten der infizierten Tiere oder indirekt durch Nahrung oder medizinische Produkte, die von infizierten Tieren stammen. Theoretisch können auch Blut- oder Organspenden FV infizierter Personen eine Gefährdung darstellen.

Bei Katzen wurde FFV erfolgreich aus Rachenabstrichen isoliert (8) und käme demnach bei Bissverletzungen in direkten Kontakt mit permissiven Zellen. Bei Rindern wurde ebenfalls diskutiert, ob BFV im Speichel vorkommt und durch Niesen von Tier zu Tier übertragen wird (67). Eine Übertragung über die Ernährung ist jedoch wahrscheinlicher. Es wurde nämlich bereits gezeigt und ebenfalls in dieser Arbeit bestätigt, dass BFV-Infektiösität in der Rohmilch vorkommt (96). Die Wahrscheinlichkeit einer erfolgreichen Interspezies-Übertragung hängt zwar von der Nähe der genetischen Verwandtschaft ab, ist also vom Affen zum Menschen wahrscheinlicher als von Katze

oder Rind, dies schließt eine solche Übertragung jedoch nicht aus. Dies zeigt sich beispielweise bei Hantaviren, die von Nagetieren auf den Menschen übertragen werden. Basierend auf den jetzigen Erkenntnissen wird der noch unbekannte Rezeptor, den FV für den Eintritt in die Zielzellen benötigen, auf sehr vielen, auch humanen Zelllinien, exprimiert und macht diese dadurch permissiv.

Die Erkenntnisse zum zoonotischen Potential der Nicht-Primaten Foamyviren sind in dem von uns verfaßten Review-Artikel **„Das zoonotische Potential animaler Foamyviren“ (125) (**S. 49**)** zusammengefaßt.

Um die Prävalenz der Nicht-Primaten-FV in ihren Wirten zu ermitteln und mögliche zoonotische Übertragungen im Menschen zu identifizieren, werden sensitive und spezifische Diagnoseverfahren benötigt, die für ein Screening großer Probenzahlen geeignet sind. Ein solches Verfahren dient zunächst der Identifizierung potentiell positiver Proben, die anschließend durch weitere Tests bestätigt bzw. ausgeschlossen werden. Die Diagnostik für FV wurde überwiegend mittels indirekter Immunfluoreszenz oder dem sogenannten Ouchterlony-Test durchgeführt. Diese Verfahren sind in Zusammenhang mit FV-Diagnostik weder sehr spezifisch noch sensitiv. Das Standard-System für die Detektion FV-spezifischer Antikörper ist momentan der Western Blot. Bislang wurden für FV-diagnostische Western Blots Antigene, die aus mit einer oder mehreren FV-Spezies infizierten Zellkulturen stammten, verwendet. Die genannten Tests sind sehr zeit- und kostenintensiv und daher nicht geeignet für einen hohen Probendurchsatz. Als Methode, mit der Möglichkeit eine große Anzahl von Seren zu untersuchen, wurde für FFV ein ELISA entwickelt, der aufgrund eines hohen Hintergrundes relativ unsensitiv ist (176). Als sensitive Methode wird die Polymerasekettenreaktion (PCR) eingesetzt, sie ist jedoch anfällig für Kontaminationen und deswegen alleine nur bedingt aussagekräftig. Virus-Isolierung ist die sicherste Methode, um eine Infektion festzustellen, sie ist aber extrem arbeitsaufwendig. Man braucht darüber hinaus Kenntnis über den Ort der Virusfreisetzung. Bei zwei Studien mit Veterinären wurden keine Marker einer FFV Infektion festgestellt (30, 176). Studien zu BFV und EFV gibt es nicht. Da bisher keine FFV- oder BFV-infizierten Menschen identifiziert wurden, finden sich auch keine Anhaltspunkte, ob eine solche Infektion mit klinischen Symptomen verknüpft ist. Katzenpathogene im Allgemeinen stehen unter dem Verdacht, ein Risikofaktor bei der Entwicklung komplexer, von vielen Faktoren abhängiger Krankheiten wie zum Beispiel Schizophrenie zu sein (153).

Dies war einer der Gründe für die Entwicklung neuer, sensitiverer Diagnose-Verfahren für FFV und BFV, die in dieser Promotionsarbeit beschrieben werden. Die ELISAs bieten insbesondere die Möglichkeit, mit vertretbarem Zeit- und Kostenaufwand viele Proben zu untersuchen **(Antibodies against Gag are diagnostic markers for feline foamy virus infections while Env and Bet reactivity is undetectable in a substantial fraction of infected cats (S. 21), Serological detection systems for identification of bovine foamy virus-infected cattle facilitate screening of cows shedding bovine foamy virus via milk (S. 28))**.

APOBEC3 Proteine als Abwehrsystem gegen retrovirale Infektionen

Zur Abwehr mikrobieller Pathogene haben höhere Organismen während ihrer Evolution ein großes Repertoire unterschiedlicher Verteidungs-Mechanismen entwickelt. Diese gehören entweder zur erworbenen, adaptiven Immunantwort wie Antikörper und zytotoxische T-Zellen, oder zum angeborenen („innate“) Immunsystem. Das angeborene Immunsystem wurde in den vergangenen Jahren verstärkt untersucht. Die dafür notwendigen Verfahren stehen erst seit kurzem zur Verfügung. Zu den Komponenten des angeborenen Immunsystems gehören unter anderem antimikrobielle Peptide, proteolytische Kaskaden, Signalmoleküle wie Interferone und spezielle phagozytierende Zellen (17, 58). Zu den neuesten Entdeckungen zählt eine Gruppe von Proteinen mit dem Namen APOBEC3. Dies sind Enzyme mit Cytosin-Desaminase-Aktivität. Bei HIV-1 wurde gezeigt, dass APOBEC3F und APOBEC3G den HIV-Replikationszyklus zum Erliegen bringen. Die APOBEC3 Proteine werden in der virusproduzierenden Zelle exprimiert und in die Viruspartikel verpackt. In der nächsten Zielzelle bewirken sie während der reversen Transkription, in dem zuerst gebildeten einzelsträngigen DNA-Strang (Minus-Strang) eine Desaminierung von Cytosinen zu Uracil. Das führt dazu, dass nachfolgend Uracil von der viralen Reversen Transkriptase als Thymin erkannt und anstelle eines Guanins ein Adenin in den plus DNA-Strang eingebaut wird. Dadurch kommt es zu einer exzessiven Anhäufung von G zu A-Mutationen im Virusgenom, was schließlich zum völligen Informationsverlust des Genoms führt. Die Uracile im proviralen DNA-Genom induzieren zudem eine Degradation durch Uracil-N-Glycosylasen. Um diesem Abwehrmechanismus zu entgehen, kodiert HIV-1 für das kleine, akzessorische Protein Vif (virion infectivity factor) (148). Vif bindet an APOBEC3G und rekrutiert einen Ubiquitinligase-Komplex, der APOBEC3 durch Polyubiquitinierung zum 26S-Proteasom steuert, wo es degradiert wird (186). Abgesehen davon hemmt Vif die Neusynthese von APOBEC3 (150). Die Abwehr der antiretroviralen Wirkung von APOBEC3 durch Vif ist weitgehend spezies-spezifisch. Vif aus HIV-1 neutralisiert nur humanes und Schimpansen-APOBEC3, nicht aber die APOBEC3-Proteine von afrikanischen Grünen Meerkatzen oder Rhesus-makaken. Umgekehrt sind die Vif-Proteine von Makaken- und Schimpansen-SIV aktiv gegen humanes APOBEC3G, nicht aber das Vif von Grünen Meerkatzen-SIV. Dies könnte eine Erklärung dafür sein, dass es keine zoonotischen Übertragungen von grünen Meerkatzen-SIV zum Menschen gegeben hat, jedoch SIV von Makaken der Ursprung von HIV-2 beim Menschen sein konnte (37).

Neben der Aktivität als Cytosin-Desaminase wirkt APOBEC3G auch nicht-enzymatisch antiretroviral. Die zugrunde liegenden Mechanismen sind noch nicht aufgeklärt (18).

In Zusammenarbeit mit C. Münks Arbeitsgruppe am Paul Ehrlich Institut zeigten wir, dass FFV durch APOBEC3 inhibiert wird und dass das akzessorische FFV-Protein Bet der molekulare Gegenspieler des felinen APOBEC3 ist. (**The antiretroviral activity of APOBEC3 is inhibited by the foamy virus accessory Bet protein,** Seite 43)

Ergebnisse und Diskussion

Antibodies against Gag are diagnostic markers for feline foamy virus infections while Env and Bet reactivity is undetectable in a substantial fraction of infected cats

Retroviren im allgemeinen und FV im speziellen gehören zu den Viren mit einem hohen zoonotischen Potential. Dies verdeutlichen inzwischen zahlreiche Berichte über die Identifizierung von Menschen, die durch den Kontakt zu Primaten mit SFV infiziert wurden (61, 177). Das Nicht-Primaten-FV aus Katzen, FFV, ist diesbezüglich kaum untersucht. Bei zwei Studien mit Veterinären wurden keine Antikörper-Träger identifiziert. Entweder haben keine Übertragungen stattgefunden oder die verwendeten Tests waren nicht sensitiv genug. Die große phylogenetische Distanz zwischen Mensch und Katze erschwert möglicherweise eine Direktübertragung. Der verwendete ELISA (176) hat eine hohe Hintergrundaktivität und Western Blots mit infizierten Zellen als Antigen sind aufgrund vieler unspezifischer Reaktionen nicht empfindlich genug. In dieser Doktorarbeit wurde deshalb erst für FFV und später auch für BFV ein sensitives und spezifisches Diagnoseverfahren entwickelt.

Der entwickelte Assay beruht auf dem Prinzip eines für humane Papillomviren (HPV) entwickelten modularen Glutathion-S-Transferase (GST) „Capture“-ELISA (146). Western Blots und frühere Untersuchungen mit anderen FV wiesen auf Gag als ideales diagnostisches Antigen hin. Zusätzlich wählten wir die Ektodomäne des Hüllproteins Env und das akzessorische Protein Bet als Antigene. Diese Proteine wurden als GST Fusionsproteine in *E. coli* exprimiert und an zuvor mit Glutathion-Casein beschichtete ELISA Platten gebunden. So wurde eine *in situ*-Aufreinigung der GST-Fusionsproteine erzielt. Anschließend wurden Serumproben inkubiert und gebundene Antikörper mittels eines an Peroxidase gekoppelten sekundären Antikörpers bzw. an Protein A gekoppelte Peroxidase detektiert. Der Assay wurde mit Referenzseren experimentell und natürlich infizierter Katzen validiert. Dabei bestätigte sich, dass in den Seren FFV-infzierter Katzen gegen Gag gerichtete Antikörper vorherrschen. In den natürlich infizierten Tieren wurden auch Bet-Antikörper konsistent nachgewiesen. Env-Antikörper waren in zwei Serumproben detektierbar, die von natürlich infizierten Katzen stammten. Die Antikörperlevel in den Seren der experimentell infizierten Katzen waren einheitlich niedriger als bei natürlich infizierten Katzen. Um die Sensitivität des Tests zu ermitteln, wurden Referenzseren mit unterschiedlich hohen FFV-Antikörperkonzentrationen seriell verdünnt eingesetzt. Starke Gag-Reaktivitäten konnten bis zu einer 1600 fachen Verdünnung der Seren klar nachgewiesen werden. Um jedoch auch niedrigere Antikörperlevel detektieren zu können, sollten die Seren nicht mehr als 1:100 verdünnt werden (Daten nicht gezeigt).

Bei einer Feldstudie mit 99 schweizer Katzen wurde eine FFV-Prävalenz von 36% bestimmt. Der „Cutoff“-Wert (Schwellenwert) für Gag-Reaktivitäten wurde aufgrund der klar bimodalen Verteilung der Antikörperreaktivitäten sowohl graphisch als auch rechnerisch ermittelt. Für Bet und Env wurde der Cutoff-Wert, aufgrund einer gleichmäßigeren Verteilung rechnerisch als Mittelwert plus drei Standardabweichungen der Gag-negativen Proben ermittelt. FFV-Infektionen waren in dieser Studie nicht klar mit einer Erkrankung assoziiert. Um eine solche Assoziation nachzuweisen und um zu zeigen, ob beispielsweise FFV und feline Immundefizienzvirus-Infektionen (FIV) häufig zusammen auftreten, werden größere epidemiologische Studien benötigt.

Einzelne Serumproben wurden parallel zu den ELISA-Daten im Western Blot analysiert. Hierbei verwendeten wir als Antigen, im Gegensatz zu in früheren Publikationen verwendeten Lysaten FFV-infizierter Zellen, durch Ultrazentrifugation angereicherte Virionen. Hierdurch wurden unspezifische Hintergrundreaktivitäten stark reduziert. Das Ergebnis war klar ablesbar: Western Blot und ELISA Ergebnisse stimmten in allen Fällen überein, wobei der ELISA bezüglich der Empfindlichkeit dem Western Blot überlegen war. Das akzessorische Protein Bet kann zudem ausschließlich im ELISA detektiert werden, weil es kein Bestandteil von Virionen ist (91).

Mit dem Konjugat aus Protein A und Meerrettich-Peroxidase werden IgG-Moleküle aller Isotypen erkannt. Um spezifisch IgM, das als erstes nach einer Infektion gebildet wird, zu detektieren, wurde ein spezifisch gegen Katzen-IgM gerichteter sekundärer Antikörper verwendet. Sequentiell nach experimenteller Infektion von Katzen genommene Serumproben zeigten die Funktionalität des IgM-spezifischen Tests (8). Der Level von FFV-Gag-spezifischen IgM-Antikörpern kumulierte bereits 14 Tage nach Infektion, wohingegen IgG-Antikörper erst langsam ab dem 14. Tag auftraten. Diese Resultate wurden im Western Blot bestätigt. Unter den Feldseren befand sich keines mit IgM-Reaktivität, was ein Hinweis darauf ist, dass kein Tier frisch infiziert war.

Der entwickelte FFV-Assay besitzt eine hohe Sensitivität und er ist aufgrund der Verwendung dreier verschiedener viraler Antigene sehr spezifisch. Diese Eigenschaften machen ihn zu einem idealen Werkzeug bei der Suche nach zoonotischen FFV-Infektionen im Menschen.

Serological detection systems for identification of bovine foamy virus-infected cattle facilitate screening of cows shedding bovine foamy virus via milk.

Der Anteil der Bevölkerung mit Kontakt zu Rindern und vor allem Produkten von Rindern, wie Fleisch und Milchprodukten, ist weitaus größer als derjenige mit Katzen als Haustiere. Eine möglichst umfassende Kenntnis über die Pathogene von Rindern ist daher wichtig. Obwohl frühere Studien Prävalenzen von bis zu 90% ermittelt hatten,

wurde das bovine Foamyvirus (BFV) im Gegensatz zu den meisten anderen Rinderinfektionen kaum untersucht.

Bei dem von uns entwickelten FFV-ELISA (siehe oben) wurden für die Detektion von BFV Gag-, Bet- und Env-Antikörpern die drei Antigene gegen die äquivalenten Antigene von BFV ausgetauscht. Für die Validierung des Assays wurden definierte aus Polen stammende Referenz-Rinderseren verwendet. Die Referenztiere waren zuvor mittels zweier „*nested*“ PCRs, eines auf BFV-infizierten Zellen basierten ELISA und eines BFV-spezifischen Agar-Immundiffusionstests auf ihren BFV-Infektionsstatus untersucht worden. Standard-„Cutoff“-Werte für alle drei Antigene wurden aus dem Mittelwert plus drei Standardabweichungen (M+3SD) der definiert BFV-negativen Proben errechnet.

Mit dem neuen BFV-ELISA wurden 89 Rinderfeldseren aus Polen analysiert. Zusätzlich lagen die Ergebnisse des „*nested*“ PCR Tests (publiziert an anderer Stelle) vor. Die Prävalenz von BFV-Gag-Antikörpern lag bei 42%. Gag-Seroreaktivitäten waren im Gegensatz zu Bet- und Env-Reaktivitäten bimodal verteilt. Da sowohl ein Referenzserum als auch eines der Feldseren Gag-Werte nahe dem Grenzwert aufwiesen, wurden die Ergebnisse unter Verwendung eines stringenteren (doppelten) Cutoff (2x(M+3SD)) neu evaluiert. Alle Gag-positiven BFV-Referenztiere blieben weiterhin oberhalb des Grenzwerts, wohingegen ein Feldserum mit leicht erhöhter Gag-Reaktivität negativ wurde. Da dieses Tier auch mittels BFV-spezifischer PCR negativ getestet wurde und keine erhöhten Bet und Env Seroreaktivitäten aufwies, wurde der BFV-Infektionsstatus negativ bewertet. Die positive Korrelation mit den Ergebnissen der diagnostischen PCR war 100% bei Verwendung des stringenten Grenzwerts und 97,4% bei Verwendung des Standard-Grenzwerts. Unabhängig vom verwendeten Grenzwert waren bei fünf ELISA-negativen Proben die PCR Ergebnisse positiv. Keine dieser Proben hatte Seroreaktivitäten im Grenzbereich, weshalb falsch-positive PCR Ergebnisse angenommen werden. Wie bei dem oben beschriebenen ELISA für FFV war Gag das diagnostische Antigen der Wahl.

Für andere Infektionen des Rinds war gezeigt worden, dass spezifische Antikörper auch in Milch detektiert werden können. Der ELISA wurde deshalb für die Untersuchung der zu den oben analysierten Serumproben gehörenden Milchproben eingesetzt. Standard- und stringente „Cutoff“-Werte für den Milchassay wurden aus den Milch-Assay-Werten der BFV-Gag-negativen Feldserumproben errechnet. Milch- und Serum- Gag-Ergebnisse stimmten unabhängig vom verwendeten „Cutoff“ in hohem Maß überein. Bei den beiden anderen Antigenen war die Übereinstimmung geringer. Entsprechend dieser Ergebnisse ist auch im Milchassay Gag das diagnostische Antigen der Wahl. Die Bestimmung von Milch-Gag-Antikörpern ist somit als Ersatz von Serumanalysen geeignet.

Anschließend wurden Seren von deutschen Rindern (n=190) untersucht. Dabei wurde eine von den polnischen Proben abweichende Verteilung der Seroreaktivitäten beobachtet. Die deutschen Seroreaktivitäten waren nicht bimodal, sondern im gesamten

Wertebereich verteilt. Bei Anwendung des stringenten „Cutoffs“ ergab sich eine Prävalenz von 6,8%. Bei 8 der 13 Gag-positiven Tiere wurde die Diagnose durch zusätzliche positive Bet Reaktivitäten bekräftigt.

Milch-Gag-Reaktivitäten der Gag-seropositiven deutschen Proben waren vollständig konsistent. Bei den Proben mit Gag-Werten zwischen Standard- und stringentem Cutoff wurden keine BFV-spezifischen Antikörperreaktivitäten in der Milch festgestellt.

Bei 18 Rindern aus Baden-Württemberg wurden parallel zu den Antikörper-Nachweisen Blut, Milch und Speichel auf BFV-Infektiösität untersucht. Die Proben wurden dazu mit BFV-permissiven Hundethymuszellen (Cf2Th) bis zu vier Wochen kokultiviert. Während dieser Zeit wurden die Zellkulturen regelmäßig mikroskopisch auf virusinduzierte cytopathische Effekte untersucht. Außerdem wurden in größeren Abständen und nach Abschluß des Experiments direkte Immunfluoreszenzen mit einem FITC-gekoppelten BFV-Referenzserum durchgeführt. Sechs der 18 Seren hatten erhöhte Gag-Seroreaktivitäten. Drei davon hatten Gag-Werte deutlich über 500 mOD_{450}, und zusätzlich auch einen positiven Milchassay Gag-Wert, und drei Gag-Serum-Werte lagen zwischen 200 und 500 mOD_{450}. Bei allen drei stark seropositiven Tieren wurde infektiöses BFV aus den PBL (Peripheral Blood Lymphocytes) Zellen isoliert und in zwei Fällen auch aus der Milch. Aus den Proben mit intermediär reagierenden und mit negativen Seren wurde in keinem Fall BFV isoliert. Das Ergebnis steht im Widerspruch zu einer früheren Veröffentlichung, in der BFV auch im Speichel infizierter Tiere gefunden worden war (Kertayadnya et al., 1988).

Der neu entwickelte ELISA für die Serumdiagnostik von BFV-Infektionen bei Rindern zeichnet sich durch eine hohe Sensitivität aus. Dies zeigt die hohe Übereinstimmung mit den Ergebnissen der empfindlichen „*nested*“ PCR. Die Verwendung multipler Antigenreaktivitäten für die Beurteilung macht auf Kreuzreaktivität beruhende falsch positive Diagnosen unwahrscheinlich. Bet-spezifische Antikörper stellen einen idealen Bestätigungsmarker dar.

Milchproben sind einfach zu gewinnen und eignen sich für eine schnelle Bestimmung klar BFV-positiver Tiere aus einem großen Kollektiv. Positive Milch-ELISA-Ergebnisse korellierten häufig mit Virusfreisetzung in die Milch.

Die durchgeführten Feldstudien verdeutlichen, dass BFV insbesondere in Polen eine hohe Prävalenz (>40%) hat. Die Unterschiede zwischen Polen und Deutschland reflektieren möglicherweise unterschiedliche Haltungs- und Fütterungsbedingungen in den zwei Ländern. Da aus Milch BFV-Infektiosität isoliert werden konnte, besteht theoretisch die Möglichkeit einer Transmission über Milch. Wenn dies auch nicht automatisch zu einer produktiven Infektion führen muß, so kann zumindest eine humorale Immunantwort induziert werden. Intermediär reagierende Seren repräsentieren möglicherweise durch Antigenaufnahme induzierte Immunantworten.

Personen, die Rohmilch BFV-infizierter Rinder zu sich nehmen, kommen in Kontakt mit BFV-Infektiösität. Ob es dabei zu Neuinfektionen kommt, wird in einer weiterführenden Studie momentan untersucht.

The antiretroviral activity of APOBEC3 is inhibited by the foamy virus accessory Bet protein

Kürzlich wurde gezeigt, dass zelluläre Cytidin-Desaminasen der APOBEC3-Familie die Genome verschiedener Orthoretroviren während der Reversen Transkription durch Mutation funktionell inaktivieren. Bei der DNA-Minusstrangsynthese werden Cytosine durch Desaminierung in Thymine umgewandelt. Dies führt zur Akkumulation von G nach A Transitionen im Plusstrang des Virusgenoms wodurch aufgrund des Verlusts korrekter Erbinformation der Replikationszyklus zum Erliegen kommt. Lentiviren verhindern dies mit Hilfe des akzessorischen Proteins Vif, das APOBEC dem proteolytischen Abbau zuführt. In dieser Veröffentlichung beschreiben wir, dass das feline Foamyvirus, ein Mitglied der Retrovirus-Unterfamilie *Spumaretrovirinae*, ebenfalls empfindlich gegenüber Genomdesaminierung durch ein hier erstmals beschriebenes felines APOBEC3 ist und dass das FV-Bet-Protein diesen antiretroviralen Mechanismus inhibiert.

CRFK Katzennierenzellen (Crandell Feline Kidney Cells) sind nicht permissiv für Bet-defizientes FFV und auch nicht für Vif-defizientes felines Immundefizienzvirus (FIV) (7, 147). In Anbetracht der neuen Erkenntnisse über die Funktion von HIV-1 Vif (siehe Einleitung) (148) wurden Bet-defiziente FFV Mutanten molekular-virologisch untersucht. Nach Infektion von CRFK-Zellen mit Wildtyp (WT) und Bet-mutierten FFV wurde ein bis zu 1000-fach reduzierter Virustiter bei den Bet-Mutanten festgestellt.

Eine Besonderheit der FV ist das Vorhandensein proviraler DNA bereits in den Virionen. Teilweise wird auch gespleißte RNA revers transkribiert und intronfreie DNA verpackt.

Vollständige Bet-WT und Bet-Mutanten-Genome wurden in CRFK-Zellen transfiziert. Drei Tage nach Transfektion wurden die neu gebildeten Viren durch Ultrazentrifugation des Zellkulturüberstands angereichert und geerntet. Mittels PCR wurde der das Intron umfassende Bereich der WT beziehungsweise Bet-mutierten Virionen-DNA amplifiziert, kloniert und sequenziert. Gespleißte Genome waren ein Garant dafür, dass nicht transfizierte Plasmid-DNA als Matrize gedient haben konnte, sondern nur neu replizierte DNA. WT-Genome waren nur sehr selten mutiert. Bet-Mutanten-Genome hatten eine deutlich erhöhte Mutationsrate mit bevorzugt G zu A-Transitionen im DNA-Plusstrang. Des Weiteren wurde gezeigt, dass diese Mutationen bevorzugt im Minus-DNA-Strang-Kontext Pyrimidin/Pyrimidin/Cytosin (mutiert nach Pyrimidin/Pyrimidin/Thymin) auftraten. Dieser Kontext ist für die APOBEC3F vermittelte Editierung bei HIV-1 typisch (57, 181). Der Befund ist ein deutlicher Hinweis auf das Vorhandensein einer APOBEC3-Desaminase in CRFK-Zellen und darauf, dass FFV-

Bet der virale Gegenspieler dieser APOBEC3-Desaminase ist. Um eine Rolle von Bet bei der Genauigkeit der Reversen Transkription auszuschließen, wurden WT und Bet-mutierte FFV-Genome in APOBEC3-freie 293T Zellen transfiziert und die Genome auf Mutationen untersucht. Die Mutationsrate war sehr niedrig und in beiden Gruppen identisch, womit eine solche Rolle Bets ausgeschlossen werden konnte.

Unser Kollaborationspartner C. Münk vom Paul-Ehrlich-Institut (PEI) identifizierte, klonierte und charakterisierte daraufhin mittels PCR mit von humanem APOBEC3G abgeleiteten, degenerierten Primern und RACE Techniken ein vollständiges felines APOBEC3 (fe3).

Um zu überprüfen, ob die oben beschriebenen Auswirkungen reproduziert werden können, transfizierten wir das klonierte feline APOBEC3 zusammen mit WT und Bet-mutierten FFV-Genomen in APOBEC3-defiziente 293T Zellen. Dies führte beim WT zu einer bis zu 10-fachen, bei dem Bet-mutierten Klon zu einer 100 bis 1000-fachen Titer-Reduktion. Gleichzeitig wurde bei den Viren mit mutiertem Bet erneut eine drastisch höhere Genomeditierung mit Guanin zu Adenin-Austauschen festgestellt. C. Münk und seine Mitarbeiter zeigten per Coimmunpräzipitation, dass Bet speziesspezifisch an fe3, nicht aber an humanes APOBEC3G bindet. Wir bewiesen wiederum, dass Bet selbst nicht in Viruspartikel verpackt wird. Ein intaktes, nicht aber ein mutiertes Bet verhinderte die Verpackung von fe3 in FFV-Virionen.

Die Daten belegen, dass fe3 für Genomhypermutation und verminderte Partikelfreisetzung von FFV mit defektem Bet verantwortlich ist. Im Gegensatz zu den Orthoretroviren findet bei FFV die fe3-vermittelte Genomdesaminierung bereits in den Virus-produzierenden Zellen während der für FV typischen früh erfolgenden Reversen Transkription statt. Bet bindet speziesspezifisch an fe3 und verhindert zum einen dessen Verpackung in Partikel und zum anderen auch auf bislang unbekannte Weise dessen schädliche mutagene Wirkung. Eine Degradation von fe3, wie sie das lentivirale Vif bei humanem APOBEC3G auslöst, wurde nicht beobachtet. Möglicherweise bewirkt Bet die Sequestrierung von fe3 in einem anderem Kompartiment der Zelle als dem, in dem die foamyvirale Partikelbildung abläuft.

Wie schon analog für die Vif-Proteine der verschiedenen SIV gezeigt wurde, neutralisiert auch FFV-Bet sehr speziesspezifisch das feline APOBEC3, nicht aber humanes APOBEC3G. Diese Speziesspezifität macht eine erfolgreiche Replikation nach zoonotischer Übertragung zum Menschen oder anderen Spezies unwahrscheinlicher. Wie jedoch für APOBEC3G gezeigt wurde, ist bereits ein einziger Aminosäureaustausch ausreichend, um dessen Speziesspezifität zu ändern (138). Minimale Veränderungen der beteiligten Proteine erlauben so unter Umständen eine dramatische Veränderung des Wirtspektrums eines Virus.

Construction and characterization of efficient, stable, and safe replication-deficient foamy virus vectors

FV sind aufgrund ihrer besonderen Eigenschaften wie 1. Apathogenität im natürlichen Wirt und nach Interspezies-Übertragung (60, 131), 2. der hohen Wahrscheinlichkeit der Integration außerhalb kodierender Genomabschnitte (115, 156), 3. der großen Verpackungskapazität (105) und 4. ihrer hohen physischen und genomischen Stabilität (142) vielversprechende Kandidaten für die Entwicklung von Vektoren für die Gentherapie. Trotz des bereits hohen Sicherheitsprofils der authentischen FV werden Modifikationen zur Erhöhung der biologischen Sicherheit untersucht. Das Ziel dieser Bemühungen ist durch Deletion möglichst großer Abschnitte des viralen Genoms die Vektoren replikationsinkompetent zu machen und das Risiko der Revertantenbildung durch unerwünschte Rekombinationsereignisse zu minimieren. Außerdem soll durch die Deletion viraler Gene die Kapazität zur Aufnahme fremder, z.B. therapeutischer Gene erhöht werden. Wichtig bei der Entwicklung von Vektoren für die Anwendung im Tier oder Menschen, ist auch die Optimierung des Vektortiters.

In einer unserer früheren Studien wurde gezeigt, dass auf FFV basierende selbstinaktivierende Vektoren (SIN) schnell replikationskompetente Revertanten erzeugen (14). In dieser neuen Studie wurden daher SIN-Vektoren mit zusätzlicher Deletion des *env* und der regulatorischen und akzessorischen *bel*-Gene entwickelt. Als Reportergene dienten beta-Galactosidase bzw. das Green fluorescent protein (GFP).

Als Nebenprojekt meiner Doktorarbeit wurden von mir Vektor-Transfektionen, Transduktionen von Katzennierenzellen (CRFK) sowie Langzeituntersuchungen zur Expressionsstabilität des Markergens und zur Vektor-Sicherheit durchgeführt. Die Versuche umfaßten klassische Zellkulturexperimente und FACS-Analysen. Dabei zeigte sich, dass der Anteil der transduzierten Zellen, die das Markergen exprimieren, leicht rückläufig ist.

Die neuen FFV-Vektoren besitzen aufgrund der Env/Bel-Deletion eine größere Verpackungskapazität als frühere Konstrukte. Auch nach mehrwöchiger Kultivierung transduzierter Zellen wurden keine replikationskompetenten Revertanten detektiert und auch eine Überinfektion mit dem WT-Virus führte nicht zur Reaktivierung. Dies verdeutlicht das sehr hohe Sicherheitsprofil der neuen Vektoren.

Darstellung der Ergebnisse als Publikationen und Publikationsmanuskripte in englischer und deutscher Sprache

1. **Antibodies against Gag are diagnostic markers for feline foamy virus infections while Env and Bet reactivity is undetectable in a substantial fraction of infected cats.** S. 21

2. **Serological detection systems for identification of bovine foamy virus-infected cattle facilitate screening of cows shedding bovine foamy virus via milk.** S. 28

3. **The antiretroviral activity of APOBEC3 is inhibited by the foamy virus accessory Bet protein.** S. 43

4. **Das zoonotische Potential animaler Foamyviren.** S. 49

5. **Construction and characterization of efficient, stable, and safe replication-deficient foamy virus vectors.** S. 62

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Antibodies against Gag are diagnostic markers for feline foamy virus infections while Env and Bet reactivity is undetectable in a substantial fraction of infected cats

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Abstract

Spumaretroviruses or foamy viruses constitute a distinct subfamily of retroviruses. The biology of foamy viruses within the authentic host, their mode of transmission, and disease potential in the authentic host or after zoonotic transmission into human or other species are almost unknown. Using feline foamy virus (FFV) as model system, we established modular enzyme-linked immunosorbent assays (ELISA) suited to determine feline IgG and IgM antibody responses against structural and non-structural FFV proteins. We validated the ELISAs with standard reference sera. In 99 cats admitted to a Swiss veterinary hospital, overall FFV Gag antibody prevalence was 36%, reactivity against Env and the non-structural protein Bet each was about 25%, and 19% of the sera were directed against all three FFV antigens. With one exception, all Bet- and/or Env-positive sera were also positive for Gag. In this small epidemiological pilot study, FFV antibodies were not significantly associated with clinical disease.

Keywords: Foamy virus; ELISA; Humoral immunity; Diagnostic marker

Introduction

Spumaretro- or foamy viruses (FVs) are a distinct group of retroviruses that gained increasing interest as novel viral vectors for gene delivery and vaccination (Linial, 1999; Rethwilm, 2003). FVs have a complex genomic organization with gag, pol, and env genes, the regulatory bel 1/tas transactivator gene, and bet (Rethwilm, 2003). Bet counteracts cellular APOBEC3-mediated restriction, is involved in particle release, and may have a role in establishing viral persistence (Alke et al., 2001; Löchelt et al., 2005; Meiering and Linial, 2002; Russell et al., 2005; Saib et al., 1995). Presently, insufficient data on FV replication in the infected individual, the sites of FV replication, the authentic target cell(s), and the extent of virus replication during life-long persistence are available.

The zoonotic potential of primate FVs is well established: Simian FV (SFV) types from chimpanzee, African green monkeys, and baboons have been detected in humans (Heneine et al., 2003). FV zoonoses were either traced back to simian caretakers who had been bitten by these primates (Heneine et al., 1998) or to Africans who were exposed to simian organs and body fluids by bush meat hunting and preparation (Wolfe et al., 2004). It is generally accepted that the prototypic human FV isolate HFV is actually a primate FV (PFV). In the few zoonotic cases known so far, no disease was associated with SFV infections and no further transmission to other humans occurred (Heneine et al., 2003). However, HFV/

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PFV may bear a disease potential since severe neurological disease consistently occurs in HFV-transgenic mice (Aguzzi et al., 1996).

In small-number studies of naturally infected cats or analysis of cats experimentally infected with wt or cloned FFV and FFV-derived vaccine vectors, we did not find evidence for a defined disease associated with FFV-infection (Alke et al., 2000; Schwantes et al., 2002, 2003). However, these studies confirmed active gene expression, replication, and progeny virus shedding in persistently infected cats clearly challenging the view that FV persistence is characterized by viral latency.

In order to study whether FFV infection is correlated with rare disease in cat, whether it may have a co-factorial role in other pathologies (Bandecchi et al., 1992; Glaus et al., 1997) and to analyze whether markers of FFV infections are detectable in man (Butera et al., 2000; Winkler et al., 1997b), sensitive and specific antibody assay systems are required. Besides virus reisolation and PCR genome detection, immunoblot reactivity against FV Gag and Bet proteins and immunofluorescence techniques are considered to be the most reliable methods to detect reactivity against FVs (Alke et al., 2000; Hussain et al., 2003; Khan et al., 1999; Winkler et al., 1997b; 1998, 1999). The only FFV Gag ELISA available at present showed considerable background reactivity (Winkler et al., 1997b). Using these diverse techniques, FFV prevalence ranged from 33% to more than 70% depending on the study and geographic region analyzed (Bandecchi et al., 1992; Daniels et al., 1999; Glaus et al., 1997; Winkler et al., 1998, 1999).

Here, we show with a novel ELISA technique that recombinant full-length FFV Gag is the diagnostic antigen of choice to identify experimental and natural FFV infections.

Results

Expression of recombinant FFV Gag, Env, and Bet fusion proteins and set-up of ELISAs

The FFV structural protein Gag, the FFV envelope-leader protein and SU ecto-domains (Wilk et al., 2001; Geiselhart et al., 2004; Lindemann and Goepfert, 2003), and the accessory Bet protein (Löchelt, 2003; Löchelt et al., 2005) were expressed as fusion proteins flanked by a N-terminal glutathione-S-transferase (GST) domain and by a C-terminal SV40-derived tag allowing immune detection by corresponding antisera (Sehr et al., 2001, 2002). The three FFV-fusion proteins were soluble and predominantly full-length and were purified from recombinant bacteria (data not shown).

ELISAs were based on a generic assay for Human Papillomavirus antibodies (Sehr et al., 2001, 2002). Recombinant FFV fusion proteins were directly adsorbed from cleared bacterial lysates to the ELISA plates as described (Sehr et al., 2001). Optimization of assay conditions was done using the FFV-positive cat reference serum 8014 and FFV-negative sera from specific pathogen-free (SPF) cats (Alke et al., 2000; Schwantes et al., 2003). Since background reactivity had been present in another FFV ELISA (Winkler et al., 1997b), the cat sera were pre-adsorbed with GST-tag-containing lysates decreasing background reactivity to 50 to 100 milli absorption units at 450 nm (mOD_{450}). The reproducibility of independently performed ELISAs was high ($R^2 = 0.987$).

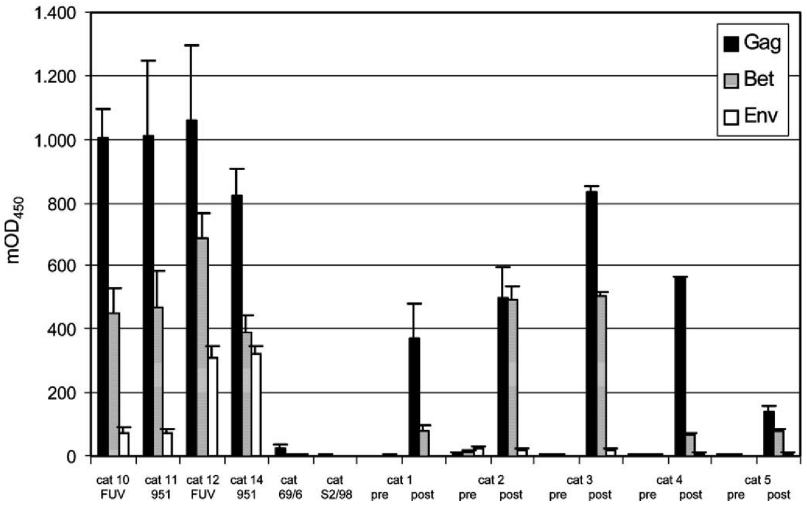

Fig. 1. Validation of FFV Gag, Env, and Bet ELISA by cat reference sera. Cats 10 and 12, and cats 11 and 14 were Australian cats naturally infected with FFV strains FUV and 951, respectively. FFV infection had been confirmed by serology, virus reisolation, and diagnostic PCR (Winkler et al., 1998). Cat 69/6 and S2/98 were FFV-negative SPF animals. Sera of SPF cats M1 to M5 were taken before (pre) and 10 weeks after (post) experimental FFV infection (Schwantes et al., 2003) and tested at 1:50 dilution. Results shown are antigen-specific absorption values (milli absorption units, mOD_{450}) with GST-tag background values subtracted. ELISAs were run against FFV Gag (black bars), Bet (grey bars), and Env (white bars) including error bars for standard deviation.

Validation of the FFV Gag, Env, and Bet ELISAs using feline reference sera

To validate reactivity against the three FFV proteins, we used reference sera 10, 11, 12, and 14 from naturally FFV-infected Australian cats (Winkler et al., 1998), pre- and post-exposition sera M1 to M5 from cats infected with empty replication-competent FFV vectors (Schwantes et al., 2003), and sera from FFV-negative SPF cats (Fig. 1). At 1:50 dilutions, FFV-negative cats 69/6 and S2/98 and pre-exposition sera were antibody-negative since the reactivity towards all three FFV antigens was always below 23 mOD_{450} In contrast, all sera taken about 10 weeks after FFV infection showed clear reactivity (more than 142 mOD_{450}) against Gag, two animals reacted strongly and three weakly with Bet, and none showed clear reactivity towards Env (all below 30 mOD_{450}). Sera from naturally FFV-infected cats (Winkler et al., 1998) showed stronger reactivity. Gag was recognized best, Env reactivity was low in two and strong in two other cats, and Bet was consistently recognized at intermediate levels. Env reactivity was not correlated with the serotype of the infecting FFV isolate: the FFV FUV-derived diagnostic antigen was similarly recognized by FFV FUV-infected cats 10 and 12 or by FFV 951-infected cats 11 and 14 (Winkler et al., 1998). In summary, the observed ELISA reactivity of the reference animals correlated well with their infection status.

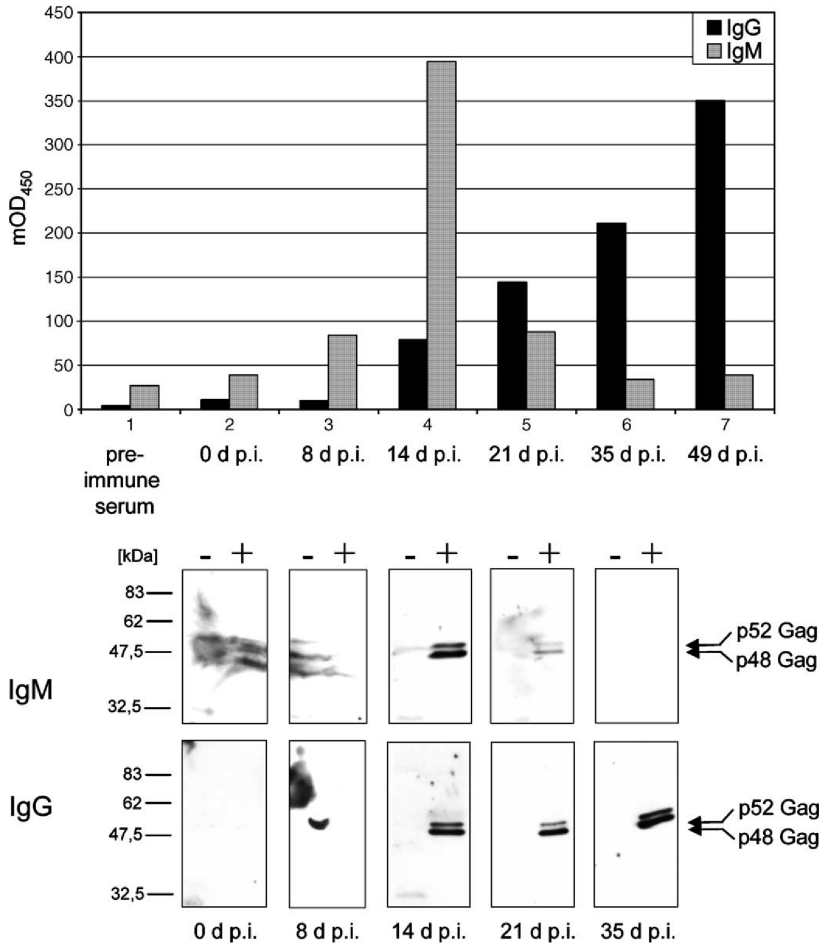

Fig. 2. Kinetics of FFV Gag-specific IgG and IgM as determined by GST-ELISA (top, single measurements) and FFV immunoblot (bottom). Sera from experimentally FFV FUV-infected cat M2 (Schwantes et al., 2003) taken before (pre) and 0, 8, 14, 21, 35, 49 days after infection (d.p.i.) were assayed for Gag reactivity using peroxidase conjugates of protein A (IgG) and goat anti-feline IgM (IgM), respectively. For immunoblots with the sera taken between 0 and 35 d.p.i., enriched FFV particle preparations (+) and mock preparations (-), respectively, were blotted after protein gel separation and the same secondary antibodies were used as above. Besides some unspecific reactivity, two forms of Gag proteins, p52 and p48, were detected.

Kinetics of anti FFV IgG and IgM in experimentally infected cats

To assess whether FFV-specific IgG and IgM antibodies can be discriminated, ELISAs were run in parallel with a protein A conjugate detecting cat IgG antibodies (Yamamoto et al., 1985) and a peroxidase-coupled antiserum specific for feline IgM. Due to the differential temporal appearance of IgG and IgM antibodies, serially taken blood samples from six cats infected with empty FFV vectors were analyzed (Schwantes et al., 2003). The sera were also subjected to IgG- and IgM-specific immunoblotting using the same secondary reagents. As shown for cat M2 (Schwantes et al., 2003), IgM reactivity peaked 14 d p.i. and then dropped rapidly whereas IgG reactivity increased steadily from day 14 (Fig. 2). The IgM-specific background reactivity was slightly larger than that for IgG but still always

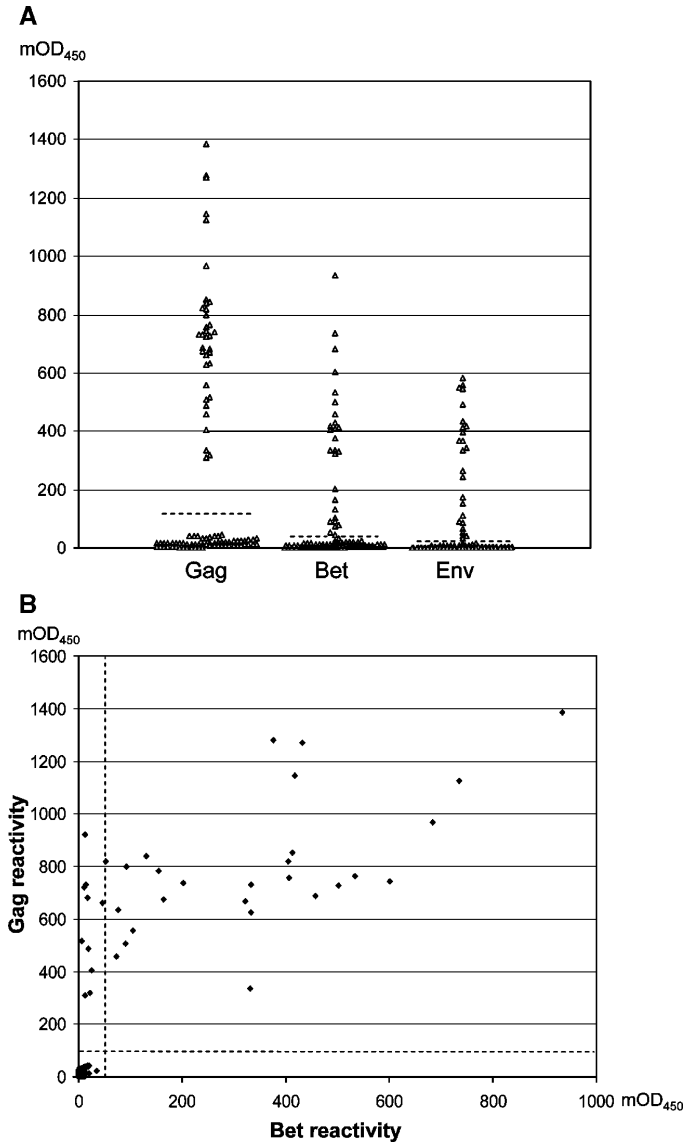

Fig. 3. Distribution of Gag, Bet, and Env antibody reactivity in 99 Swiss cat sera. In panel A, ELISA reactivity of all 99 sera towards FFV Gag, Bet, and Env is shown in dot-plot presentations. Dashed lines indicate the calculated cut-off values. In panel B, Gag (vertical axis) and Bet reactivity (horizontal axis) is presented in a two-dimensional plot. Each point represents a data pair of an individual serum. The upper right sector shows double-positive, the lower-left double-negative, the lower-right sector would display sera positive for Bet only, and the upper left sector represents sera positive for Gag only.

below 50 mOD_{450}. The ELISA results paralleled the immunoblot data and showed a sensitivity allowing IgM detection already at 8 d.p.i.

Pattern of FFV-specific IgG antibodies in sera of hospitalized cats

Sera of 99 Swiss domestic cats taken to hospital for diverse reasons were assayed for IgG antibodies against FFV Gag, Env, and Bet. Reactivity towards Gag was strongest and most frequent and showed a bimodal distribution (Fig. 3A). Gag reactivity of 63 sera was below 45 mOD_{450} and above 300 mOD_{450} for the remaining 36 sera. A stringent cut-off value of 112 mOD_{450} was calculated as 2 x (mean + 3 SD). Bet and Env antibodies showed not such a bimodal distribution of reactivity. Over 50% of these sera displayed very low values of less than 10 mOD_{450}, the remaining sera were evenly distributed among higher values. Bet and Env reactivities of Gag-negative sera were all very low (Fig. 3B and data not shown) suggesting that Gag negativity is correlated with negativity for Env and Bet. Thus, stringent cut-off values for Bet and Env were calculated from only the Gag-negative sera (excluding positive outliers) resulting in values of 46 and 21 mOD_{450} for Bet and Env, respectively.

ELISA reactivity of sera gradually declined upon serial dilution and remained above the cut-off at dilutions of 1:100 to 1:1.600 for Gag, 1:400 to 1:800 for Bet, and 1:100 to 1:800 for Env (not shown). Thus, 1:50 dilutions are required to detect all FFV-positive sera.

Of all 99 sera, 37 showed FFV antibodies, of which 36 reacted with Gag, 26 with Bet, and 25 with Env (Table 1). About half (19/37) of the antibody-positive sera reacted with all three antigens whereas only 6 displayed single reactivity against Gag or Env. The remaining sera contained Gag plus Bet or Gag plus Env antibodies whereas no Bet plus Env only serum was detected. The strengths of reactivity of double-positive sera were grossly correlated, with Env or Bet reactivities >200 mOD_{450} mainly coinciding with Gag reactivities

Table 1
FFV antibody patterns in hospitalized Swiss cats

Total number of sera	99
Number of triple-negative sera	62
Number of positive sera	
For any antigen	37
For Gag	36
For Bet	26
For Env	25
Number of single-positive sera	
For any antigen	6
For Gag	5
For Bet	0
For Env	1
Number of double-positive sera	
For any combination of two antigens	12
Gag+Bet	7
Gag+Env	5
Bet+Env	0
Number of triple-positive sera (Gag + Bet + Env)	19

>600 mOD_{450} (Fig. 3B and data not shown). The combined reactivity of cats against Gag and Bet (26/36) is similar to that seen for SFV-infected chimpanzees (Hahn et al., 1994). Only a single serum was slightly Env-positive but negative for Gag and no serum was Bet-positive in the absence of Gag antibodies. This antibody pattern makes Gag the diagnostic antigen of choice for the serological detection of FFV-infected cats.

None of the 99 Swiss cat sera exhibited IgM reactivities against Gag (data not shown) indicating that the FFV infections observed here were not recent. Selected cat sera were also analyzed by immunoblotting using purified FFV particles as antigen. For Gag antibodies, data from ELISA and immunoblot were fully concordant while the Env ELISA was superior when compared to immunoblots (data not shown). In addition, Bet antibodies are known to poorly react in immunoblots (Alke et al., 2000) suggesting the Bet ELISA to be better suited for Bet antibody detection.

Discussion

We describe novel ELISAs for the sensitive and specific detection of antibodies against both known FFV serotypes. The specificity of the ELISAs was confirmed using defined reference sera from naturally and experimentally infected cats (Winkler et al., 1998; Schwantes et al., 2003). Gag, Bet, and Env antigens are recognized with higher sensitivity in the novel ELISAs than in immunoblots. Gag was shown to be the diagnostic antigen of choice. The new FFV Gag ELISA is superior to a previous one which showed substantial background reactivity (Winkler et al., 1997b), a critical issue when analyzing sera with a low-level of reactivity e.g. from recently infected animals or from humans who might be zoonotically infected with FFV.

Utilization of multiple antigens and the capacity to discriminate early and transient IgM responses from long-lasting IgG reactivity allow detection of antibodies as marker for past or previous/current infections. In addition, the FFV ELISAs established here allow direct determination of antibody patterns in order to potentially associate individual pattern with disease, the kinetics of infection, and the host–virus interplay. Such sero-epidemiological studies for FFV will certainly extend our understanding of FV infection and replication in general. The direct comparison of feline reactivity towards Gag, Env, and Bet revealed that Gag reactivity is strongest. This clearly corresponds to the general observation that reactivity towards Gag, either in ELISA or immunoblots is diagnostic for FV infection in primates (including zoonotically infected human) and cats (Alke et al., 2000; Hussain et al., 2003; Khan et al., 1999; Winkler et al., 1998). Strong Gag reactivity obtained with all positive sera clearly differentiates Gag-negative from Gag-positive sera as shown by the bimodal distribution of Gag reactivity (Fig. 3A). This bimodal distribution allowed easy calculation of the Gag cut-off value. Since Bet and Env did not display such clear pattern of reactivity, their cut-off values were determined from the Gag-negative sera. A single Gag-negative serum slightly exceeded the calculated Env cut-off.

Since this serum was clearly negative in immunoblots (data not shown) and since reactivity against Bet and Env generally ranged from strong to very low, this animal is considered FFV-negative and the Env reactivity unspecific. As all Gag-specific reactivity was strong, background and specificity problems are not relevant for this diagnostic antigen thus making Gag the antigen of choice to diagnose FFV infection by serology.

The small-scale study performed did not reveal any significant association of a defined disease and FFV infection. Thus, larger epidemiological surveys are required including detailed information on the cats (age, sex, disease status, other infections etc.) to clarify whether FFV infection does induce disease or whether FFV infection is associated with feline immunodeficiency virus infections (Glaus et al., 1997; Bandecchi et al., 1992). In addition, such studies may reveal whether the pattern of sero-reactivity towards different antigens differs during infection. For instance, the Australian cats (Winkler et al., 1998) showed in general higher levels of reactivity than FFV vector-infected animals 60 days p.i. (Schwantes et al., 2003; Fig. 2) which may indicate that Bet and Env reactivity reaches high levels only after long-term infection or which may point to differences between the Australian field virus and the molecular cloned FFV used as vaccine vector.

Materials and methods

Molecular cloning and recombinant proteins

The complete FFV FUV (Winkler et al., 1997a) *gag* ORF was amplified by PCR with primers Gag-s and Gag-as (Table 2) thereby introducing restriction sites for *BamHI* and *SalI*. The FFV *bet* gene was amplified from a Bet expression plasmid (Alke et al., 2001) with primers Bet-s and Bet-as introducing sites for *BglII* at the 5' and *SalI* at the 3' end. The ectodomain of the Elp-SU part of FFV Env (Wilk et al., 2001; Geiselhart et al., 2004) was amplified with primers Env-s and Env-as introducing *EcoRI* and *SalI* sites. All PCRs were done using Herculase Hotstart proof reading DNA polymerase (Stratagene, Heidelberg, Germany) at 95 °C for 2 min plus 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 75 s. PCR products were digested with restriction enzymes cleaving at the introduced sites. Purified DNA fragments were fused in frame between the 5' GST domain and the 3' SV40 tag (KPPTPPPE-PET) of correspondingly digested pGEX4T3tag derivatives (Sehr et al., 2002). Clones were identified by restriction enzyme digestion and DNA sequencing.

For fusion protein expression, E. coli BL21 cells were transformed with pGEX-X-tag plasmids and recombinant proteins were purified as described (Sehr et al., 2001, 2002).

GST capture ELISA and cut-off definition

The ELISAs were performed essentially as described (Sehr et al., 2001, 2002). 96-well microtiter plates (Thermo Labsystems, Dreieich, Germany) were coated with glutathione casein, pre-adsorbed with blocking buffer (0.2% (w/v) casein in PBS, 0.05% (v/v) Tween 20), and then reacted with 100 pl cleared E. coli lysates containing the GST-tag or GST-Xtag fusion proteins (0.25 µg/µl total protein in blocking buffer).

Sera from naturally and experimentally infected cats (Alke et al., 2000; Schwantes et al., 2003) or from 99 cats with unknown immune status collected at a veterinarian hospital in Zurich, Switzerland, were pre-incubated in blocking buffer containing 2 µg/µl total protein from a GST-tag expressing E. coli BL21 (Sehr et al., 2001) at a dilution of 1:50 (for protein A peroxidase conjugate, Sigma, Munich, Germany, 1:5000 dilution), or 1:100 (for anti cat IgM peroxidase conjugate, Bethyl, Montgomery, USA; 1:10,000 dilution). Pre-absorbed sera were incubated for 1 h at RT in the coated ELISA plate wells, washed, and incubated for 1 h at RT with either conjugate. Substrate reaction and quantification were done as described (Sehr et al., 2001). Unless otherwise noted, all incubations were performed with a volume of 100 µl/well.

For each serum, the background absorbance with GST-tag was determined and subtracted from the absorbance with the GST-X-tag protein to calculate its specific reactivity against the FFV antigens. Measurements were done in duplicate on different plates and the mean value of the specific reactivity of the duplicate was taken as the readout.

Cut-off values were calculated from the group of Gag-negative sera as 2 X (mean + 3 SD) excluding positive outliers.

Immunoblot analyses of recombinant proteins and FFV particles

FFV particles from cell culture supernatants of FFV-infected CRFK cells were enriched by ultracentrifugation through a 20% (w/v) sucrose cushion (30,000 X *g*, 2 h, 4 °C), and served as antigen for immunoblot analyses (Wilk et al., 2001).

Table 2
PCR primers for generating FFV Gag, Bet, and Env GST fusion proteins

Primer	Sequence	Restriction site
Gag-s	5'-CGAGTCGGATCCATGGCTCGAGAATTAAATCCTCTCC	*BamHI*
Gag-as	5'-GCATGAGTCGACATCTTTACCCCCTTTCTTTCCACCG	*SalI*
Bet-s	5'-CGAGTCAGATCTATGGCTTCAAAATACCCGGAAGAAG	*BglII*
Bet-as	5'-GCATGAGTCGACTTCAGAGTCAGATGACTCAGATGTTG	*SalI*
Env-s	5'-CGAGTCGAATTCTCAATGGAAAGAAGCAATAACACATCC	*EcoRI*
Env-as	5'-GCATGAGTCGACTTGTCTTCTACCTTTCTTTCTTTCACAAG	*SalI*

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Virology (submitted)

Serological Detection Systems for Identification of Bovine Foamy Virus-Infected Cattle Facilitate Screening of Cows Shedding Bovine Foamy Virus Via Milk.

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Abstract

The biology of foamy viruses, their mode of transmission and disease potential in their natural host and after interspecies transmission are almost unknown. To gain insights into the prevalence of bovine foamy virus (BFV) and its zoonotic potential, enzyme-linked immunosorbent assays (ELISA) were established to determine antibody responses against Gag, Env and the non-structural Bet proteins in bovine serum and milk. In Polish cattle, strong Gag reactivity was most frequent (41.5%) and strongly associated with Bet antibodies, Env antibodies were less frequent. German cattle showed an overall BFV antibody prevalence of 6.8 %. Besides clearly BFV-positive animals, a substantial number of weakly reacting cattle was identified. BFV-specific antibodies were also detectable in milk.

BFV was isolated from PBLs and milk cells of BFV-positive cattle but not from antibody-negative or weakly reacting animals. The implications of these findings for the potential interspecies transmission of BFV to humans will be discussed.

Introduction

Spumaretro- or foamy viruses (FVs) are a distinct subfamily of retroviruses that are under development as novel viral vectors for gene delivery and vaccination (Josephson et al., 2002; Linial, 1999; Rethwilm, 2003; Schwantes et al., 2003). Similar to the much better studied lentiviruses, FVs have a complex genetic makeup (Rethwilm, 2003) encoding the canonical retroviral Gag, Pol, and Env proteins and the regulatory Bel1/Tas transactivator. In addition, the accessory Bet protein counteracts cellular APOBEC3-mediated restriction and may have a role in particle release and in establishing viral persistence (Alke et al., 2001; Löchelt et al., 2005; Meiering and Linial, 2002; Russell et al., 2005; Saib et al., 1995). In contrast to the well-established molecular biology of FVs, insights into FV replication in the infected individual, the sites of FV replication, the authentic target cell(s), the potential disease association, and the extent of virus replication during life-long persistence are limited at present.

Interspecies transmission of simian FVs (SFV) from chimpanzee, African green monkeys, and baboons into humans has been repeatedly detected (Heneine et al., 2003) Human populations with an endemic FV are not known at present, and thus the prototypic human FV isolate HFV is considered to be a primate FV (PFV). Interspecies infections occur either in simian caretakers who had been bitten by these primates (Heneine et al., 1998), in Africans exposed to simian organs and body fluids during bush meat hunting and preparation (Wolfe et al., 2004), and in humans with close contact to pet simians or temple monkeys (Jones-Engel et al., 2006; Schillaci et al., 2005). In the few human cases of interspecies FV infections, no disease has been associated with these events and no further transmission to other humans has occurred (Heneine et al., 2003). The containment of FV interspecies transmissions may be in part due to the species-specific protective function of Bet that efficiently inactivates APOBEC3 proteins of the cognate host but not of distantly related animals including humans (Löchelt et al., 2005; Russell et al., 2005). However, any interspecies transmission independent of whether it results in a productive or only a restricted replication may bear a disease potential since severe neurological disease consistently occurs in HFV-transgenic mice (Aguzzi et al., 1996).

The first isolation of a FV from cattle was in 1969 (Malmquist et al., 1969). Since then, several studies have shown that BFV, also designated bovine spumaretro- or syncytial virus, is prevalent in a relatively high percentage of cattle (Appleby, 1979; Greig, 1979; Jacobs et al., 1995; Malmquist et al. 1969). BFV-specific antibodies were detected by immunodiffusion and immunofluorescence and proviral BFV DNA was identified by different PCR techniques (Granzow et al., 1983; Malmquist et al., 1969; Materniak et al., 2006; Pamba et al., 1999). The reported prevalence values vary considerably which may be either due to geographic differences or different detection methods that do not allow direct comparison of the data. For other FVs, PCR genome detection, sero-reactivity against Gag and Bet proteins in blotting or ELISA, and immunofluorescence techniques are considered to be the most reliable detection methods (Alke et al., 2000; Hussain et al., 2003; Khan et al., 1999; Romen et al., 2006; Winkler et

al., 1998). Finally, virus isolation is the gold standard to diagnose infections.

Here we present a fast and reliable antibody screening platform suited for bovine milk and serum thus allowing broad screening for BFV prevalence. Surveillance of cattle appears mandatory since infectious BFV can be isolated from raw milk pointing to the risk of BFV transmission to men.

Results

Expression of recombinant BFV Gag, Env, and Bet fusion proteins and set-up of ELISAs

The BFV structural protein Gag, the envelope-leader protein and SU ecto-domains (Env), and the accessory Bet protein were expressed as fusion proteins flanked by a N-terminal glutathione-S-transferase (GST) domain and a C-terminal SV40-derived tag (Sehr et al., 2001; 2002). The three BFV-fusion proteins were soluble and predominantly full-length (data not shown).

ELISAs were based on previously established generic assays for feline FV (FFV) antibodies (Romen et al., 2006). Recombinant BFV fusion proteins were adsorbed from cleared bacterial lysates to the ELISA plates as described (Sehr et al., 2001).

Validation of the BFV Gag, Env, and Bet ELISAs using bovine reference sera

To validate sero-reactivity against the three BFV proteins, we used reference sera from 12 naturally BFV-infected and 10 uninfected Polish cattle. Their BFV-infection status had been before unequivocally established by three independent assays: two BFV DNA PCRs (Pamba et al., 1999), an antibody ELISA based on BFV-infected cells (to be published elsewhere), and a BFV-specific agar gel immuno-diffusion assay (data not shown).

In the novel ELISAs, sera were used at 1:50 dilutions. Sera from BFV-negative cattle exhibited some baseline reactivity towards all three BFV antigens, reaching up to 198 mOD_{450} against Gag in one serum (Fig. 1). However, all sera from BFV-positive animals showed much higher reactivity against Gag (>750 mOD_{450}), followed by Bet (>350 mOD_{450}), and 9/12 animals showed reactivity against Env above 150 mOD_{450} (Fig. 1). Standard cut off values calculated from all negative reference sera as the mean value plus three standard deviations (M + 3SD) were 218 mOD_{450} for Gag, 217 mOD_{450} for Bet and 124 mOD_{450} for Env with no outliers. The comparably high background values in the BFV ELISAs may be related to the fact that a Protein-G conjugate was used for detection of bovine IgG instead of a Protein-A conjugate used in a corresponding FFV ELISA for cats (Romen et al., 2006).

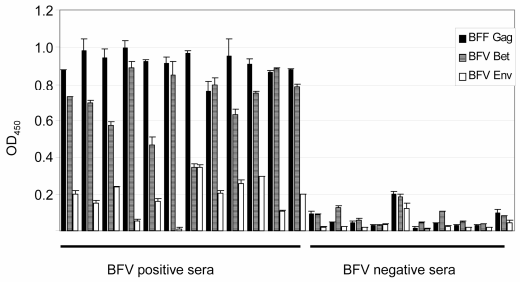

Fig. 1. Validation of BFV Gag, Bet, and Env ELISAs using Polish cattle sera. Ten BFV-negative and 12 BFV-positive cattle reference sera were analyzed. Positive and negative sera were clearly distinguishable regarding sero-reactivity against Gag and Bet. Env antibodies were not consistently present in BFV-infected cattle and had in general lower titers.

None of the reference sera showed elevated Bet or Env reactivity in the absence of Gag reactivity (Fig. 1). In summary, the ELISA reactivity of the reference animals for Gag and Bet correlated absolutely with their BFV-infection status as determined by the other detection methods (PCRs, cell-based ELISA, agar gel immuno-diffusion assay) while Env-specific antibodies were only found in 9/12 BFV-positive animals. The number of multiple reactivity of the BFV reference sera is slightly higher than in FFV-infected cats (Romen et al., 2006).

The reproducibility of independently performed ELISAs with two different Gag antigen batches was high (R^2=0,97).

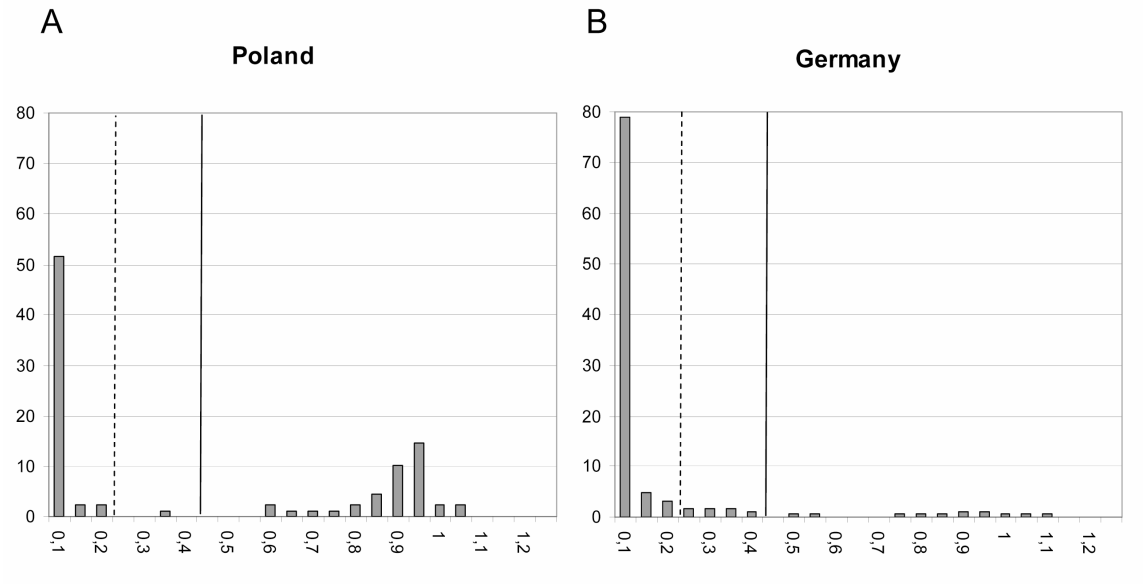

Fig. 2. Histogram plot of Gag antibody reactivity in serum samples of cattle from Poland (n=89) (A) and Germany (n=190) (B) (Y-axis: percentage of animals; X-axis: OD_{450} values). Whereas Gag reactivity in Polish cattle showed a bimodal distribution with most sera having either very low or strong reactivity, there was a substantial number of weak reactors (between standard and stringent cut-off) detectable in German cattle. Standard and stringent cut offs are indicated as dashed and full lines.

BFV-specific antibodies in field sera of 89 Polish cattle

Using the newly developed ELISA, we analyzed field sera from 89 Polish cows that had been randomly collected and tested before by nested BFV-PCR. BFV Gag reactivity was bimodal with peaks at about 100 mOD_{450} and greater than 600 mOD_{450} (Fig. 2A). Using the standard cut off value of 218 mOD_{450} for Gag, 38 sera (42.7%) scored positive. One serum from each the BFV-negative reference animals (198 mOD_{450}) and the Polish field study (308 mOD_{450}) displayed reactivity close to the standard cut off. When applying a more stringent (doubled) cut off of 2x(M + 3SD, 436 mOD_{450}), all BFV-positive reference cattle still scored positive while the single weakly-reacting field serum felt below the cut off. This animal was negative in i. BFV-PCR, ii. Bet and Env serum ELISAs, and iii. milk ELISAs for any BFV antigen (data not shown and see below).

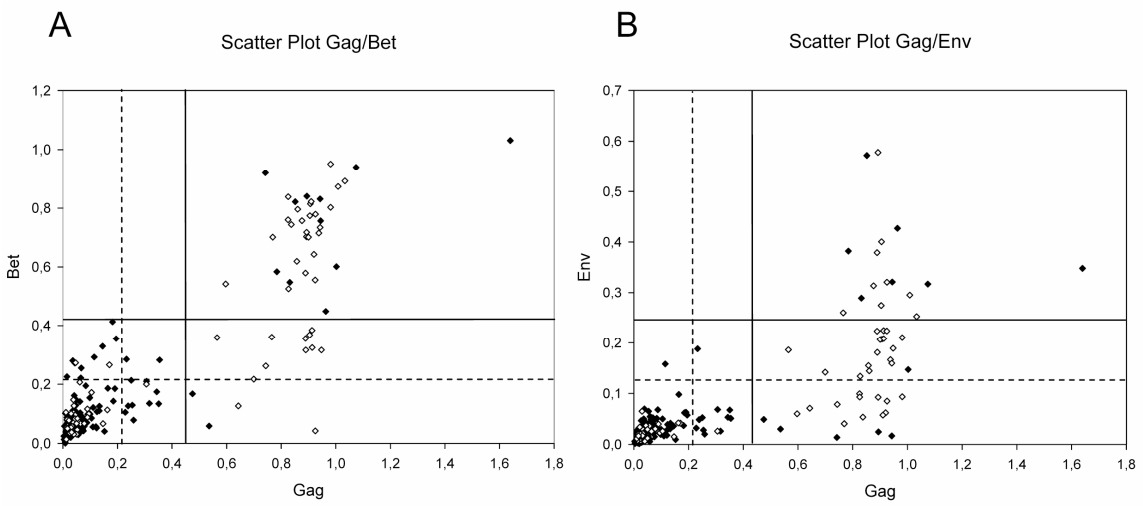

Fig. 3. Association of Gag plus Bet and Gag plus Env sero-reactivities of 89 Polish (white diamonds) and 190 German (black diamonds) cattle depicted in two-dimensional scatter plots. Each diamond represents a data pair of an individual serum (OD_{450} values). Dashed and full lines indicate standard and stringent cut off values, respectively.

Thus, Gag sero-reactivity above the stringent cut off is referred as strong-positive indicative for BFV infection while animals with reactivity between standard and stringent cut off are designated weak positive with an unclear BFV infection status. Based on these values, 41.5% of the tested Polish cattle were considered BFV-infected.

Bet and Env reactivity did not exhibit a bimodal distribution. Reactivity against these antigens may have limited diagnostic value as previously seen for FFV-infected cats (Romen et al., 2006). Using the standard and the deduced stringent cut off values for Bet and Env (Fig. 3A and B), a clear correlation of strong Gag and strong Bet reactivity was detectable (25/37) while 10 strongly Gag-positive animals had weak Bet reactivity. The stability index (R-squared value) for Gag and Bet reactivity was 0.77. In contrast, only 9 strongly Gag-positive animals were strongly Env-positive while 16 showed weak Env antibody titres. No Env reactivity was detected in Gag-negative animals whereas two Gag-negative animals scored weakly positive for Bet.

Six animals were strongly positive for all three antigens, 22 were strongly double-positive (19 Gag plus Bet and three Gag plus Env), while nine sera showed only strong Gag reactivity.

All of the strong Gag-positive sera and 97.4% of all Gag-positive sera were also BFV DNA PCR-positive (Table 1). Five ELISA Gag-negative animals were PCR-positive. None of these animals exhibited threshold sero-reactivity to any of the BFV antigens indicating that the PCR results might be inaccurate. These data confirm that Gag is the diagnostic antigen of choice while Bet and Env reactivity has supportive value.

Table 1
Consistency of ELISA and second independent BFV detection assays in respect of the cut offs used.

	> standard cut off	reactivity second assay	> stringent cut off	reactivity second assay
Poland	38	97.4%, 37/38 (PCR pos.)	37	100%, 37/37 (PCR pos.)
South Germany	18	50%, 3/6 (virus isolation)	9	100%, 3/3 (virus isolation)
North Germany	6	66.7%, 4/6 (IIF pos.)	4	75%, 3/4 (IIF pos.)

	< standard cut off	reactivity second assay	< stringent cut off	reactivity second assay
Poland	51	90.2%, 46/51 (PCR neg.)	52	90.4%, 47/52 (PCR neg.)
South Germany	126	100%, 12/12 (no virus isolation)	135	100%, 15/15 (no virus isolation)
North Germany	40	97.5% (IIF neg.)	42	97.6% (IIF neg.)

Milk serology and comparison to blood serology

We then tested whether the matched milk samples of these 89 Polish cows contain BFV specific antibodies as had been shown for other cattle infections with bovine leukaemia and bovine viral diarrhoea virus and for *Neospora caninum* (Carli et al., 1993; Hall et al., 2006; Niskanen et al., 1989). Milk was used at a dilution of 1:5. A comparison of 15 representative serum and milk pairs is shown in Fig. 4. A clear but non-linear correlation was in general obvious for Gag (see below) while Bet and Env reactivity showed a higher degree of variation between serum and milk. Cut off levels for all three antigens were calculated from the 52 BFV sero-negative Polish cows. For milk antibodies, standard cut offs were 186 for Gag 192 for Bet, and 107 mOD_{450} for Env, stringent cut offs were 372, 384, and 214 mOD_{450}, resp.. Of the 37 strongly Gag sero-positive animals, one showed weak and 34 strong Gag reactivity in the milk (Table 2). The correlation of serum versus milk data for Bet and Env was less stringent. None of the sero-negative cows scored positive for BFV antibodies in milk.

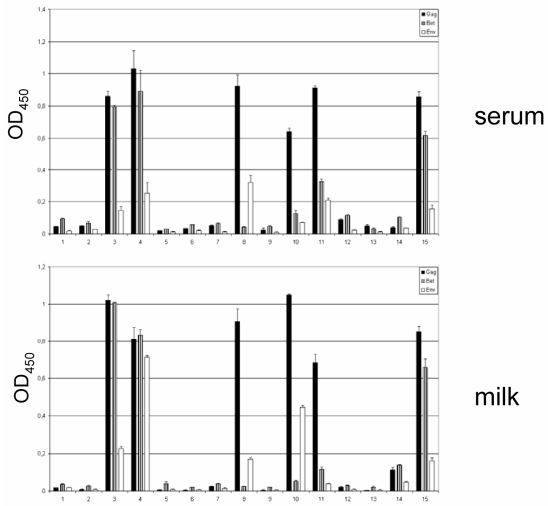

Fig. 4. BFV-specific antibodies in paired serum and milk samples of 15 representative Polish cattle. Gag and Bet antibodies were consistently detected in both samples whereas Env antibody pattern showed a less strong agreement. (Gag : black bars; Bet : grey bars; Env : white bars)

BFV-specific antibodies in sera and milk of 190 German cattle

Using the cut off values as defined for the Polish reference animals, we analyzed sera and milk samples of 144 German cows from 11 farms in South Germany taken during general examination by veterinarian disease surveillance and 46 cattle sera from Northeast Germany. The histograms indicate significant differences in the distribution of Gag reactivity in Poland versus Germany (Fig. 2). The overall prevalence of strong BFV Gag reactivity in Germany (13 out of 190 animals, 6.8%) is much lower than in Poland. Out of the 13 strongly Gag-positive sera, eight had also strong Bet and seven strong Env reactivity. Among the 13 Gag-positive sera, only one had intermediate Env reactivity. In addition, nine German animals were weakly Gag-reactive, one had weak Gag plus weak Bet reactivity, and another displayed weak reactivity against all three BFV antigens.

For the sera from Northeast Germany, immuno-fluorescence assays using BFV-infected cells done in parallel showed good agreement for negative but only moderate consistency for Gag-positive sera of 66.6 to 75% depending on the cut off value used (Table 1). In total, 18 selected cows from South Germany were studied for BFV isolation (for details, see below): only from animals with strong Gag reactivity, BFV was recovered. In contrast, Gag-negative animals or those with weak Gag antibodies did not allow BFV isolation showing high assay consistency for strongly BFV Gag-positive and Gag-negative sera. BFV prevalences ranged in individual South German herds from 0/12 up to 3/10 BFV-positive animals.

Table 2
Comparison of serum and milk reactivities of 89 Polish cattle.

	Number of milk reactants / consistency to stringent serum reactants	
	standard milk cut off	stringent milk cut off
Gag	37 / 35	34 / 34
Bet	29 / 23	21 / 19
Env	15 / 7	6 / 3

Virus isolation from blood, milk and saliva

Blood, milk and saliva samples from 18 cows from South Germany were studied for BFV isolation: three were strongly Gag-positive (900–1,000 mOD_{450}) and contained Gag-specific antibodies in the milk, three cows had no milk reactivity but two displayed weak Gag reactivity and another had weak Gag plus Bet reactivity, and 12 were clearly Gag-negative. Saliva, enriched PBLs, and milk cells were co-cultivated with permissive dog thymus cells (Cf2Th). These cultures were examined for signs of BFV infection (induction of multi-nucleated syncytia) for up to 4 weeks. The cultures with PBLs from the three strongly positive cows developed syncytia 14 d p.i. and BFV infection was confirmed by direct immuno-fluorescence (Fig. 5). Importantly, BFV was also isolated from two milk cell preparations of ELISA sero- and milk-antibody positive cattle (syncytia formation and IF). The cultures with all other samples remained virus free, indicating that these animals were not producing infectious virus (no viremia) at the time of sampling or the isolated virus amount did not exceed infection thresholds. Under the conditions used, saliva samples were negative for BFV contrary to previous reports (Johnson et al., 1988). This result has to be viewed with caution since saliva co-cultivation was in general difficult and only possible in the presence of high amounts of antibiotics and repeated media changes.

Discussion

We have developed novel ELISAs for the sensitive and specific detection of antibodies against three BFV proteins. The ELISAs were validated using defined reference sera from naturally infected cattle. Gag was shown to be the diagnostic antigen of choice. This clearly corresponds to the general observation that reactivity towards Gag, either in ELISA or immunoblots is diagnostic for FV infection in primates (including zoonotically infected humans) and cats (Alke et al., 2000; Hussain et al., 2003; Khan et al., 1999; Romen et al., 2006; Winkler et al., 1998). According to our data, the stringent Gag cut off is a valuable parameter to positively identify productively BFV-infected animals as shown by the excellent agreement of strong Gag reactivity and additional tests to identify BFV-infected cattle.

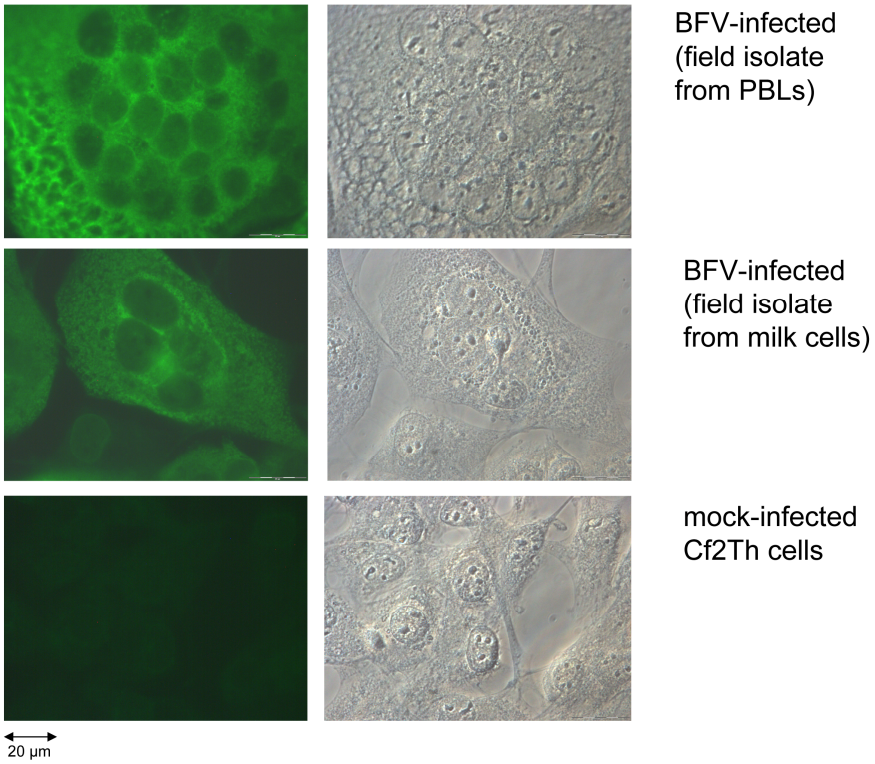

Fig. 5. Direct immunofluorescence (left-hand panel) and phase contrast microscopic images (right-hand panel) of Cf2Th cells infected with BFV from cattle PBLs (top panel) and raw-milk cells (middle panel). The lower panel represents uninfected controls. The immunofluorescence staining of cells displaying BFV-induced cytopathic effects (syncytia, top and middle panel) was done with a FITC-labelled BFV reference serum (Granzow et al., 1983).

Similarly, the standard Gag cut off appears well suited for the unambiguous negative identification of non-infected, BFV-negative cattle (Table 1). However, one Polish and a number of German cattle have been identified with weak Gag reactivity. These intermediate reactants show mainly singular weak reactivity against Gag or, less frequently, additional low-level reactivity against BFV Bet and/or Env. Although only few of these animals have been tested, BFV could not be isolated from their blood that is otherwise a reliable source of virus to identify persistently FV-infected animals. Thus, it might be that these animals are either at a very early stage of infection or that these animals are not productively infected or have cleared the virus. We assume, however, that the weak reactivity may be due to the fact that these animals were solely exposed to BFV antigens but were not productively BFV-infected. German calves are frequently fed with dry milk products and the weak sero-reactivity detected might have resulted from exposition to such milk-derived BFV antigens in the form of inactivated viruses or virus-producing cells, for instance via ingestion of dry milk products. The situation in Poland appears to be different in that calves included in this study have been fully nursed and a high percentage has thus been productively infected by BFV associated with high BFV-specific reactivity. It will be necessary to further characterize and define the group of weakly reacting animals with respect to BFV infection and serology.

The frequency of Bet antibodies in sera of BFV positive cattle was similar to that of FFV-infected cats (Romen et al., 2006). Bet and Env reactivity may be a well-suited additional marker to characterize the group of weakly reacting animals. In addition, the use of three diagnostic antigens may also allow defining different stages of infection, an issue that should be analyzed in more detail.

The difference in BFV prevalence in German (about 7%) and Polish (42%) cattle and published data (Granzow et al., 1983) may be attributed to differences in livestock husbandry. Polish cattle are often kept in herds of variable size but with close contact to each other. In Germany, cattle are often kept under semi-industrial conditions without much contact between the animals. In addition, mother-offspring transmission of BFV via milk may be more common in Poland than in Germany (see above). In line with this assumption and previous reports (Appleby et al., 1979; Lucas et al., 1986), BFV transmission may be restricted to young animals: a second examination of the cattle from a German farm one year after the first did not identify newly infected animals (data not shown). In summary, our data indicate that i. BFV contagiosity is comparably low, ii. the conditions under which German cattle is kept may restrict infection, and iii. that only calves are fully susceptible for BFV, most probably via nursing. In the present study we did not analyze the potential interplay between BFV and other pathogens of cattle, a question that can now be addressed using the novel BFV ELISAs.

We show that milk is a suitable surrogate for serum without much forfeit of sensitivity. This feature and the fact that milk can be sampled easily makes milk highly suited for large-scale screenings and livestock monitoring. In addition, we confirmed previous reports that infected cows shed infectious BFV via milk. It is currently open, whether the infection is transmitted via free BFV particles or via infected cells as known for other milk-borne infections. Consistent with a cell-associated transmission, BFV was recovered from the cellular fractions of raw, untreated milk whereas the antibody-containing whey did not allow BFV isolation. The relative ease with which infectious BFV was recovered from raw milk and blood, for instance without interferon suppression as suggested for the isolation of SFVs (Falcone et al., 1999) is important regarding BFV transmission since it indicates a substantial infectivity present in milk or PBLs. Among cattle, nursing may be thus the most important route of BFV spread as has been already discussed by others (Lucas et al., 1986). Via raw milk and related milk products but also other cattle-derived materials, humans are also exposed to BFV. In developed countries, milk is typically pasteurized before it gets to the consumer. However a growing number of people consumes untreated raw milk products and are thus at an increased risk of interspecies infections including BFV (Leedom, 2006).

The BFV ELISAs established here allow direct determination of antibody pattern to potentially link individual pattern with disease, the kinetics of infection, and the host-virus interplay. Such sero-epidemiological studies for BFV will certainly extend our understanding of FV infection and replication in general and the zoonotic potential of this milk-borne virus.

Materials and methods

Molecular cloning and recombinant proteins

The BFV *gag* gene was amplified by PCR with primers Gag-s and Gag-as (Table 3) using cloned full-length infectious DNA (Renshaw et al., 1991). The BFV *bet* gene was amplified from a BFV particle cDNA preparation as template with primers Bet-s and Bet-as. The ectodomain of the Elp-SU part of BFV Env was amplified with primers Env-s and Env-as. In all cases, the primers introduced an *Eco*RI site at the 5' and a *Sal*I site at the 3' end of the amplicons. All PCRs were done using Herculase Hotstart proof reading DNA polymerase (Stratagene, Heidelberg, Germany) at 95°C for 2 min plus 30 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 75 s. PCR products were digested with restriction enzymes cleaving at the introduced sites. Purified DNA fragments were fused in frame between the 5' GST domain and the 3' SV40 tag (KPPTPPPEPET) of correspondingly digested pGEX4T3tag derivatives (Sehr et al., 2002). Clones were identified by restriction enzyme digestion and DNA sequencing.

For fusion protein expression, *E. coli* BL21 cells were transformed with pGEX-X-tag plasmids and recombinant proteins were purified as described (Sehr et al., 2001; 2002).

Table 3
PCR primers for generating BFV Gag, Bet and Env GST fusion proteins.

Primer	Sequence	Restriction site
Gag s	5'- CGAGTCGAATTCAATGGCTCTTAATGACTTCGACCCTATAGC	*Eco*RI
Gag as	5'- GCATGAGTCGACAGATGATTGCCCTTGATTTCCACTTGAAGTGG	*Sal*I
Bet s	5'- CGAGTCGAATTCGATGGCTAGCGGTGGAACGCCGGAGAAAGC	*Eco*RI
Bet as	5'- GCATGAGTCGACTTGAGATGTCTTCAGATCCAGGTCTTCTTCAC	*Sal*I
Env s	5'- CGAGTCGAATTCACAATGGAAGGGAGCTATTGAGAGC	*Eco*RI
Env as	5'- GCATGAGTCGACTCCGTCCTGCGTTTTATTCTGATAAC	*Sal*I

GST capture ELISA

ELISAs were performed as described (Romen et al., 2006). Briefly, 96-well microtiter plates (Thermo Labsystems, Dreieich, Germany) were coated with glutathione casein, blocked with blocking buffer (0.2 % (w/v) casein in PBS, 0.05 % (v/v) Tween 20) and then reacted with 100 µl cleared *E. coli* lysates containing the GST-tag or GST-X-tag fusion proteins (0.25 µg/µl total protein lysate in blocking buffer).

Sera from naturally BFV-infected and uninfected cattle were preincubated at a dilution of 1:50 in blocking buffer containing 2 µg/µl total protein from GST-tag expressing *E. coli* BL21 (Romen et al., 2006), milk was preincubated at a dilution of 1:5. Serum and milk samples were incubated for 1 h at RT in the coated ELISA plate wells, washed, and incubated for 1 h at RT with protein G peroxidase conjugate (Sigma, Munich, Germany, 1:5.000 dilution). Substrate reaction and quantification were done as described (Sehr et al., 2001). Unless otherwise stated, all incubations were performed with a volume of 100 µl/well.

For each serum, the background absorbance with GST-tag was determined and subtracted from the absorbance with the GST-X-tag protein to calculate its specific reactivity against the BFV antigens. Measurements were done in duplicate on different plates and the mean value of the specific reactivity of the duplicate was taken as the readout.

Animals

Serum samples used as reference material were obtained from 3-6 years old Friesian Holstein cows from two herds from North Poland. Polish field sera were from 4-8 years old Friesian Holstein cows from 3 different herds from North Poland. The size of the herds was between 120-180 animals, all animals analyzed were clinically healthy.

Virus isolation from PBLs, milk cells and saliva

Fresh EDTA blood was centrifugated, the plasma was collected for ELISA testing and the buffy coat cells were removed (Alke et al., 2000) and incubated with ACT erythrocyte lysis buffer (140 mM NH4Cl, 16.5 mM Tris/HCl; (Michel et al., 2002)). After erythrocyte lysis, the PBLs were incubated with BFV-permissive dog thymocytes (Cf2Th cells) and passaged for at least three weeks.

Milk samples were centrifugated, the liquid phase was collected for ELISA testing and pelleted milk cells resuspended in PBS were used for cocultivation as described above.

Saliva was harvested with cotton plugs from the mouth of cattle. The plugs were incubated in cell culture medium containing the antibiotics penicillin, streptomycin, gentamycin, amphotericin and the fungicide nystatin (Alke et al., 2000). After low-speed centrifugation, the super-natant was applied to Cf2Th cells and cultured as described.

During three to four weeks of passages, Cf2Th cells were examined for cytopathic effects by phase contrast microscopy and additionally by direct immunofluorescence.

Direct immunofluorescence

Cf2Th cells from cocultivation experiments were seeded on glass coverslips. After two d, the coverslips were, washed with PBS and cells were fixed at least 20 min in ice cold 100% methanol / 0.02% EGTA. Cells were rehydrated for 20 min. in PBS and unspecific protein-binding sites were blocked with 3% BSA in PBS for 30 min. A FITC-conjugated, bovine polyclonal BFV reference antibody (diluted 1:500 in PBS with 0,3% BSA) was incubated for 60 min followed by three 5 minute PBS wash steps (Granzow et al., 1983). Cover-slips were washed in double-distilled water and ethanol and then mounted.

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The antiretroviral activity of APOBEC3 is inhibited by the foamy virus accessory Bet protein

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Genome hypermutation of different orthoretroviruses by cellular cytidine deaminases of the APOBEC3 family during reverse transcription has recently been observed. Lentiviruses like HIV-1 have acquired proteins preventing genome editing in the newly infected cell. Here we show that feline foamy virus (FFV), a typical member of the foamy retrovirus subfamily *Spumaretrovirinae*, is also refractory to genome deamination. APOBEC3-like FFV genome editing in APOBEC3-positive feline CRFK cells only occurs when the accessory FFV Bet protein is functionally inactivated. Editing of *bet*-deficient FFV genomes is paralleled by a strong decrease in FFV titer. In contrast to lentiviruses, cytidine deamination already takes place in APOBEC3-positive FFV-producing cells, because edited proviral DNA genomes are consistently present in released particles. By cloning the feline APOBEC3 orthologue, we found that its homology to the second domain of human APOBEC3F is 48%. Expression of feline APOBEC3 in APOBEC3-negative human 293T cells reproduced the effects seen in homologous CRFK cells: Bet-deficient FFV displayed severely reduced titers, high-level genome editing, reduced particle release, and suppressed Gag processing. Although WT Bet efficiently preserved FFV infectivity and genome integrity, it sustained particle release and Gag processing only when fe3 was moderately expressed. Similar to lentiviral Vif proteins, FFV Bet specifically bound feline APOBEC3. In particles from Bet-deficient FFV, feline APOBEC3 was clearly present, whereas its foamy viral antagonist Bet was undetectable in purified WT particles. This is the first report that, in addition to lentiviruses, the foamy viruses also developed APOBEC3-counteracting proteins.

cytidine deamination | virion infectivity factor | spumaretrovirus | restriction factor | zoonosis

The replication of viruses, their spread and disease potential in the infected individual, as well as their transmission to new hosts of the same or another species are tightly controlled by the interplay of several host- and virus-encoded functions. In the simplest case, the presence or absence of a single host function required for virus replication determines the permissiveness of the host. In many instances, however, resistance is actively mounted by the host through the expression of inactivating factor(s) directly interfering with the replication of the pathogen (1). As a consequence of the dynamic host/virus coevolution, virus-encoded defense strategies have been developed that are then also subjected to coevolutionary adaptation processes on either sides. An advanced understanding of the underlying molecular processes of antiviral resistance and virus counter-defense are essential for preventive and therapeutic purposes and also for basic research issues (2).

A case in point is the fact that the host-induced lethal editing of retroviral genomes by cellular APOBEC3 deaminases is specifically prevented by the HIV virion infectivity factor (Vif) (3–8). In this system, human APOBEC3F and -3G cytidine deaminases (hu3F and hu3G) specifically edit cytidine residues of the single-stranded DNA intermediate of retroviral reverse transcription leading to HIV genome degradation or hypermutation and subsequent error catastrophe. The protective effect of the HIV Vif accessory protein depends on strong and specific binding to cellular APOBEC3 proteins and their subsequent targeting to proteasome-mediated degradation (3,4,9–11). Both processes prevent packaging of APOBEC3 into HIV particles thus leading to the maintenance of genome integrity and replication competence. Although different APOBEC3 proteins can edit a variety of retroviral genomes and can even interfere by unknown means with the replication of the distantly related hepadnaviruses (12), the Vif-mediated defense strategy appears to be of high specificity. The Vif protein of the closely related simian immunodeficiency virus from African Green Monkey (SIV_{agm}) does not protect against hu3G and, vice versa, nonhuman APOBEC3 proteins are resistant against HIV Vif-induced inactivation (3,13), although a certain degree of cross-protection has been observed (14, 15). In selected cases, the species-specific APOBEC3–Vif interaction is determined by a single amino acid residue (16–19). Contrary to the APOBEC3-neutralizing activities of primate lentivirus Vif proteins, virtually nothing is known about APOBEC3-counteracting strategies used by simple retroviruses that do not have the capacity to encode a Vif-like protein.

Foamy viruses (FVs), the only members of the subfamily of *Spumaretrovirinae,* have complex genomes with the capacity to encode a Vif-like function. However, several features of FV replication are clearly different from the *Orthoretrovirinae*, e.g., the timing of reverse transcription and particle formation (reviewed in ref. 20); therefore, potential mechanisms to cope with the danger of genome editing are worth investigating for these viruses, too.

Here we show that the FFV-encoded accessory Bet protein inhibits cytidine deamination of the viral genome and, in particular, interferes with the antiretroviral activity of the feline APOBEC3 (fe3) protein described here. FFV Bet binds to fe3 but not to hu3G. Although fe3 is present in FFV particles derived from *bet*-deficient genomes, Bet is undetectable in WT FFV particles, as HIV Vif might be (21, 22). The importance of these findings to our understanding of virus-induced targeting of APOBEC3, as well as the effects on FV replication, will be discussed.

Materials and Methods

Cell Culture and cDNA Preparation. FFV-permissive feline CRFK cells, FeFAB cells, 293T cells, and FFV virions were propagated

Abbreviations: Vif, virion infectivity factor; FV, foamy virus; PBMC, peripheral blood mononuclear cell; HA, hemagglutinin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY971954).

and used as described (23). Feline peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated whole blood by Histopaque-1077 (Sigma) gradient centrifugation and cultured after activation with PHA (3 μg/ml) for 3 days in RPMI medium 1640 containing 15% FBS, 5 X 10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and 100 units of human recombinant IL-2 per ml at 37°C and 5% CO_2. For cDNA preparation, total RNA was isolated by using the RNeasy mini kit (Qiagen) according to the manufacturer. Total RNA (5 μg) was used to generate cDNA by using SuperScript III reverse transcriptase (Invitrogen).

Plasmids and DNA Transfection. FFV WT and Bet mutant plasmids pFeFV-BBtr and pFeFV-MCS and the eukaryotic FFV Bet expression plasmid have been described (23, 24). In pFeFV-BBtr, the 387-residue WT Bet is truncated after amino acid 116, whereas in pFeFV-MCS, few residues are exchanged and inserted at the same site. To increase gene expression, both Bet mutations were cloned into the CMV-IE promoter-driven FFV pCF-7 (25), resulting in mutants pCF-BBtr and pCF-MSC. The expression vector for hemagglutinin (HA)-tagged hu3G (phu3G-HA) (3) was a gift of Nathaniel R. Landau (The Salk Institute for Biological Studies, La Jolla, CA). Feline APOBEC3 (fe3) was identified by using 5' and 3' RACE reactions (5'/3'-RACE kit, Roche Diagnostics) employing mRNA from CRFK cells, the forward fAPO3F9 (5'-TGGAGGCAGCCTGGGAG-GTG-3') and reverse fAPO3F16 (5'-CTTGAGGGAGGAGG-GAGGATG-3') primers, and *Pwo* polymerase (Roche Diagnostics). Thirty cycles were run at 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min. PCR products were cloned into pCR4Blunt-TOPO (Invitrogen), sequenced, and transferred into the *EcoRI* sites of pcDNA3.1(+) (Invitrogen) generating pfe3. Similarly, expression plasmid pfe3-HA encoding C-terminal HA-tagged fe3 was made by using forward fAPO3F18 (5'-TAGAAGCT-TACCAAGGCTGGCGAGAGGAATGG-3') and reverse fAPO3F19 (5'-AGCTCGAGTCAAGCGTAATCTGGAACA-TCGTATGGATACCTAAGGATTTCTTGAAGCTCTGC-3') primers, sequenced, and cloned into the *HindIII* and *XhoI* sites of pcDNA3.1(+). DNA transfection into CRFK cells was done with Lipofectamine 2000 according to the manufacturer (Invitrogen), 293T cells were transfected by Ca-phosphate precipitation (26).

The fe3 cDNA PCR product was inserted into the *BamHI* and *SalI* sites of bacterial expression plasmid pGEX4T3, and the glutatione S-transferase-tagged fe3 fusion protein was purified by glutathione Sepharose chromatography as described (27) and used for antibody induction in rabbits (28).

Virological Methods. FFV particles were prepared from infected CRFK cells 3 or 5 days after infection. Particles were enriched from cell culture supernatant by sedimentation through 20% sucrose and resuspended in PBS as described (29). Particles were digested with the subtilisin protease to remove proteinaceous contaminants not incorporated into the virions (26).

Preparation of Particle-Derived Proviral DNA. To remove contaminating plasmid DNA, enriched FFV particles were treated for 2 h at 37°C with DNaseI according to the supplier (MBI Fermentas, St. Leon-Rot, Germany). The DNase was subsequently inactivated by adding EDTA to 2.5 mM, Proteinase K (Roche Diagnostics) to 0.2 mg/ml and incubation for 45 min at 72°C. Proteinase K was inactivated for 10 min at 98°C.

PCR Amplification, Cloning, and Analysis of Proviral FFV DNA. Virion-encorporated FFV DNA was amplified with sense primer 5'-CTTCTGGTTTGGACCTTACC-3' and antisense primer 5'-GTTTTAGTAAGTGTAGCGGCGA-3' using the proof-reading Herculase DNA polymerase according to the manufacturer (Amersham Pharmacia). A total of 34 reaction cycles were run at 94°C for 30 s, 56°C for 40s, and 75°C for 2 min. This PCR allowed amplification of unspliced FFV proviral DNA of 615 nt and spliced FFV proviral DNA of 330 nt and identification of the *bet* mutations. Reaction products were cloned by using the TOPO cloning kit as per the manufacturer's instructions (Invitrogen). Clones were identified by restriction enzyme digestion, and plasmid DNA was sequenced by using the DNA sequencer 377 (Applied Biosystems).

Immunoprecipitation and Western Blot Analysis. For coimmunoprecipitation of FFV-Bet and fe3 or hu3G, 293T cells were transfected with 2 μg of fe3-HA or human APOBEC3G-HA expression plasmid pfe3-HA or phu3G-HA and 2 μg of pFFV-Bet. After 2 days, cells were lysed in TLB (20 mM Tris, pH 7.4/137 mM NaCl/10% glycerol/2 mM EDTA, pH 8/1% Triton X-100/50 mM Na-β-glycerophosphate and protease inhibitors) and lysates cleared by centrifugation. For immunoprecipitation of fe3-HA or hu3G-HA, supernatants were incubated with anti-HA-beads (Roche Diagnostics) for 60 min at 4°C and washed five times with TLB. After boiling in electrophoresis sample buffer, samples were subjected to SDS/PAGE and immunoblotting (23). The FFV Bet, Env leader protein, and cat 8014 antisera have been described (23). Membranes were reacted with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia) and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia). For immunoblotting, identical amounts of cell extracts were used as determined by Roti-Quant protein quantification (Roth, Karlsruhe, Germany).

Results

Bet-Mutated FFV Genomes Are Edited in Feline CRFK Cells. We recently reported that CRFK cells display a nonpermissive phenotype when infected by *bet*-defective FFV (23). Similarly, Vif-minus feline immunodeficiency virus (FIV) is replication deficient in CRFK cells (30). In light of recent findings on the function of lentivirus Vif, we questioned whether expression of an APOBEC3-like cytidine-deaminase in CRFK cells might be involved in the restriction of *bet*-deficient FFV.

Table 1. Sequence characteristics of CRFK cell-derived, encapsidated FFV DNA genomes from WT and Bet mutant FFV proviruses

	pFeFV-7 wt		pFeFV-MCS		pFeFV-BBtr	
	Spliced	Unspliced	Spliced	Unspliced	Spliced	Unspliced
No. of sequences analyzed	8	8	12	14	8	12
Mutations G–A/other	0/0	1/3	37/1	31/3	14/1	41/5
Clones without G – A editing	8	7	0	3	0	4
Minimal no. of G – A per clone	0	1	1	1	1	2
Maximal no. of G – A per clone	0	1	9	11	3	9
Average no. of G – A per clone	0	0.1	3.1	2.2	1.8	3.5
G – A exchanges per 100 nucleotides	0	0.02	1.01	0.38	0.60	0.59

Table 2. Sequence context of minus strand C to T editing events in transfected Bet-mutant proviral DNA

Sequence exchange	Frequency, % In CRFK cells (*n* = 123)	Frequency, % In fe3-expressing 293T cells (*n* = 57)
CCC to CCT	0	0
CTC to CTT	2	9
TCC to TCT	14	21
TTC to TTT	68	56
XPyC to XPyT	8	9
PyXC to PyXT	8	5
PuPuC to PuPuT	0	0

Pu, purin base; Py, pyrimidine base; X, any base.

Therefore, we reexamined the replication of the previously described FFV *bet* mutants pFeFV-MCS and pFeFV-BBtr in CRFK cells (23). In clone pFeFV-MCS, only a few amino acids in the central part of Bet had been changed, and clone pFeFV-BBtr is characterized by a truncation of Bet at the same site (23). As described (23), the changes in *bet* resulted in a 10^2- to 10^3-fold reduced titer of the mutants compared to WT FFV (data not shown). To identify the cause for the reduced titer, *de novo* synthesized FFV genomes were analyzed for the presence of APOBEC3-mediated C to U deamination of the DNA minus-strand resulting in G to A exchanges on the plus strand. For these studies, we took advantage of two specific features of FV reverse transcription: a substantial fraction of FV particles already contains full-length proviral DNA and part of this DNA specifically lacks the *bet* intron (see ref. 31). These intron-deficient, *bet*-spliced FFV DNAs are only generated after replication of the plasmid-encoded FFV genomes, and therefore cannot be derived from input DNA. CRFK cells were transfected with WT and *bet*-mutated FFV genomes. Released particles were purified 3 days later by sedimentation through sucrose and subjected to DNaseI digestion to remove plasmid DNA. The encapsidated, protected DNA was extracted and amplified by using PCR primers that allowed direct amplification of spliced and unspliced FFV DNA and confirmation of the introduced *bet* mutations. The sequencing data for spliced and unspliced FFV DNAs are summarized in Table 1. FFV WT genomes displayed a low mutation frequency with no preference for G to A exchanges. In contrast, G to A substitutions were highly enriched in DNAs from both *bet* mutants, independent of whether spliced or unspliced DNA was sequenced. The number of G to A exchanges varied between 1 and 11 per sequence (Table 1). Whereas all spliced cDNAs from both mutants contained at least one G to A exchange, some unspliced and thus even longer cDNAs of mutants pFeFV-MCS and pFeFV-BBtr did not. It is likely that these unmodified, full-length sequences were derived from input plasmid DNA and not from reverse-transcribed genomes despite the DNaseI digestion.

When analyzing the minus strand for the sequence context in which the changes occurred, 68% were TTC to TTT changes, 14% were TCC to TCT exchanges, and in the remaining clones, at least one pyrimidine residue (NPyC or PyNC) preceded the altered C nucleotide (Table 2). PyPyC to PyPyT mutations are typical for APOBEC3-mediated editing of retroviral genomes (32, 33). In summary, these data indicate that CRFK cells express an APOBEC3-like deaminase (see below) and that FFV Bet counteracts this editing activity.

To exclude the possibility that mutagenesis of *bet* interferes with the fidelity of FFV reverse transcription, we transfected WT

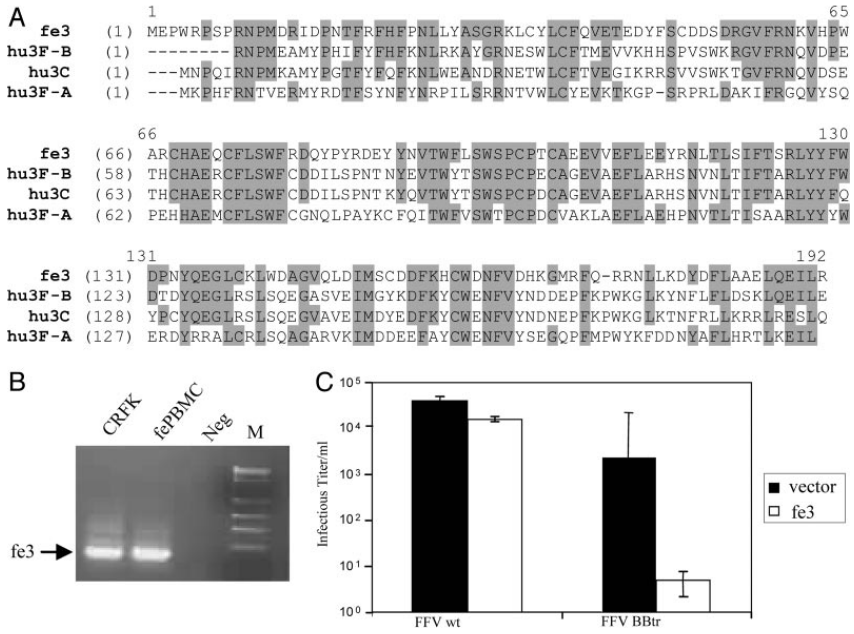

Fig. 1. The FFV Bet protein counteracts the antiviral activity of feline APOBEC. (A) Alignment of the amino acid sequence of feline APOBEC3 (fe3), human APOBEC3C (hu3C), and human APOBEC3F domain 1 (hu3F-A) and domain 2 (hu3F-B). Identical residues are marked. (B) Analysis of fe3 expression by RT-PCR of total RNA from feline CRFK cells and feline PBMCs. Neg, no cDNA added. (C) WT (pFeFV-7) and Bet mutant (pFeFV-BBtr) FFV genomes were cotransfected with pUC18 control DNA (vector) and pfe3 expression plasmid into 293T cells. The titer was determined by using FeFAB titration cells (23).

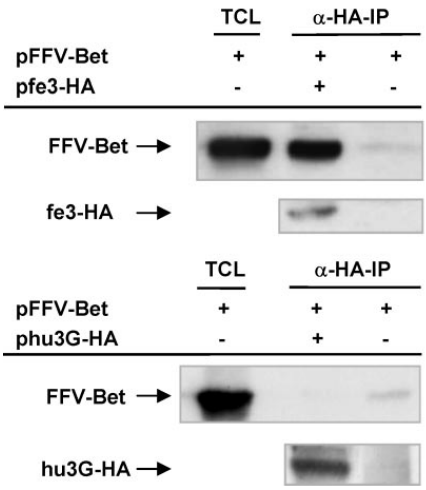

Fig. 2. FFV Bet coimmunoprecipitates with feline APOBEC3, but not with human APOBEC3G. 293T cells were transfected with FFV Bet alone or with expression plasmids encoding HA-tagged fe3 or hu3G. As positive controls, total cell lysates (TCL) were prepared 2 days aftertransfection and FFV Bet was detected by immunoblotting using α-FFV-Bet antiserum (lane 1, upper line in each panel). For detection of APOBEC-associated FFV-Bet, α-HA-immunoprecipitations (α-HA-IP) were performed and FFV Bet was detected by immunoblot analysis (lanes 2 and 3, upper line). α-HA-immunoprecipitation was controlled by a α -HA immunoblot (lower line in each panel).

pFeFV-7 and mutant pFeFV-BBtr into APOBEC-negative 293T cells and analyzed reverse-transcribed genomes from released particles for mutations. Under these conditions, the frequency and types of mutations were similar for WT and *bet*-mutated FFV genomes excluding a direct effect of Bet on the fidelity of the FFV RT (see below).

Characterization of Feline APOBEC3. To identify APOBEC3 expression in CRFK cells, degenerate primers derived from exons 3 and 6 of hu3G were used to amplify and clone the central part of the corresponding CRFK cell-derived feline APOBEC3 cDNA. The full-length feline APOBEC3 (fe3) cDNA was subsequently constructed by 5'- and 3'-RACE techniques. The 192-aa-long fe3 shows significant homology to the second (48.5%) and first (38.1%) domain of hu3F and to the single domain of hu3C (46.4%) cytidine deaminases (Fig. 1*A*). hu3F and fe3 consistently have a similar editing preference for the trinucleotide TTC (see Table 2), whereas hu3G prefers CCC (7, 34). When diagnostic PCR primers were used, substantial fe3 expression was detectable in CRFK cells and in PHA-activated feline PBMCs (Fig. 1*B*); the fe3 cDNA derived from PBMC was identical to that from CRFK cells.

Fe3 Reduces the Titer of *bet*-Deficient FFV and Induces Genome Editing. The effect of fe3 coexpression with WT and *bet*-deficient FFV genomes was studied after transfection of 293T cells. For this purpose, the FFV titers were determined 2 days after transfection by using FeFAB reporter cells (23). Cotransfection of pfe3 reduced the WT FFV titer of pFeFV-7 up to 10-fold, whereas a 10^2- to 10^3-fold reduction in titer was detected with the Bet-truncated pFeFV-BBtr mutant (Fig. 1*C*). This finding clearly demonstrates that FFV Bet efficiently counteracts the antiviral activity of feline APOBEC3.

As described for CRFK cell-mediated FFV genome editing, a total of 29 FFV DNA genomes released from WT and *bet*-mutant pFeFV-BBtr cotransfected with either pfe3-HA or pUC18 was analyzed. Fe3 overexpression in 293T cells resulted in 0.05 G to A exchanges per 100 nucleotides for the WT FFV genome compared with 0.13 G to A exchanges per 100 nucleotides when pUC control DNA was coexpressed. As expected, editing of the Bet mutant pFeFV-BBtr increased editing to 1.05 G to A exchanges per 100 nucleotides when fe3 was coexpressed, whereas few G to A exchanges (0.08 G to A exchanges per 100 nucleotides) occurred without fe3. The sequence context of the G to A exchanges by fe3 coexpression in 293T cells (Table 2, right column) is similar to that seen in CRFK cells expressing the endogenous fe3 deaminase activity. This finding indicates that the majority of FFV genome editing in CRFK cells can be attributed to the cloned fe3 or a closely related feline cytidine deaminase.

FFV Bet Specifically Binds to Feline APOBEC3. Because the fe3-encoded deaminase showed a Bet-dependent phenotype on FFV titer and genome editing, we analyzed by coimmunoprecipitation assays whether fe3 is specifically bound by FFV Bet. An FFV Bet expression plasmid was cotransfected into 293T together with plasmid pfe3-HA or control DNA cells, and lysates were subjected to coimmunoprecipitation using anti-HA beads, allowing detection of the HA-tagged fe3 protein (Fig. 2). Similar to HIV-1 Vif (3, 13), FFV Bet was coprecipitated by fe3-HA, whereas FFV Bet was not detected when the HA-tagged human APOBEC3G (hu3G-HA) protein was used, although it was clearly present in the lysate. These data demonstrate a species-specific binding of FFV Bet to the homologous fe3 but not the heterologous hu3G protein.

FFV Bet Is Not Incorporated in Virions. To determine whether the abundantly expressed cytoplasmic Bet that efficiently interacts with host cell-encoded fe3 is a component of viral particles, immunoblotting studies were performed. Particles were harvested from the supernatant of CRFK cells 5 days after WT FFV infection. The virions were subjected to subtilisin digestion to remove any Bet that was merely attached to the surface but not incorporated into FFV particles (26). Whereas low amounts of Bet were detectable in undigested FFV particles, subtilisin treatment completely eliminated Bet-specific signals, indicating that Bet was only copurified with virus particles (Fig. 3*A*). The conditions of subtilisin treatment were controlled by following digestion of the 16-kDa ectodomain of the FFV Env leader protein (Elp) to the 9-kDa membrane-protected product (Fig. 3*B*) (26). In lentiviruses, the APOBEC3-protecting Vif protein is found in released virions in most laboratories, but other groups have failed to detect Vif in virions (21, 22).

Fe3 Interferes with FFV Particle Release and Accumulates in Particles from *bet*-Deficient Genomes. We analyzed whether fe3 expression affects FFV gene expression and release or composition of particle. To this end, WT pCF-7 and Bet mutant pCF-BBtr proviruses were cotransfected with decreasing amounts of plasmid pfe3-HA into 293T cells (Fig. 4). In cellular extracts, HA-tagged fe3 was clearly detectable as a discrete band of 22 kDa (Fig. 4*A*). The overall expression level of fe3-HA was not altered in WT versus mutant Bet-expressing cells. The expression level of FFV Gag was also not affected by fe3-HA coexpression; however, the processing of the FFV p52 Gag precursor to the p48 Gag cleavage product was consistently reduced on overexpression of fe3-HA (Fig. 4*B*), whereas Pol processing appeared normal (data not shown). The observations that fe3 stability is not affected by Bet and that increasing amounts of fe3 interfere with Gag but not with Pol processing were confirmed in independent experiments (data not shown).

We then analyzed the cell culture supernatants for WT and mutant particle release. When the Gag-reactive cat serum 8014

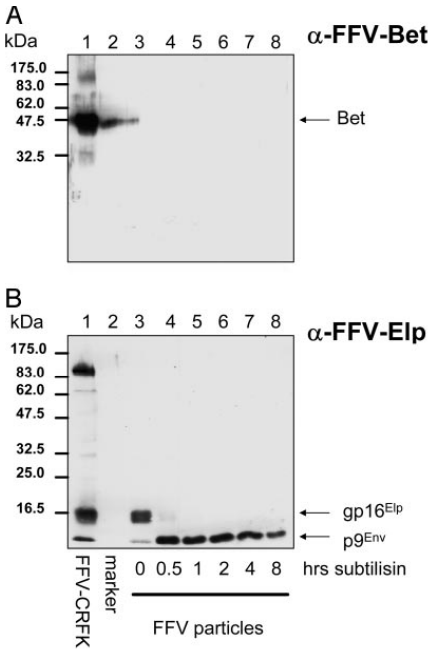

Fig. 3. FFV Bet is undetectable in purified FFV particle preparations. FFV particles enriched from supernatants of FFV-infected CRFK cells by sedimentation through a sucrose cushion were digested with subtilisin for different time points as indicated below the blot and analyzed by immunoblotting. In parallel, antigen from FFV-infected CRFK cells and molecular mass markers were applied (lanes 1 and 2). The blot in A was reacted with a serum against FFV Bet, the blot in B was probed with an Elp antiserum. The positions of FFV Bet, the Env leader protein gp16^{Elp}, and the Env signal peptide p9^{Env} (26) are indicated at right, those of marker proteins are given at left.

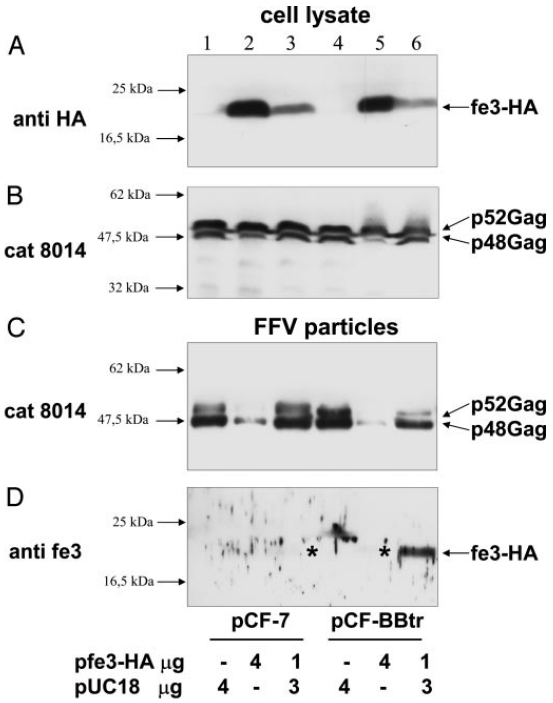

Fig. 4. Effect of fe3 on FFV Gag expression and particle release. 293T cells were cotransfected with 4 μg of WT pCF-7 and mutant pCF-BBtr plus either pUC18 control DNA or 4 or 1 μg of pfe3-HA expression plasmids as indicated below the blots. Two days after transfection, cellular extracts (*A* and *B*) and released FFV particles (*C* and *D*) were harvested and analyzed by immunoblotting using an HA-specific antibody (*A*), cat serum 8014 (*B* and *C*), and the fe3-specific antiserum (*D*). The positions of marker proteins are given at the left margin. The positions of fe3-HA and p48 and p52 Gag are indicated. The asterisks mark faint fe3-HA bands in WT (lane 3) and mutant particles (lane 5) only visible after overexposure (data not shown). For virions from *bet*-deficient genomes, twice the amount was loaded (*C* and *D*, lanes 4–6).

was used, cotransfection of high amounts (4 μg) of pfe3-HA strongly reduced release of particles derived from WT and *bet*-deficient proviruses, whereas lower amounts of fe3 only affected release from *bet*-deficient FFV genomes (Fig. 4*C*). A parallel blot reacted with the fe3-specific serum clearly revealed low amount of fe3-HA in particles from *bet*-deficient FFV genomes (Fig. 4*D*). In WT particles, miniscule amounts of fe3-HA were detectable only after overexposure of the blot (marked by asterisks in lane 3). For pCF-BBtr-derived virus, the amount of fe3-HA detected paralleled the release of particles: the low-level release with high fe3 concentrations resulted in only trace amounts of fe3 in the particle fraction, whereas moderate particle budding (at 1 μg of pfe3-HA DNA) was paralleled by an increased fe3 release. These data show that WT Bet inhibits fe3 packaging into FFV particles.

Discussion

We report here that *bet*-deficient genomes of FFV, a representative member of the retroviral *Spumaretrovirinae* subfamiliy, are edited in a cell type-dependent manner. We show that *bet*-deficient FFV genomes are susceptible to APOBEC3-mediated genome editing in feline CRFK cells. The cytidine deamination of FFV genomes already takes place in the virus-producing cell and not exclusively in the newly infected cell as shown for different orthoretroviruses including lentiviruses (3, 5–8). Co-immunoprecipitation assays and cotransfection of fe3 expression plasmids with WT and *bet*-mutated FFV genomes indicate that Bet, in addition to its other known functions (23, 35–37), counteracts cellular APOBEC3 activities. Infectivity of Bet-deficient FFV is reduced not only by genome deamination but also by an APOBEC3-induced reduction of particle release.

Studying APOBEC3-mediated FV genome editing and viral countermeasures, we identified a previously undescribed feline orthologue of the APOBEC3 deaminase family. The fe3 gene consists of a single APOBEC3 domain that displays significant homology (48.5%) to the second domain of hu3F. This genetic relatedness is consistent with the hu3F-like editing context described here for fe3. Fe3 mRNA is substantially expressed in feline PBMCs and CRFK cells.

Expression of fe3 is consistent with the nonpermissive phenotype of CRFK cells toward replication of *bet*-mutated FFV, and fe3 or a related APOBEC3 deaminase might also restrict replication of Vif-deleted FIV in CRFK cells (30). We assume that the APOBEC3-inactivating function is also required for FFV replication in its presently unknown target cells in cats explaining why Bet is maintained *in vivo* and *in vitro* (20, 25, 31).

APOBEC3-mediated genome deamination is considered to be an efficient barrier against lentiviral interspecies transmission events because the Vif proteins tend to counteract only APOBEC3 proteins of the cognate host species (3). The binding

characteristics presented here for FFV Bet parallel these findings. Thus, genome deamination of FFV in humans after a zoonotic transmission is not likely to be prevented by FFV Bet. In preliminary experiments, we even found strong editing of the WT FFV genome by human and African green monkey APOBEC3G with a concomitant reduction in viral titers and similar effects of fe3 on the primate (human) FV (data not shown). This may explain why zoonotic transmission of FFV has not been detected (38).

The data presented indicate that FFV Bet binds to fe3. Together with the high-level cytoplasmic expression of Bet in all cell culture systems studied (31), this may point to an active sequestration of APOBEC3 away from the sites of FV particle assembly. This active sequestration of fe3 is in line with the observation that functional inactivation of Bet correlated with accumulation of fe3 in released virus particles. The alternative mechanism, that Bet may direct APOBEC3 proteins to proteasome-mediated degradation as is well documented for Vif (4, 9–11), appears unlikely because intracellular fe3 levels were not affected by FFV Bet. The fact that subtle mutations of Bet in clone pFeFV-MCS destroyed its protective potential as severely as truncating Bet at the same site indicates that this central part of Bet either directly affects its function, e.g., during APOBEC3 binding, or that this sequence is absolutely required for proper protein folding. The high concentrations of Bet may be not only required for APOBEC3 sequestration, but also to the other Bet functions, e.g., in establishing and maintaining persistence (35), reactivation from latency (36), intercellular trafficking (37), or particle release (23).

We cannot provide an explanation for why overexpression of fe3 reduced only intracellular Gag processing and particle release, two processes that may be functionally linked. However, WT Bet can partially suppress this reduction in Gag processing provided that fe3 is expressed at only moderate levels. Because the replication pathway of FVs is in part reminiscent of that of hepatitis B virus, the APOBEC3-mediated effect on FV particle release may be related to the presently unknown inhibitory mechanism of hu3G directed against hepatitis B virus pregenome packaging (12, 39).

The most distinguishing feature in the APOBEC3G-mediated editing of FV genomes in contrast to orthoretroviruses is the timing of deamination: in orthoretroviruses, editing only occurs in the newly infected cell. In contrast, deamination of FFV genomes by fe3 is already clearly detectable in genomes packaged into released particles. We obtained similar data for the primate FV (data not shown) that can be edited by different APOBEC3 deaminases (40). However, we cannot exclude the possibility that editing also takes place after virus release or further increases during postentry reverse transcription (41). The early onset of FV genome editing is most probably related to the fact that FV reverse transcription already starts before or during particle formation and release in the virus-producing cell. This finding may explain our observation that only low amounts of fe3 are present in particles from *bet*-deficient FFV genomes, because, in FVs, the virus-producing cell, and not the newly infected cell, is the major site of APOBEC3 action.

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Das zoonotische Potential animaler Foamyviren

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Zusammenfassung

Die Infektion des Menschen mit einem Tierpathogen birgt die Gefahr, dass sich die zoonotischen Erreger im Wirt ausbreiten und ihr Wirtsspektrum so verändern, dass es zu einer Übertragung von Mensch zu Mensch kommen kann. Hier besteht also ein ernstes Risiko für die Weltgesundheit. Ein solches Szenario ereignete sich beispielsweise bei der Infektion vieler Menschen mit den Affen Immundefiziensviren (SIV), die zur Entstehung der humanen Immundefizienzviren (HIV) und nachfolgender AIDS Pandemie führte.

Auch bei der SARS Coronavirus Epidemie gibt es gute Hinweise auf einen Wirtswechsel eines tierischen Virus.

Die Gefahr von zoonotischen Übertragungen tierischer Viren auf den Menschen hat durch die Veränderungen im Lifestyle sogar noch zugenommen. In diesem Review wird mit der Kenntnis gut dokumentierter Fälle zoonotischer Übertragung verschiedener Affen-Fomy Viren auf den Menschen das Potential zoonotischer Infektion von Rinder-, Katzen- und Pferde-Foamy Retroviren diskutiert.

Abstract

Human infections by pathogens originating from animals are a permanent threat. In particular the potency of transmission within the human population is the prerequisite for an epidemic or pandemic distribution of the new pathogen. This scenario has happened in the past on several independent occasions with the simian immunodeficiency viruses, which led to the HIV (AIDS) pandemic. The same appeared to have happened with the SARS coronavirus epidemic, although the authentic animal host has not yet been defined. This review summarizes well documented cases of transmission of various simian foamyviruses to man and discusses the zoonotic potential of non-human foamyvirus from cats, cattle and horses.

Einführung

Foamyviren (FV), die auch als Spuma- oder Spumaretroviren bezeichnet werden, sind die am wenigsten untersuchte Gruppe innerhalb der Familie der Retroviren (Rethwilm, 2003). Während der letzten Jahre ist das Interesse an FV aus verschiedenen Gründen gewachsen. Foamyviren werden zum einen als vielversprechende Vektoren für die gezielte Verabreichung und Expression therapeutischer Gene oder Antigene, z.B. für die Transduktion menschlicher hämatopoetischer Stammzellen, oder als Impfstoff-Vektoren angesehen (Vassilopoulos et al., 2001; 2003a, Schwantes et al., 2003). Zum anderen wurde wiederholt gezeigt, dass Foamyviren aus wildlebenden oder in Gefangenschaft lebenden Affen, (Simian Foamyviren, SFV), mehrfach auf den Menschen übertragen worden sind (Heneine et al., 1998; Schweizer et al., 1997). Schließlich ist anzuführen, dass sich die Foamyvirus-Replikation, Morphogenese und Partikelstruktur von der anderer Retroviren unterscheidet (Fig. 1A; Rethwilm, 2003; Linial and Eastman, 2003; Lecellier and Saib, 2000; Linial, 1999). Diese Foamyvirus-spezifischen Unterschiede führten zu einer neuen systematischen Einordnung innerhalb der Familie der Retroviren, die jetzt in zwei Unterfamilien unterteilt ist: die Spumavirinae und die Orthoretrovirinae. Die Orthoretrovirinae umfassen alle anderen bekannten Retroviren, wie z.B. die hochpathogenen humanen Immundefizienzviren (HIV), die humanen T-Zell-Leukämie-Viren (HTLV) und Tierpathogene, wie das Virus der infektiösen Anämie der Einhufer, die felinen und bovinen Leukämie-Viren, die Immundefizienzviren von Rindern und Katzen sowie das enzootische ovine Nasaltumorvirus (Rethwilm, 2003).

Das bekannteste Foamyvirus ist das so genannte humane Foamyvirus (HFV), welches das erste Retrovirus war, das, noch vor der Entdeckung und Isolierung des HTLV-1 und HIV, aus einem Menschen isoliert wurde (Achong et al., 1971). Es ist das am gründlichsten untersuchte Virus dieser Gruppe und wird deshalb auch als Prototyp-Foamyvirus bezeichnet. Nach intensiven Studien und einer lang andauernden Debatte ist allgemein akzeptiert, dass das ursprüngliche HFV-Isolat seinen Ursprung im Schimpansen hat und dass alle dokumentierten Foamyvirus-Infektionen des Menschen wahrscheinlich von Nichthuman-Primaten (NHP) erworben wurden (Heneine et al., 1998; Herchenröder et al., 1994). Während das zoonotische Potential der SFV aus NHPs relativ gut erforscht ist, ist zur Zeit nur sehr wenig über das zoonotische Potential der Foamyviren von Haus- und Nutztieren bekannt. Da gerade in der nördlichen Hemisphäre der Kontakt zu bovinen, felinen und equinen Foamyviren (BFV, FFV, EFV) sehr viel wahrscheinlicher ist, als der mit Foamyviren von NHPs, soll nach einer Zusammenfassung der molekularen und allgemeinen Biologie der FV das zoonotische Potential dieser Viren diskutiert werden.

Molekularbiologie der Foamy Viren

Wie HIV und HTLV sind auch die Foamyviren aufgrund ihrer genetischen Organisation, ihrer Strategien der Genexpression und ihrer Wechselwirkungen mit dem infizierten Wirt und den Wirtszellen, komplexe Retroviren (Fig. 1, Löchelt, 2003). Sieht man aber von diesen Ähnlichkeiten ab, so haben Foamyviren zahlreiche einzigartige

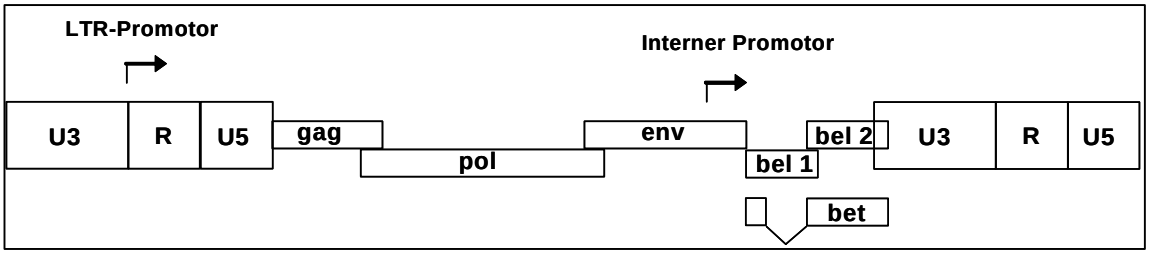

Fig. 1: Genetische Karte von Foamyviren am Beispiel des FFV. Die FFV Gene *gag*, *pol*, *env*, *bel1* und *bel2* sind als Kästen dargestellt. Die regulatorischen „langen terminalen Repetitionen" (LTR) sind in die U3, R, and U5 Regionen unterteilt (nicht maßstabsgerecht). Der Promotor in der 5' LTR und der interne Promotor (IP) sind durch geknickte Pfeile dargestellt, die Pfeilspitze weist in die Transkriptionsrichtung. Das Bet Protein besteht, wie schematisch anggedeutet, aus Bel1- und Bel2-Domänen.

Eigenschaften, die sie klar von den klassischen Onkoretroviren und den Lentiviren unterscheiden. Das Foamyvirus-Genom ist eine lineare, positiv orientierte RNA mit einer Länge von 11 bis 13 kB. Zusätzlich zu den für alle Retroviren charakteristischen Genen *gag*, *pol* und *env* und den regulatorischen Elementen in beiden LTRs enthalten Foamyviren bis zu drei zusätzliche offene Leserahmen (bel1 bis bel3), die hauptsächlich von einem internen Promotor exprimiert werden (Fig. 1, zusammengefasst von Löchelt, 2003). Das Vorhandensein dieses funktionellen und essentiellen internen Promotors (IP) ist ein charakteristisches Merkmal der Foamyviren (Löchelt et al., 1993). Der LTR Promotor und der IP benötigen für die Genexpression und Infektiosität den Bel1- Transaktivator. Vom IP wird, wegen seiner erhöhten Basalaktivität, die Foamyvirus-Genexpression gestartet und kontrolliert (Löchelt, 2003, Meiering and Linial, 2002). Die Nutzung von zwei Promotoren erlaubt eine zeitliche Regulierung der Expression früher regulatorischer und später Strukturproteine.

Die Funktion(en) der übrigen Bel-Proteine und des stark exprimierten Bet-Proteins, das sowohl Bel1- als auch Bel2-Sequenzen enthält, sind weitgehend unbekannt (Alke et al., 2001). Antikörper gegen Bet haben zumindest für das HFV und die SVF einen diagnostischen Wert.

Die proteolytische Prozessierung der Foamyvirus-Gag-Proteine ist einzigartig unter den Retroviren. Bei freigesetzten Foamyvirus-Partikeln (Fig. 2A) werden die drei Gag-Domänen Matrix (MA), Capsid (CA) und Nukleocapsid (NC) nicht durch die virale Protease voneinander getrennt, es wird lediglich ein C-terminales Peptid von 4 kDa abgespalten (Zemba et al., 1998; Pfrepper et al., 1999; Fig. 2B).

Ein weiteres Charakteristikum der Foamyviren ist, dass die Foamyvirus-Pol-Proteine - unabhängig von Gag - von einem gespleißten Transkript synthetisiert werden (Fig. 1, Bodem et al., 1996; Enssle et al., 1996; Yu et al., 1996). Aufgrund des Fehlens eines Gag-Pol- Fusionsproteins muss das Pol-Protein spezifisch in neu gebildete Virionen eingebaut werden (Löchelt und Flügel, 1996).

Eine weitere einzigartige Eigenschaft ist das Einsetzen der reversen Transkription der RNA- Genome bereits in der Virus-produzierenden Zelle, vor dem Freisetzen der Virionen (Moebes et al., 1997; Yu et al., 1999). Aus diesem Grund enthält eine beträchtliche Anzahl freigesetzter Foamyvirus-Partikel

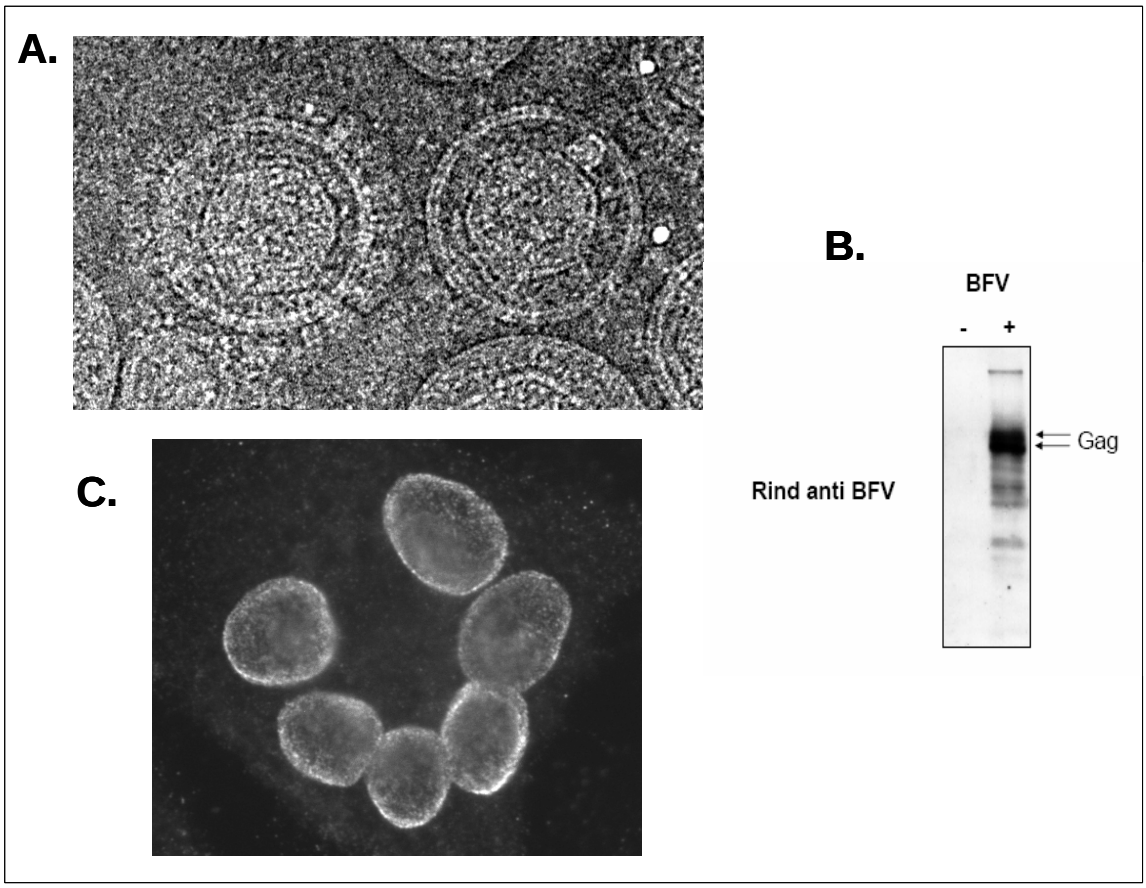

Fig. 2: FV Morphologie und Foamyvirus-spezifischer zytopathische Effekte. **A.** Cryo-electronenmikroskopische Aufnahme freigesetzter FFV Partikel. Die Oberflächen-Glykoproteine und die innere Anordnung der Gag-Proteine mit der unter der viral Membran gelegenen MA-Schicht und dem polygonalen Kapsid sind klar sichtbar (siehe auch (172). **B.** Nachweis und Reaktivität BFV-spezifischer Antikörper aus Rindern. Als Antigen wurden Partikelpräparationen aus den Überständen BFV- (+) und Schein-infizierter (-) Cf2Th Zellen aufgetragen. Spezifische, virale Proteinbanden, insbesondere die Gag Doppelbande bei 59 und 55 kDa, sind deutlich erkennbar. **C.** Kleine, frühe FFV-induzierte vielkernig Syncytien (Zellfusionen) infizierter und nicht-infizierter CRFK Zellen. FFV Gag Proteine, die an der Kernmembran und in cytoplasmatischen Aggregaten vorliegen, wurden durch indirekte Immunfluoreszenz mit einen Gag-spezifischen Antiserum nachgewiesen. Das Bild in A wurde freundlicherweise von John Briggs und Brent Gowen zur Verfügung gestellt.

sowohl RNA-Genome als auch genomische, provirale DNA.

Im Gegensatz zu den Orthoretroviren sind bei Foamyviren die Env-Oberflächenproteine für die Partikel-freisetzung erforderlich (Baldwin and Linial, 1998; Pietschmann et al., 1999). Dies liegt wahrscheinlich an einer spezifischen Interaktion der Gag-Matrix-Domäne mit dem Env-Leader-peptid (Wilk et al., 2001).

Sequenzanalysen verschiedener Foamy-virus-Genome zeigten, dass *pol* das am höchsten konservierte Gen ist, wogegen *gag* stärker divergiert als *env* (Tobaly-Tapiero et al., 2000). Bei allen anderen Retroviren ist *env* weniger konserviert als *gag* (Wang and Mulligan, 1999).

Innerhalb der FV-Spezies sind die Genome selbst nach zoonotischer Über-tragung hoch konserviert und zeigen nur kleinste genetische Variationen (Phung

et al., 2001; Schweizer et al., 1999). Nur für das Katzen-Foamyvirus sind zwei Serotypen bekannt, deren spezifische Unterschiede überwiegend auf die externe Oberflächendomäne von Env beschränkt sind.

Biologie der Foamy Viren

Foamyviren besitzen einen weiten Gewebs-Tropismus und sind *in vitro* in einer Vielzahl verschiedener Zelltypen unterschiedlicher Spezies propagiert worden (Fig. 3; Hill et al., 1999). Die große Palette permissiver Zellen mag auf eine weite Verbreitung des viralen Rezeptors hindeuten. Foamyviren replizieren in Zellkultur meist lytisch (Fig. 2C), allerdings wurde in bestimmten Zellen auch eine persistierende Replikation ohne Zellschädigung beschrieben (Meiering et al., 2001). In natürlich infizierten Wirten verursachen Foamyviren eine persistierende Infektion ohne offensichtliche Krankheitssymptome. Aufgrund dieser scheinbar gutartigen Persistenz im natürlich infizierten Wirt, werden Foamyviren als apathogen angesehen (Saib, 2003). Ein Zusammenhang einer FFV-Infektion mit Nierenversagen bei Katzen wurde diskutiert; die Relevanz dieses Befundes ist jedoch unklar. Obwohl sie unter natürlichen Bedingungen apathogen sind, besitzen Foamyviren doch ein beträchtliches Krankheitspotential, wie an HFV-transgenen Mäusen gezeigt wurde (Aguzzi, 1993). In diesen Mäusen konnten durchweg stark pathologische Zellveränderungen in Form von Syncytienbildung im Gehirn sowie neurologische Symptome festgestellt werden (Tschopp et al., 1996). Allerdings stellen transgene Mäuse lediglich ein experimentelles System dar, welches nicht zwangsläufig die authentische Situation reflektiert.

Experimentell oder natürlich auftretende Foamyvirus-Infektionen sind durch eine starke und polyspezifische humorale Immunantwort gegen verschiedene Struktur- und Nichtstrukturproteine charakterisiert (Fig. 2B). Reaktivitäten gegen Gag, Pol, Env und Bet beginnen innerhalb von Wochen nach der Infektion, nehmen mit der Zeit zu und bleiben lange Zeit auf relativ hohem Niveau stabil (Fig. 2B). Dies ist ein Hinweis, dass das Immunsystem fortwährend oder periodisch durch alle viralen Antigene stimuliert wird (Alke et al., 2000). Dieser Befund deutet auf eine konstante virale Genexpression (Fig. 3) mit kontinuierlicher Produktion viraler Infektivität hin.

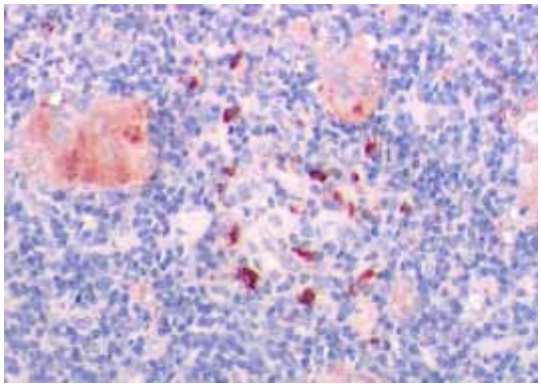

Fig. 3: Immun-histochemischer Nachweis von FFV Bet Protein in Thymusgewebe experimentell FFV-infizierter Katzen. Die Pfeile weisen auf Bet-positive Zellen hin.

Im Einklang mit der Idee einer produktiven FV-Persistenz, im Gegensatz zu einer klassischen viralen Latenz, mit einer mehr oder weniger eingeschränkten viralen Genexpression, ist die Tatsache, dass streunende, mit FFV natürlich infizierte Katzen in einer Studie immer positiv bezüglich FFV-spezifischer DNA, Antikörper und viraler Infektiosität getestet wurden (Winkler et al., 1998). Die Reaktivitäten gegen Gag, und bei HFV auch Bet, gelten als diagnostische Marker (Hahn et al., 1994). Der Beitrag der zellulären

Immunantwort bei der Kontrolle der Persistenz sowie die Induktion einer Immunität der Schleimhaut sind zur Zeit noch unbekannt (Schwantes et al., 2003).

Das zoonotische Potential der Affen-Foamy Viren

Nach der Isolierung des prototypischen HFV aus dem Nasopharyngskarzinom eines afrikanischen Patienten (Achong et al., 1971) wurde eine intensive Suche nach HFV-Infektionen bei verschiedenen kranken und gesunden Bevölkerungsgruppen gestartet (Ali et al., 1996; Schweizer et al., 1995, Wolfe et al., 2004). Zum heutigen Zeitpunkt ist kein übereinstimmender und allgemein akzeptierter Zusammenhang zwischen der Detektion von HFV oder Foamyvirus spezifischen Infektionsmarkern und einer definierten Erkrankung des Menschen bekannt. Es wurden anfänglich Korrelationen zur Basedow'schen Krankheit, chronischer Thyreoditis und anderen Erkrankungen des Menschen postuliert, die aber nicht bestätigt werden konnten (Lagaye et al., 1992; Saib et al., 1994; Schweizer et al., 1994). Es gilt als gesichert, dass die Prävalenz des HFV-Isolats in der menschlichen Bevölkerung im Gegensatz zu anfänglichen Befunden, die auf geographische Unterschiede von Regionen relativ hoher Inzidenz hindeuteten, extrem gering ist (Ali et al., 1996; Schweizer et al., 1994; 1995).

Bei der Sequenzierung der aus Schimpansen isolierten SFV_{cpz}, wurde offensichtlich, dass das HFV-Isolat und verschiedene SVF_{cpz}-Isolate nahezu identisch sind (Herchenröder et al., 1994). Deshalb wurde davon ausgegangen, dass dieses einzelne HFV-Isolat das Ergebnis einer von Schimpansen ausgehenden FV-Kontamination der Zellkulturen war, die zur Propagierung des HFV-Isolats dienten. Diese Interpretation erwies sich jedoch als voreilig, da serologische Studien unter Verwendung von Seren aus Tierpflegern, die beruflich NHP ausgesetzt sind, in 2-4 % der Fälle eine klare Reaktivität gegen HFV besaßen, wohingegen Zeichen für zoonotische Infektionen mit anderen Affenretroviren nicht erbracht werden konnten oder extrem selten waren (Brooks et al., 2002; Heineine et al., 1998; Sandstrom et al., 2000). Bei diesen und anderen Studien wurde gezeigt, dass zoonotische Übertragung nicht nur mit SFVcpz, sondern auch mit den SFV aus grünen Meerkatzen, Pavianen, Gorillas, Mandrillen und Makaken möglich ist (Wolfe et al., 2004; Brooks et al., 2002; Heineine et al., 1998; Sandstrom et al., 2000). Besonders der Befund, dass es wiederholt Übertragungsereignisse von genetisch und antigenetisch unterschiedlichen SFVs gab, zeigt deutlich, dass SFVs ein beträchtliches zoonotisches Potential besitzen, das signifikant höher ist als das der anderen überprüften NHP-Retroviren (Heineine et al., 1998). Ausgehend von den bekannten Fällen scheint es, dass tiefe Bisswunden und möglicherweise infektiöser Speichel der Tiere, die Zubereitung von Affenfleisch und die Transplantation von Organen aus Pavianen zu SFV-Übertragungen führten (Wolfe et al., 2004; Allan et al., 1998; Heineine et al., 1998). Die erworbenen Viren induzieren eine persistierende Infektion beim Menschen mit anhaltenden SFV-spezifischen Antikörpern und der Verbreitung intakter und beschädigter proviraler Genome (Callahan et al., 1999). In einigen Fällen konnten sogar infektöse Viren reisoliert werden (Boneva et al., 2003; Schweizer et al., 1997).

Bei keiner der bekannten mit HFV/SFV infizierten Personen konnten -

abgesehen von der Tatsache einer lang andauernden SFV- Infektion Symptome einer SFV- induzierten Erkrankung festgestellt werden (zusammengefasst bei Heneine et al., 2003). Im Gegensatz zu den ursprünglich infizierten Personen, die klar alle Zeichen einer FV- Infektion aufwiesen, wurden weder Familienmitglieder noch Empfänger von Blutprodukten positiv auf eine SFV- Infektion getestet (Boneva et al., 2002; 2003). So scheint es, dass infizierte Menschen Endwirte darstellen. In Anbetracht der sehr niedrigen Zahl gut dokumentierter Fälle muss man allerdings vorsichtig sein, voreilige Schlüsse über das Krankheitspotential zu ziehen.

Die für die Detektion zoonotisch erworbener SFV benutzten Reagenzien basieren primär auf Proteinen (für ELISA und Immunoblots) und auf DNA-Sequenzen des HFV-Isolats (für PCR Amplifikation) (Heneine et al., 1998; Schweizer et al., 1995). Dies kann dazu führen, dass Infektionen mit entfernter verwandten SFVs dem Nachweis entgehen. Ein kürzlich entwickelter Immunoblot geht dieser Problematik, durch Verwendung einer Kombination verschiedener HFV- und SFV- abstammender Antigene, aus dem Weg (Hussain et al., 2003). In diesem Zusammenhang kann erwähnt werden, dass jüngst Anzeichen von SFV-Infektionen bei afrikanischen Buschjägern detektiert wurden, was ein Hinweis darauf ist, dass auch das Jagen und Schlachten infizierter Tiere zu zoonotischer Übertragung von SFV führen kann (Wolfe et al., 2004). In Anbetracht des weit verbreiteten Konsums von „Buschfleisch“ in Zentralafrika, birgt dieser Teil der Welt ein erhöhtes Risiko der Entstehung humaner FV. Der Eintritt von HIV in die menschliche Bevölkerung erfolgte nach denselben Mechanismen. Die Ko-Infektion mit diesem oder einem anderen immunsuppresiven Erreger kann das Zoonoserisiko von SFV sogar erhöhen (Weiss, 2001).

Das Potential einer zoonotischen Übertragung der Rinder, Katzen und Pferde Foamy Viren

Bis zum jetzigen Zeitpunkt wurden drei Nicht-Primaten-Foamyviren, das bovine (BFV), das equine (EFV) und das feline (FFV) Foamyvirus, vornehmlich *in vitro* und in geringerem Ausmaß *in vivo*, studiert und charakterisiert (zusammengefasst von Saib, 2003). FV aus anderen Spezies wie Seelöwen und Hamstern werden hier nicht weiter betrachtet, da momentan nur sehr begrenzt Informationen dazu vorliegen. In den Industrienationen der westlichen Welt ist der Kontakt und das Risiko einer zoonotischen Übertragung von SFV völlig begrenzt auf wenige Laborarbeiter und Tierpfleger. Im Gegensatz dazu ist in diesen Ländern beinahe die gesamte Bevölkerung den Pathogenen von Haus- (Pferde und Katzen) und Nutztieren (Rinder) ausgesetzt. Die Gefahr einer Übertragung besteht entweder beim Kontakt mit FFV-, BFV- oder EFV- infizierten Tieren oder indirekt durch Nahrung oder medizinische Produkte, die direkt oder indirekt von infizierten Tieren stammten. Die hohe Prävalenz von BFV und FFV in deren natürlichen Wirten (zwischen 30 und mehr als 70 % der ausgewachsenen Tiere sind seropositiv) macht Kontakte zwischen Foamyvirus-infizierten Tieren und dem Menschen sehr wahrscheinlich (zusammengefasst von Saib, 2003). In gegenwärtig durchgeführten Untersuchungen von Rinderseren aus Sachsen wurden einige Tiere serologisch eindeutig BFV-positiv gestestet (Fig. 2B), bei weiteren Tieren lag ein Anfangsverdacht vor, der sich

nicht verifizieren ließ. Diese Situation zeigt, dass neuartige diagnostische Systeme für breit angelegte epidemiologische Untersuchungen eindeutig erforderlich sind. Diese Einschätzung kann auch im vollen Umfang auf die FFV- und EFV-Diagnostik übertragen weden.

Das Risiko einer zoonotischen Interspezies-Übertragung ist jedoch nicht allein vom Ausmaß und der Art der Exposition mit einem heterologen Pathogen abhängig, sondern auch vom beteiligten Virus. Sind die vom Virus kodierten Proteine die primären Ziele des adaptiven, humoralen und zellulären Immunsystems, werden die durch den Wirt festgelegten Strukturen, z.B. das Glykosylierungsmuster und Virion-assozierte Wirtsproteine, direkt durch Mechanismen der angeborenen Immunität oder durch passiv angepasste kreuzreaktive Immunmechanismen identifiziert (Burton, 2002). So gesehen nimmt das Risiko einer zoonotischen Übertragung mit der Nähe der genetischen Verwandtschaft des authentischen mit dem neuen Wirt zu. Dadurch sind z.B. aufgrund von konservierten Glykosylierungsmustern Übertragungen vom Affen auf den Menschen viel wahrscheinlicher also solche von weit entfernten Spezies wie Katzen, Rindern und Pferden (Saib, 2003). Bei FFV scheint der direkte Kontakt zu infizierten Katzen der wahrscheinlichste Weg einer potentiellen zoonotischen Übertragung zu sein. Da infektiöse FFV reproduzierbar von oralen Abstrichen infizierter Katzen gewonnen werden konnten, bergen auch Katzenbisse an sich ein Risiko der Übertragung, weil durch die Verletzung der Haut Kontakt zu Virus- empfänglichen Zellen z.B. Leukozyten möglich wird. Jedoch wird auch nicht-aggressives soziales Verhalten wie Lecken als Übertragungsweg von Katze zu Katze angenommen (Winkler et al., 1999). Zwei Studien an Veterinären und an anderen, berufsmäßig in Kontakt zu Katzen kommenden, Personen, haben keinen Hinweis auf zoonotische Infektionen mit FFV ergeben (Butera et al., 2000; Winkler et al., 1997). Jedoch könnte diese offensichtliche Resistenz gegenüber einer Übertragung, zum Beispiel bei Kindern, mit einem sich erst entwickelnden Immunsystem, oder Personen mit geschwächtem Immunsystem, weniger effektiv sein. Auf den Risikofaktor, den ein Kontakt von Kindern, mit noch nicht voll entwickeltem Immunsystem, zu Katzen und ihren Pathogenen, ist schon hingewiesen worden. Torrey und Yolken (1995) bringen solche Infektionen mit multifaktoriellen, komplexen Erkrankungen oder Krankheitskomplexen in Zusammenhang.

Ähnlich ist auch die potentielle Übertragung von EFV auf diejenigen begrenzt, die direkten Kontakt zu Pferden haben. Ob EFV in der Mundhöhle der Pferde vorkommt, was eine Grundvorraussetzung für eine durch Bisse verursachte Infektion wäre, ist nicht bekannt. Ein weiterer möglicher Kontakt zu EFV könnte beim Schlachten und durch Pferdefleischkonsum auftreten.

Für BFV gelten dieselben, oben genannten, sowie zusätzliche Möglichkeiten der Übertragung. Zunächst nimmt man an, dass die BFV-Ausbreitung vorherrschend über die Verbreitung BFV- kontaminierten Speichels erfolgt. Die BFV-Übertragung ist nicht von aggressivem Verhalten, wie Bissen, abhängig. Es wurde gezeigt, dass für eine Übertragung innerhalb derselben Spezies soziale Kontakte und das Einatmen von Aerosolen (Lecken und Niesen) ausreichen (Johnson et al., 1988).

Dadurch ist die Wahrscheinlichkeit einer zoonotischen Übertragung deutlich erhöht. Weiterhin konnte gezeigt werden, dass Milch BFV-ähnliche Partikel und infektiöses BFV enthält. Somit könnten auch Rindfleisch, und andere vom Rind stammende Lebensmittel, möglicherweise infektiöses BFV enthalten. Das Risiko, durch Verletzungen beim Schlachten, oder durch das Einatmen von Aerosolen, BFV zu erwerben, ist in Betracht zu ziehen. Auch medizinische Produkte, die aus Zellkulturen stammen, denen Rinderserum zugesetzt wurde, oder direkt vom Rind stammende Therapeutika, stellen ein Risiko für eine BFV-Kontamination dar. Deshalb sollte sorgfältiges Testen und vorheriges Inaktivieren dieser Produkte verpflichtend sein. Ähnliche Übertragungswege sind auch für den Erreger der bovinen spongiformen Enzephalitis (BSE) vorgeschlagen worden (Ludwig et al., 2003).

Schließlich werden zur Zeit Foamyvirus-Vektoren, die vom prototypischen HFV, SFV und FFV abgeleitet sind, für den Gebrauch im Menschen konstruiert. Weiterhin wurden bereits FFV-Vektoren erfolgreich in Katzen als Vakzinevehikel eingesetzt (Mergia and Heinkelein, 2003; Vassilopoulos et al., 2001; 2003b: Schwantes et al., 2003). In Anbetracht der erwarteten Anwendung Foamyvirus-basierter Vektoren bei Nutztieren muss das zoonotische Risiko sehr sorgfältig evaluiert werden.

Zusammenfassung und Ausblick

Zum jetzigen Zeitpunkt weisen keine diagnostischen und experimentellen Daten auf eine zoonotische Übertragung von FFV, BFV und EFV auf den Menschen hin. Die jüngsten zoonotischen Übertragungen anderer Viren haben jedoch das öffentliche Bewusstsein für solche Szenarien, mit unvorhersehbaren und sogar fatalen Konsequenzen, geweckt. Es ist allgemein akzeptiert, dass Zoonosen, vor dem Hintergrund der Veränderungen der Lebensgewohnheiten, eine neue Herausforderung für die Öffentlichkeit und internationale Gesundheitssysteme darstellen, und dass eine wissenschaftliche Aufarbeitung eine Grundvoraussetzung dafür ist, dass angemessene Lösungen für diese Bedrohungen gefunden werden (Ludwig et al., 2003). Die gewonnenen Kenntnisse über das zoonotische Potential der Primaten-Foamyviren, die zur Verfügung stehenden medizinischen Technologien und die Verfügbarkeit von Daten und Reagenzien für die Entwicklung neuer und sensitiver Detektionssysteme, machen es bei dem hohem wissenschaftlichen und medizinischen Interesses möglich, diese offenen Fragen nun adäquat anzugehen.

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Gene Therapy (accepted)

Construction and Characterization of Efficient, Stable, and Safe Replication-Deficient Foamy Virus Vectors

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Summary

Since serious side effects affected recent virus-mediated gene transfer studies, novel vectors with improved safety profiles are urgently needed. In the present study, replication-deficient retroviral vectors based on feline foamy virus (FFV) were constructed and analyzed. The novel FFV vectors are devoid of almost the complete *env* gene plus the internal promoter – accessory *bel* gene cassette including the gene for the viral transcriptional transactivator Bel1/Tas. In these Bel1/Tas-independent vectors, expression of the lacZ (β-galactosidase) marker gene is directed by the heterologous, constitutively active human ubiquitin C promoter (ubi). Env-transcomplemented vectors have un-concentrated titers of more than 10^5 transducing units/ml. The vectors allow efficient transduction of a broad array of diverse target cells which can be increased by repeated vector exposure. However, the number of lacZ marker gene expressing cells decreased slightly upon serial passages of the transduced cells. Vectors carrying a self-inactivating SIN deletion of the TATA box and most parts of the viral promoter were not rescued by wt FFV whereas those with the intact or a partially deleted promoter were readily reactivated. This finding indicates that the viral promoters are in fact non-functional, pointing to a highly advantageous safety profile of these new FFV-ubi-lacZ-SIN vectors.

Introduction

Although the promise of vector-mediated gene transfer has been recently challenged by serious side effects, this branch of modern molecular medicine has still a great potential for further application (1). This can, however, be only achieved when present problems are rationally addressed and solved by appropriate innovations. In addition, it is required to design and optimize the most appropriate vector system for a given medical application implicating that in the future diverse viral and non-viral gene delivery systems constitute the toolbox of molecular medicine. Efficacy and safety profiles of each considered vector system will determine whether it will enter preclinical and clinical evaluation and application (1).

Among the currently used viral vectors, retroviruses hold a great, perhaps the greatest promise due to the stable transduction of target cells. However, the stable integration into the host genome may also lead to unwanted alterations or even oncogenic transformation of the corresponding target cells (2).

Novel retroviral vectors based on primate- and non-primate foamy viruses (FV) which constitute the *Spumavirinae* subfamily of the retroviruses have currently been established and intensively examined by us and others (3-13). For instance, FV vectors based on the human/primate FV allow efficient, stable, and long-term transduction of human haematopoietic cells without detectable side effects (11,12).

FVs are considered to be apathogenic in their natural animal hosts and after zoonotic transmission into humans (14, 15). Therefore, FV-based vectors offer an advantageous safety profile (14, 16, 17). Along with this argument, FV DNA integration properties are favourable for safe and stable transduction of target cells and malignant transformation of FV-infected or transduced cells has never been described (18, 19). Additionally, replication-competent FFV-based vaccine vectors induce a partially protective immunity against a highly pathogenic challenge virus in cats (6).

In one of our previous studies, we have demonstrated that FFV self-inactivating (SIN) vectors rapidly give rise to replication-competent revertants (RCRs) indicating that additional genetic deletions are required to generate safe vectors (4). In a following study, we determined that the essential *cis*-acting sequences required for FFV-based vectors are located upstream and around the *gag* start codon and inside *pol*. The *env* gene turned out to be dispensable for vector function, a characteristic similar to other studied FVs (9, 20). In order to optimize the vector function, we focus in the present study on the construction and characterization of FFV vectors deleted in *env* or in almost the whole *env-bel* genomic region. The *bel* genes (Fig. 1A) encode the viral Bel1/Tas transactivator and Bet that counteracts APOBEC3-mediated host restriction (21, 22). Importantly, only the *env-bel*-deleted vectors carrying SIN-deleted LTR promoters turned out to be suited for the safe and long-term transduction of target cells.

Results

We recently showed that i. FFV SIN vectors rapidly generate RCRs indicating that additional deletions are

required to generate safe vectors and that ii. essential *cis*-acting sequences required for FFV-based vectors are located upstream and around the *gag* start codon and within *pol* (4, 20). Here we concentrate on the characterization of *env*-*bel*-deleted FFV vectors and those with an additional SIN deletion.

Characterization of env-deleted vectors

We first analyzed FFV *env*-deleted vectors for titers and long-term transduction efficiencies. For this purpose, the Env-deleted vectors pCF-Bet-GfpΔEnv and pCF-Bet-Gfp-SINΔEnv (Fig. 1, C and D) containing either the intact or the promoter- and TATA-box-deleted FFV SIN-LTR were co-transfected with the Env-expression plasmid pBC-FFV-Env into 293T cells. Vector titers of cell-free supernatants were determined 2 d later using FFV-FAB titration cells (4, 5). Using 3µg of pCF-Bet-GfpΔEnv or pCF-Bet-Gfp-SINΔEnv plus 3µg pBC-FFV-Env for cells grown in 6 cm dishes, vector titers of about 10^4 transducing units (TU)/ml were obtained.

We then analyzed whether the *env*-deleted FFV vectors allow stable, long-term transduction of permissive CRFK cells and whether RCRs are generated upon extended culture of vector-transduced cells. In repeated experiments using CRFK cells transduced by vectors pCF-Bet-GfpΔEnv and pCF-Bet-Gfp-SINΔEnv, no RCRs and no cytopathic effects were detectable after at least nine serial passages of transduced cells (data not shown). At the same time, we determined the duration of Gfp expression in order to analyze the stability of marker gene expression. During the first passage, 10% of the transduced cells were positive for Gfp auto-fluorescence. The number of positive cells decreased after the 2nd passage to below 5% and readily declined upon further passages (data not shown).

In summary, *env*-deleted FFV genomes direct the production of vector particles, which are able to transduce CRFK cells without detectable levels of RCRs. However, these *env*-deficient vectors do not result in a stable, long-term transduction of target cells.

Particle release depends on Env

We next analyzed protein expression and particle release of pCF-Bet-GfpΔEnv in the presence of different amounts of the Env expression construct pBC-FFV-Env. Two d after co-transfection into 293T cells, cell culture supernatants were harvested for preparation of vector particles and cell extracts were analyzed by immunoblotting (Fig. 2) using cat 8014 reference serum (23). Efficient proteolytic processing of $p52^{Gag}$ to $p48^{Gag}$ was detectable (upper panel) in cells transfected with the parental replication-competent vector pCF-Bet-Gfp (Fig. 1, B) and in all samples transfected with the *env*-deleted vector pCF-Bet-GfpΔEnv. Immunoblot analyses of released vector particles (Fig. 2, lower panel) showed efficient particle release from cells co-transfected with 5 or 2µg pBC-FFV-Env (lanes 1 and 2). Minimal particle release was still detectable with 0.2µg pBC-FFV-Env (lane 3) whereas in the complete absence of Env (lane 5), no particles were released into the supernatant. Similar to the human/primate FV, these data for FFV confirm that the release of

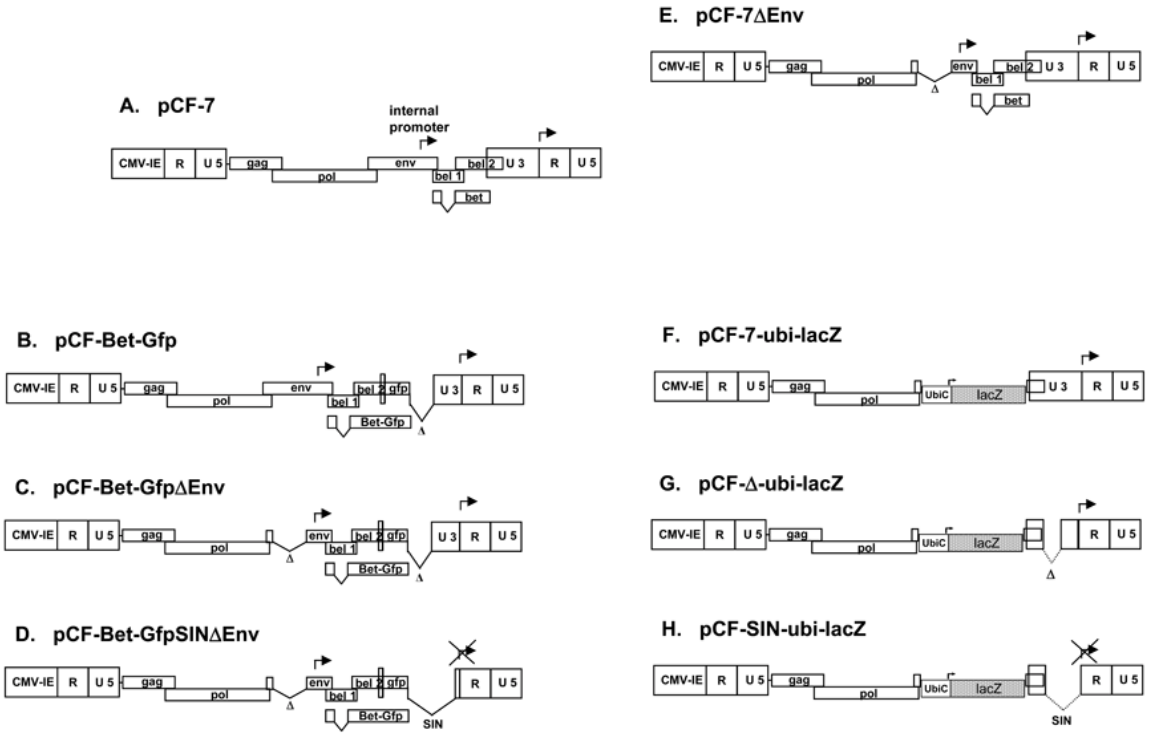


Figure 1. Schematic presentation of the different replication-competent and replication-deficient FFV vectors used. In A, the authentic replication-competent and CMV-IE promoter-driven FFV vector pCF-7 is shown. FFV genes and the LTRs subdivided into the U5, R, and U3 regions, are marked by open boxes. The FFV LTR and internal promoters are marked by rectangular arrows and the structure of the bet gene is shown. The replication-deficient vector pCF-ΔEnv (E) is derived from pCF-7 by deleting env sequences as indicated by a broken line (Δ). In B, the pCF-7 derived replication-competent vector pCF-Bet-Gfp with the gfp marker gene marked by an open box and the partial U3 deletion in the 3' LTR marked by a broken line (Δ) is shown (5). Derived from this construct are the env-deleted vectors pCF-Bet-GfpΔEnv and pCF-Bet-Gfp-SINΔEnv (C and D). In panels F to H, the ubi-lacZ vectors pCF-7-ubi-lacZ, pCF-Δ-ubi-lacZ, and pCF-SIN-ubi-lacZ with the intact, truncated, and functionally deleted LTR promoter and the ubi-lacZ cassette inserted are shown.

FV particles depends on the amount of co-expressed Env and that no FV particles are released in the absence of Env (24, 25).

We further analyzed whether the amount of released particles correlates with the vector titers as measured with FFV-FAB cell titration (26). For this purpose, pCF-Bet-GfpΔEnv was co-transfected with decreasing amounts of the Env expression plasmid pBC-FFV-Env. Two d p.t., the vector titers were determined and normalized to co-expressed secreted alkaline phosphatase (SEAP). In line with the immuno-blot results (Fig. 2), the vector titers showed in repeated experiments a clear Env-dependence (Table 1). With high amounts of Env (5µg pBC-FFV-Env DNA), vector titers were in the range of the parental replication-competent vector pCF-Bet-Gfp ($9*10^4$ TU/ml).

Transcomplementation with different Env-expression plasmids

We next analyzed whether the *env*-deficient provirus pCF-7ΔEnv derived from the FFV serotype FUV (Fig. 1, E)

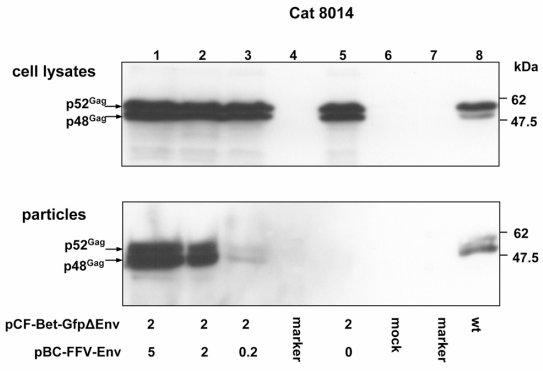


Figure 2. Immuno-blot analysis of cell lysates and particles from transfected 293T cells. 293T cells were transfected with the replication-deficient vector pCF-Bet-GfpΔEnv and different amounts of the pBC-FFV-Env expression plasmid as given below the blots. Two d p.t., cells were lysed and particles harvested from the supernatants. Cellular extracts (upper panel) and particles (lower panel) were analyzed by immuno-blotting with FFV cat reference serum 8014.(8) The positions of molecular mass markers are shown in kDa at the right hand margin, names of the detected FFV Gag proteins are given at the left hand margin. wt: pCF-Bet-Gfp, mock: non-transfected cells.

can be pseudotyped or complemented with the following FV Env proteins: (i) FFV-FUV serotype Env from pCInFUV7Env DNA, (ii) human/ primate FV Env from plasmid pCInHFVEnv, and (iii) a chimeric FFV Env consisting of the 44 N-terminal residues from FFV-FUV Env and the following 938 amino acids from the FFV serotype 951 from plasmid pCInFFV7/951Env (27, 28). The heterologous human/primate FV Env allowed almost no transduction whereas the chimeric FUV/951 Env resulted in titers two logs lower than the authentic FUV Env from pInFUV7Env or pBC-FFV-Env (10^4 - 10^5 FFU/ml). These results show that type- specific interactions between N-terminal Env leader protein sequences and Gag are required as recently proposed although other factors may be also required (29, 30).

We also tested whether FFV particles can be pseudotyped with the vesicular stomatitis virus glycoprotein VSV-G (31). In repeated experiments, no marker gene transfer using VSV-G-pseudotyped FFV vectors was detectable confirming previous studies using simian/primate FVs (24).

Table 1
Env-dependence of FFV vector particle release

pCF-Bet-GfpΔEnv (μg)	pBC-FFV-Env (μg)	mean vector titer[a] (TU/ml)
2	5	8×10^4
2	1	2×10^4
2	0.2	3×10^2
2	0	4×10^0

[a] mean vector titers normalized to co-expressed SEAP activity

Characterization of ubi-lacZ vectors

To increase vector safety and to allow insertion of larger foreign genes, additional vector sequences were removed. For this purpose, almost the complete *env* gene encompassing the internal promoter, the *bel1/tas* gene, and large parts of *bet* were deleted and replaced by a 4.7 kb marker gene cassette consisting of the constitutively and broadly active human ubiquitin C (ubi) promoter and the lacZ marker gene (32, 33). Clone pCF-7-ubi-lacZ carries the intact 3'-LTR while pCF-Δ-ubi-lacZ bears the partially deleted but functional U3 promoter. In contrast, pCF-SIN-ubi-lacZ is devoid of any FFV promoter due to the *env* and SIN deletions (Fig. 1, F to H). In all these clones, *pol* was left intact whereas in corresponding clones marked by the suffix -SB, the three C-terminal amino acids of Pol were deleted.

To analyze whether the new ubi-lacZ genomes allow efficient production of vector particles, 4µg of the different vector DNAs together with 2µg of pBC-FFV-Env were co-transfected into 293T cells. Two d later, vector titers were determined by staining for lacZ expression in the transduced cells. FFV-ubi-lacZ vector titers of 10^4 to 10^5 TU/ml were detected independent of whether the LTRs were intact, partially deleted, or SIN-deleted. Titers were similar to those of replication-competent vectors.

To analyze viral protein expression and particle release from transfected 293T cells, we performed immuno-blot analyses with cell lysates and released particles (Fig. 3, A). In cells transfected with 2µg of the parental clone pCF-7 (Fig. 1, A), efficient proteolytic processing of $p52^{Gag}$ to $p48^{Gag}$ was detectable (Fig. 3, A, upper panel, lane 9). In contrast, reduced amounts of the processed Gag-protein $p48^{Gag}$ were detectable in cells co-transfected with the different ubi-lacZ vectors plus the Env-expression plasmid (upper panel, lanes 1 to 6). Pol-expression and -processing was also reduced in the ubi-lacZ vectors compared to the parental virus (data not shown). Immuno-blot analyses of released vector particles (lower panel) showed efficient vector release from cells transfected with the parental FFV vector while less amounts of ubi-lacZ vector particles were detectable.

In summary, the novel FFV ubi-lacZ vectors direct the production of functional vector particles. However, the efficiency of vector protein processing and release are reduced.

Optimization of vector titers

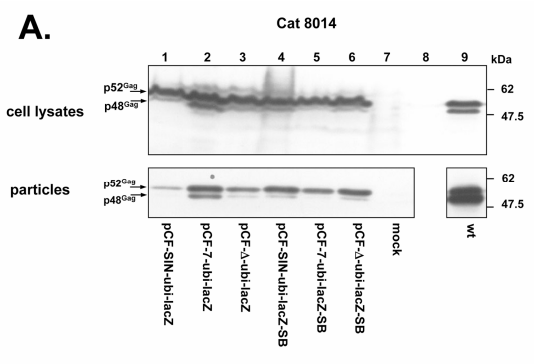
To obtain higher vector titers, we studied the transfection conditions by cotransfection of 2µg of the vector genome pCF-SIN-ubi-lacZ with different amounts of pBC-FFV-Env and determined the titers 2 d later. As already observed with the *env*-deleted vectors, the ubi-lacZ vector-mediated gene transfer is Env-dependent. At least 1 µg pBC-FFV-Env was necessary to obtain vector titers above 10^4 TU/ml. Increasing the amount of Env was in general accompanied by further increased titers (data not shown). Co-expression of the accessory Bet protein (34) in the vector-producing 293T cells did not reproducibly increase vector titers.

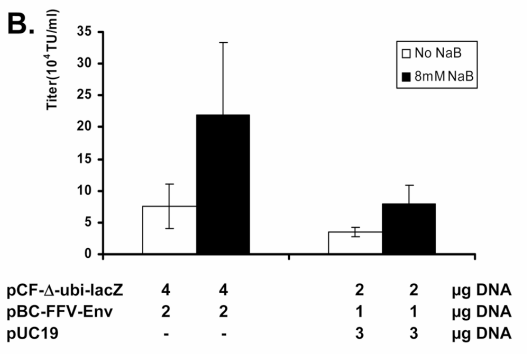
We then analyzed whether addition of sodium-butyrate (NaB) to the transfected cells does increase the vector titers. For this purpose, NaB was added to the 293T cell medium at a final concentration of 8 mM from 20 to 48 h after cotransfection of pCF-Δ-ubi-lacZ and pBC-FFV-Env (Fig. 3, B). Vector titers were determined using CRFK cells and compared to controls without NaB. In independent experiments and using different DNA concentrations, NaB increased vector titers two- to three-fold.

Optimization of transduction efficiencies

We next analyzed whether transduction efficiency can be increased by multiple rounds of vector expositions. For this purpose, CRFK cells were transduced with multiplicities of infection (MOI) of 0.1 to 0.2 either once or repeatedly (up to five times). Transduction rates increased mainly after the 2^{nd} and 3^{rd} consecutive transduction whereas

further treatments yielded only minor effects (Fig. 3, C). As expected, transductions at high MOI showed only minor increases since already a high percentage of cells had been transduced (data not shown). These data correlate with those for other viral vectors (35). Thus, multiple transductions may be especially suited for cells with a low sensitivity towards FFV vector transduction or low-concentrated vector stocks.





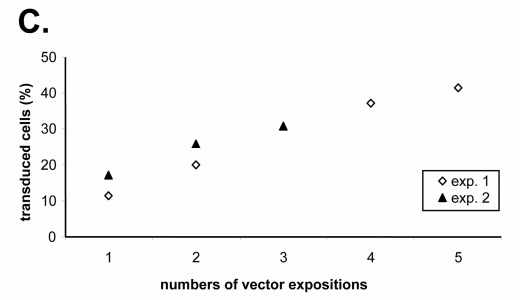


Figure 3. Optimization of ubi-lacZ vectors. **A.** Gene expression and particle release from ubi-lacZ vector transfected 293T cells. 293T cells were transfected with the replication-deficient ubi-lacZ vectors pCF-7-ubi-lacZ, pCF-Δ-ubi-lacZ, and pCF-SIN-ubi-lacZ and the corresponding vectors lacking three Pol residues (marked by the suffix –SB) as given below the blots. All vector genomes were trans-complemented with the FFV Env clone pBC-FFV-Env. Two d p.t., supernatants were harvested for particle preparation and the cells were lysed. Cellular extracts (upper panel) and particles (lower panel) were analyzed by immuno-blotting with cat serum 8014. The positions of molecular mass markers are shown in kDa at the right hand margin, names of the detected FFV Gag proteins are given at the left hand margin. wt: pCF-7, mock: untransfected cells. **B.** Enhancing effect of NaB on FFV vector production. 293T cells were transfected with 4µg pCF-Δ-ubi-lacZ plus 2µg pBC-FFV-Env (left) and 2µg pCF- Δ-ubi-lacZ plus 1µg pBC-FFV-Env plus 3µg pUC18 DNA (right) and NaB was added to a final concentration of 8mM from 20 to 48 h p.t. (solid bars) or mock-treated (light bars). Thereafter, vector titers were determined using CRFK target cells and expressed as TU/ml. Error bars are given. **C.** Enhancing effect of multiple transductions on marker gene transfer efficiency. In two independent experiments (diamonds and triangles), vector pCF-Δ-ubi-lacZ was generated by transient transfection as described and used for low MOI (0.1 to 0.2) transduction of CRFK cells. Vector transductions were repeated twice a day. All transduced samples were fixed and stained 2 d after the last transduction. The efficiency of marker gene transfer is expressed as percentage of stained cells.

Long- term transduction with ubi-lacZ vectors

We next analyzed the stability of transgene expression in ubi-lacZ vector-transduced CRFK cells. These cells transduced with the different ubi-lacZ vectors were split twice a week for up to 10 weeks. In regular intervals, parts of the culture were assayed for lacZ expression and the percentage of stained cells was determined. Comparing the initial number of marker gene expressing cells to the final number at the end of the study, in two out of three cases, a two- to three fold reduction of the number of positive cells was apparent. The third study did not show a decline of transduced cells. Vector-derived RCRs were not detected in any of these long-term studies.

pCF-SIN-ubi-lacZ vectors are not reactivated by wt FFV super-infection

The ubi-lacZ FFV vector genomes are flanked by LTRs upon integration into the target cell genome. Thus, we studied whether the integrated vectors are reactivated by wt FFV super-infection. For this purpose, CRFK cells transduced with the ubi-lacZ vectors with the intact, partially deleted, and SIN LTR (and untreated controls) were wt FFV-infected. Supernatants were harvested 2 d after super-infection when strong syncytia developed. The clarified supernatants were then added to fresh CRFK cells (reporter culture) to determine whether lacZ expression indicative for vector reactivation had occurred. All reporter cultures developed syncytia which were induced by wt FFV (Fig. 4, D to F). FFV infection of CRFK cells transduced with intact and ΔU3 LTR ubi-lacZ vectors resulted additionally in lacZ transduction and dark-blue stained nuclei in the reporter cultures indicative of vector rescue (Fig.4, E and F) similar to that obtained directly after transduction with the pCF-SIN-ubi-lacZ (Fig. 4, C). The titers of reactivated vectors were about 10^4 TU/ml. In contrast, the LTR promoter-deficient pCF-SIN-ubi-lacZ vector did not yield any detectable marker gene transfer (panel D) indicating that no vector reactivation occurred. The controls of untreated or only FFV-infected CRFK cells (A and B) as well as reporter cultures from ubi-lacZ-transduced but not FFV super-infected cells (data not shown) did not show stained nuclei. These data were confirmed in two independent experiments.

Transduction of different cell types with ubi-lacZ vectors

Finally, diverse human and non-human cells from different organs were assayed for transduction by vectors pCF-7-ubi-lacZ and pCF-SIN-ubi-lacZ. Several of these cells were transduced with high or intermediate efficiency (Table 2). Cells permissive for FFV replication showed in general also a considerable permissiveness towards FFV vector transduction. Remarkably, most murine cells showed low permissiveness indicating that mouse cells express restriction factors counteracting transduction by FFV vectors.

Discussion

In the present paper we describe the construction and functional characterization of novel replication-deficient retroviral vectors based on the FFV genome. The vectors constructed display a highly advantageous safety profile and are suited for transduction of a broad range of different human and

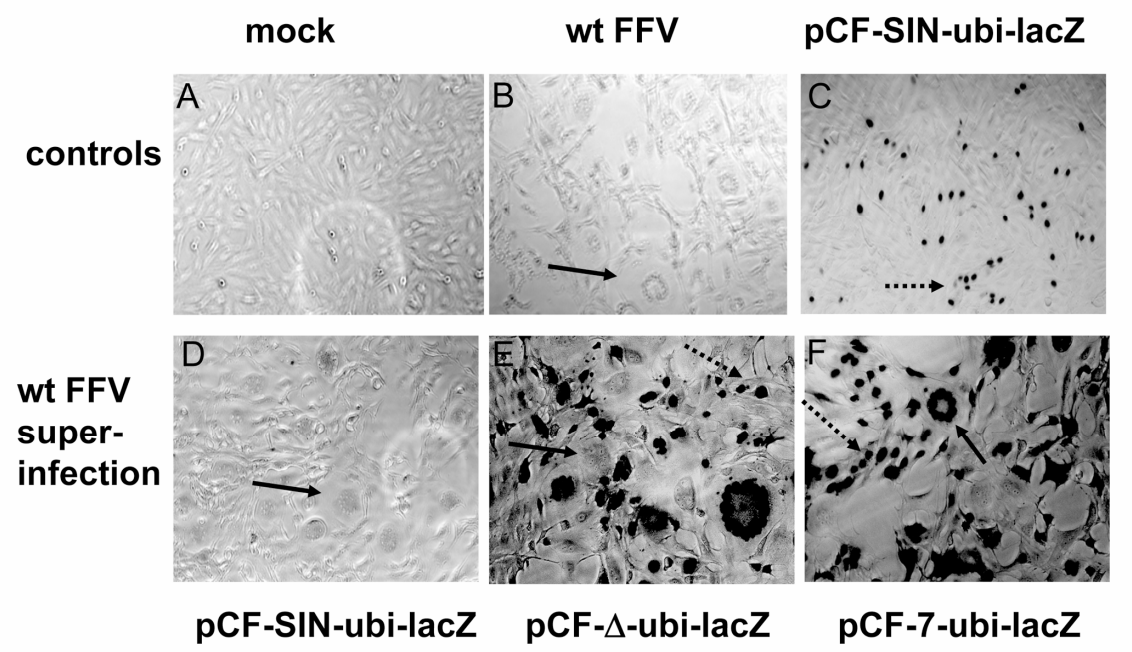


Figure 4. The pCF-SIN-ubi-lacZ vector genome is not reactivated by wt FFV. Vectors pCF-7-ubi-lacZ, pCF-Δ-ubi-lacZ, and pCF-SIN-ubi-lacZ were generated using standard methods and used to transduce CRFK cells at a MOI of 0.3. The transduced cells were super-infected with FFV after the fourth passage. Two d after super-infection, the cell culture supernatants were harvested, cleared by low-speed centrifugation and used to transduce/infect CRFK cells as a reporter culture. As controls, CRFK cells were mock-infected (A), FFV-infected (B), and pCF-SIN-ubi-lacZ-transduced (C). Two d later, reporter and control cultures were fixed and stained for lacZ expression. All reporter cultures (D to F) developed syncytia similar to the FFV-infected control (B). Reporter cultures from pCF-Δ-ubi-lacZ- and pCF-7-ubi-lacZ-transduced cells (E and F) showed in addition strong nuclear lacZ staining in syncytia and single cells. In contrast, pCF-SIN-ubi-lacZ reporter cells showed syncytia without any evidence for lacZ marker gene transduction (D) similar to the FFV-infected controls (B). As expected, pCF-SIN-ubi-lacZ-transduced cells displayed nuclear staining without syncytia induction (C). Images were taken at a 250-fold magnification. The solid arrows mark syncytia and the dashed arrows point to single cells with specifically lacZ-stained nuclei.

non-human cells. This limited target cell specificity may be even advantageous provided that vector targeting can be achieved by other means (e.g. by direct injection into the tumor) or when this is not strictly required (e.g. in case of ex vivo transduction regimen).

The *env-bel*-deficient ubi-lacZ vectors are much safer than previous FFV vectors: (4) i. the generation of RCR has not been detected using these vectors, ii. ubi-lacZ vectors with the SIN deletion in the LTR are not reactivated by super-infection with wt FFV, and iii. the absence of significant amounts of SIN LTR-directed viral transcripts was confirmed by reverse transcription PCR-based expression studies (data not shown). This indicates that the SIN-deleted 5'-LTR did not direct expression of genomic vector transcripts. We conclude that also the 3'-LTR with the corresponding SIN deletion is transcriptionally silent. Thus, the pCF-SIN-ubi-lacZ vector is unlikely to direct expression of flanking cellular sequences from the viral LTR promoters. With respect to vector

Table 2 Susceptibility of different cell types towards FFV vector transduction.

species	cell line	cell type	transduction efficiency[a]
human	293T	epithelial	+
	Hela	epithelial	-
	HT-1080	fibrosarcoma	++
	LoVo	epithelial	++
	UROtsa	epithelial	++
	T24/83	epithelial	++
	HT1197	epithelial	(+)
	RT112	epithelial	+
	UM-UC-3	epithelial	+
	primary	gingiva fibroblasts	+++
mouse	B16F10	skin melanoma	-
	EL4	lymphoblasts	-
	A9	fibroblasts	-
	3T3	fibroblasts	+
	CMT93	epithelial	+
	L929	fibroblasts	(+)
	primary	T-cells	-
cat	KE-R	fibroblastoid	++
	CRFK	epithelial	+++
hamster	BHK-21	fibroblasts	++
sheep	PO	epithelia-like	++
dog	Cf2Th	fibroblastoid	++

[a] transduction efficiencies are expressed as follows: -: no transduction; (+): below 1%; +: up to 5%; ++: up to 20%; +++: above 20%.

safety, the complete inactivation of the SIN LTR in the FFV ubi-lacZ vectors adds up to the low preference for FV integration into transcribed genes as it is known for lentivirus-based vectors (18, 19, 36).

To further enhance the safety profile of these FFV vectors it is reasonable trying to avoid run-through transcription from the heterologous ubi promoter and to molecularly characterize the integration site preference of FFV (see also below).

In addition, a scenario of vector reactivation by infection with the parental wt FFV is unlikely considering the application of a feline-derived vector in men, a setting where also a preexisting immunity against the vector is unlikely. Even when the FFV ubi-lacZ vectors are used during pre-clinical trials in cats (or in the course of cat gene therapy), simple recombination events between the integrated and multiply engineered vector genome and

the wt exogenous parental virus are not sufficient for creating RCRs confirming the advantageous safety profile of the FFV *env-bel*-deficient vectors. The functional characterization of these novel FFV-based vectors in the authentic, immuno-competent, and not inbred host, the cat, is challenging but feasible. For instance, we recently showed that replication-competent vectors that are directly related to the replication-deficient ubi-lacZ vectors are efficient at expressing a heterologous vaccine antigen without any side effects (6). In order to use the current vectors in animal experiments, we employed the constitutively active human ubi C promoter for transgene expression since this promoter displays long-term activity in several cell types from men and a broad range of animals (37). In addition, mapping of vector integration sites in the authentic animal model will become possible considering the success of the cat genome sequencing project (38). Currently, no information is available on the integration site specificity and frequency of FFV in infected or transduced cells. Since FV infection is known to induce superinfection resistance and since FFV does not retrointegrate upon infection, we assume that in general a single provirus is integrated per transduced cell (39, 40).

The expected whole cat genome sequence may also explain why the number of marker gene expressing cells decreased over time as observed in two out of three experiments: vector integration into heterochromatic areas of the genome with the subsequent suppression of transgene expression might be the underlying mechanism. We are currently underway to address this issue by using inhibitors of DNA methylation and by the utilization of alternative reporter genes. Alternatively, the nuclear localized LacZ enzyme expressed by our vectors could have toxic or detrimental effects as discussed recently thus leading to a counter-selection against transduced cells (41).

Our study reveals that not only the closely related FVs of simian and higher primates but also the distantly related FFV cannot be pseudotyped by heterologous surface glycoproteins of different origin. Even the Env protein from the human/primate FV did not allow efficient marker gene transduction, underlining the concept that highly specific Gag-Env interactions are required for proper particle assembly although other reasons can not be formally excluded (29, 30). In addition, we did not find a significant effect of Bet on the vector titer since the 293T cells used for vector production are APOBEC3-deficient and thus, the deaminase-counteracting function of Bet is not required (22). Additional effects of Bet on FFV vector particle release were not observed in this study.

FV vectors have intrinsic advantages, e.g. the high physical and genetic stability, apathogenicity of the parental viruses, capacity for the uptake of larger foreign DNA sequences, and reduced dependence on replicating cells (9). To fully appreciate the potential of the novel FFV ubi-lacZ vectors, improvements in vector production and titer as well as a better understanding of target cell and integration site specificity are required.

Material and methods

Virus and cells

293T cells and FFV-FAB titration cells were grown as reported previously (26). Transfection of recombinant DNA into 293T cells was done by calcium co-precipitation (42). The infectivity of FFV-derived replication competent vectors and the transduction efficiencies of replication-deficient vectors were assayed using FFV-FAB cells and CRFK cells grown in 24-well plates (26).

To normalize transfection efficiencies of different vector constructs, 1µg of the SEAP plasmid pCMV-SEAP was co-transfected (43). Two d later, an aliquot of the supernatant was harvested and SEAP chemoluminescent detection was performed as recommended (Roche, Mannheim, Germany). Vector titers were normalized to co-expressed SEAP activity and are expressed relative to the replication competent vector pCF-Bet-Gfp.

To analyze long-term transduction of FFV vectors, vector stocks from transfected 293T cells were used to transduce permissive CRFK cells (4). Transduced cells were serially passaged twice a week and the supernatants were analysed for RCRs by infection of FFV-FAB cells (4). In parallel, duration of Gfp expression from Gfp transducing vectors was determined by microscopy of the transduced CRFK cultures.

For the transduction of different target cell lines, cells were grown in 6 well plates and transduced with 1 ml of vector particle containing cell culture supernatants. Three d after transduction, the efficiency was determined by lacZ staining.

To purify FFV particles, cell culture supernatants from transfected 293T cells were harvested 2 d post transfection (p.t.), and cleared by low-speed centrifugation. Particles were sedimented through 2 ml of 20% (wt/vol) sucrose in PBS for 2 h at 28,000 rpm in a SW 41 rotor (Beckman, Munich, Germany) and resuspended in protein lysis buffer (30).

Molecular cloning

To delete the major parts of *env* (from RNA position 5340 to 7676) without deleting the internal promoter, FFV sequences were amplified in a standard PCR with primers 8746S (5'-TCAGTCGACTCATCCTGAGTTAACGCGTACGACAGACTGTGGCA-TACC-3'), which contains a recognition site for *Sal*I (underlined) and 9390AS (5'-CAACAATTTTACTGGTATGC-3') using pCF-Bet-Gfp as template (4, 5). To obtain the *env* deleted vectors pCF-7ΔEnv, pCF-Bet-GfpΔEnv and pCF-Bet-Gfp-SINΔEnv the vectors pCF-7, pCF-Bet-Gfp, and pCF-Bet-Gfp-SIN and the PCR product were digested with *SalI* and *Bsu36I* and ligated.

For construction of ubi-lacZ vectors, the plasmid pGem7-ubi-lacZ containing the ubi promoter upstream of the *lacZ* gene was first constructed. For this purpose, plasmid pGem1nlslacZ was digested with *SalI* and *BamHI* to excise the *lacZ* gene (33). The ubi promoter-containing plasmid #17.XhoI (32) was digested with *XhoI* and *BamHI* and the *lacZ* gene was inserted downstream of the ubi promoter.

The ubi-lacZ expression cassette was inserted by two slightly different methods into the replication-competent vectors pCF-7 and pCF-ΔU3 and the replication-deficient vector pCF-SIN (4, 5). In the first series of ubi-lacZ vectors called pCF-7-ubi-lacZ, pCF-Δubi-lacZ

and pCF-SIN-ubi-lacZ, the *pol* gene was left intact and 138 bp of the 5' end of *env* were maintained in the vector genome. The deletion ranges from the *Acc65*I site (RNA genome position 5383) at the 5' end of *env* to the *BamH*I site in *bel2* about 250bp upstream of the 3'-LTR (RNA genome position 9022). The vector genomes pCF-7, pCF-ΔU3 and pCF-SIN were digested with *Acc65*I, blunt ended with Klenow enzyme, and digested with *BamHI*. The plasmid pGem7-ubi-lacZ was digested with *Sal*I, blunt ended with Klenow enzyme and digested with *BamH*I. Thereafter, the ubi-lacZ fragment was ligated with the vector genomes.

In the second type of ubi-lacZ vectors called pCF-7-ubi-lacZ-SB, pCF-Δ̃ubi-lacZ-SB and pCF-SIN-ubi-lacZ-SB, the deletion in the vector genome ranges from the *Sal*I site in the 3' end of *pol* (RNA genome position 5323) to the *BamHI* site in *bel2*. In these vectors, the last three codons of *pol* were deleted and only 79bp of the 5' end of *env* were maintained. For clonings, plasmid pGem7-ubi-lacZ and the vector genomes pCF-7, pCF-ΔU3 and pCF-SIN were digested with *Sal*I and *BamH*I and the ubi-lacZ cassette was ligated into the vector genomes.

Immuno-blot analyses

Transfected cells were harvested by lysis in 1% SDS. Immuno-blotting of proteins separated on denaturing gels and detection of specifically bound antibodies by enhanced chemo-luminescence was done as described previously (44). Cat 8014 antiserum was used as previously described (23, 45).

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