Foamy Virus-Host Interactions

Shannon Murray

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ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

University of Washington
Graduate School

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Shannon Murray

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Chair of the Supervisory Committee:

Maxine Linial

Reading Committee:

Maxine Linial

Michael Emerman

Nancy L Haigwood

Nancy Haigwood

Date: 3/01/2007

Signature Shannon Murray

Date March 8, 2007

University of Washington

Abstract

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Chair of the Supervisory Committee:
Professor Maxine Linial
Molecular and Cellular Biology

Foamy viruses (FV) are retroviruses but, unlike the orthoretroviruses, have no disease association. The studies reported in this dissertation address foamy virus–host interactions in order to understand why foamy virus infection is nonpathogenic. I evaluate hypotheses that could explain this apparent anomaly, namely, that viral latency or the host immune system limits potential pathogenic effects. Utilizing naturally FV-infected rhesus macaques (*Macaca mulatta*), I determined that viral replication in hosts is limited to the oropharyngeal tissues. Further, I found that FV infection is not characterized by latency, and that robust viral replication is detectable in the oral cavity, with the highest levels in samples obtained from oral swabs. Surprisingly, host immune responses, which are characterized in this research, do not contribute significantly to limiting viral replication in the blood or other tissues. This was most convincingly demonstrated by the utilization of simian immunodeficiency virus (SIV)-infected macaques that were severely immunosuppressed and depleted of $CD4^+$ T cells. In these SIV-infected macaques, there was an expansion of FV replication to only one tissue—the jejunum—and not to any other tissues that were also highly depleted of $CD4^+$ T cells. Thus, systemic immune control of FV infection appears to be insignificant, and it is likely that SIV-induced changes unique to the jejunum account for FV

replication there. Finally, I identified the cell type that is permissive for FV replication *in vivo*: superficial epithelial cells in the outermost layers of the oral mucosal epithelium, many of which appeared to be actively shedding. This cellular site of replication offers a plausible explanation of the innocuous character of FV infection, since this cell type turns over rapidly and is relatively dispensable to its host. Limitation of viral replication to a superficial site is likely to minimize damage to its host while increasing the efficiency of virus transmission via shed cells in saliva. Taken together, these findings have contributed to an explanation of the nonpathogenic outcome of FV infection and allowed for the development of a model of within-host spread of FV infection.

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Abbreviations and Acronyms

APOBEC3	apolipoprotein B-editing enzyme catalytic polypeptide 3
as	anti-sense
BAL	bronchoalveolar lavage lymphocytes
BHK	baby hamster kidney
BME	Betamercaptoethanol
CMV	Cytomegalovirus
CPE	cytopathic effect
DC	dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
dpi	days post-infection
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FFPE	formalin-fixed, paraffin-embedded
FV	foamy virus
GALT	gastrointestinal-associated lymphoid tissue
GST	glutathione *S*-transferase
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HPV	human papilloma virus
HTLV	human T cell leukemia virus
ICC	intracellular cytokine
IFN	interferon
IHC	immunohistochemistry
IP	internal promoter
ISH	in situ hybridization
KSHV	Kaposi sarcoma herpesvirus
lcmd	Laser-capture microdissection
LTR	Long terminal repeats
MLV	murine leukemia virus
NBF	neutral buffered formalin
NHP	nonhuman primates
ODmax	the maximum optical density
ONPRC	Oregon National Primate Research Center
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFV	prototype foamy virus
p.i.	post infection
PMBC	peripheral blood mononuclear cells
qPCR	quantitative PCR

qRT PCR	quantitative reverse transcriptase PCR
RM	rhesus macaque
RPMI	Roswell Park Memorial Institute Medium
RT	reverse transcriptase
s	sense
SAIDS	simian auto-immune deficiency syndrome
SEB	staphylococcal enterotoxin B
SFAB	simian foamy virus-activated β-gal
SFV	simian foamy virus
SFVmac	macaque simian foamy virus
sIgA	secretory IgA
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
SPF	specific pathogen-free
SU	surface
TF	telomerized fibroblast
Tg	transgenic
Th1, Th2	T helper 1, T helper 2
TNF	tumor necrosis factor alpha

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The support, inspiration and encouragement from my partner, Carl Chatzky, has made it possible for me to concentrate on my research, while every day being more inspired by the constant process of learning. Carl has helped create an environment of scientific inquiry and growth.

Louis Picker has been a mentor whose scientific insight I have truly learned from and been amazed by. Without his advice and scientific support, which I deeply appreciate, I could not have pursued the research described here.

Maxine Linial gave me the space to pursue diverse areas of research, which was necessary in order to do the initial exploratory studies, and to engage in independent research.

There are many whom I would like to thank for their support during different aspects and phases of my development as a scientist, but those who have been most influential are the friends and colleagues who have reminded me that creativity and imagination are integral to the scientific process. Those individuals include Alena Gallegos, Erica Andersen-Nissen, and Joshua Walker. I would also like to thank Martha Jackson for assistance in improving my critical thinking and writing abilities.

"Imagination is more important than knowledge, for knowledge is limited while imagination embraces the entire world." – Albert Einstein

Dedication

I dedicate this to Carl Chatzky, and to all those who see education, science and the interest of humanity as inextricably linked.

On the University of Washington Campus, next to the Student Union Building, there is a monument to the "Voluntarios Internacionales." Inscribed on this monument are "You are history, you are legend" and the lines, "40,000 international volunteers came to the defense of the Spanish Republic [1936-1939] when Hitler, Mussolini, and Franco attacked. Among them were some 3,000 young Americans, the Abraham Lincoln Brigade . . . Eleven students from the University of Washington joined in that struggle." I also dedicate this to those students, and their spirit of world cooperation and justice.

Chapter I: Background

Introduction

Foamy virus (FV), also known as spumavirus, is the only genus of the *Spumaretrovirinae* subfamily of retroviruses (Table 1.1). This subfamily was recently reclassified from a genus due to its divergence from the only other retroviral subfamily, the orthoretroviruses (86). FV have been found in nonhuman primates (NHP), cats, cows, and horses as well as in humans who have acquired the infection from NHP. In all hosts, FV establish a lifelong, persistent infection with no associated pathologies. However, in vitro they replicate to high titer and cause characteristic rapid cytopathic effects in many cell types. This apparent contradiction has not yet been explained.

A FV was first described in 1954 as a “transmissible agent” causing cytopathicity in rhesus macaque-derived kidney cell cultures (35). The giant, multinucleated ‘foamy’ syncytia and highly vacuolized appearance characteristic of infected cell cultures led to the further identification of the syncytia-inducing agent as a virus (129). However, FV appeared under the electron microscope as immature virions, unlike the mature virions observed in orthoretrovirus-infected cultures. Further study indicated that the FV proviral genome contains typical retrovirus elements, including long terminal repeats (LTRs), and the Gag, Pol, and Env proteins. However, FV also encode two unique nonstructural proteins, Tas (transcriptional activator of spumavirus) and Bet (between envelope and *tas*).

The FV genome and assembly pathway also have features similar to another family of reverse-transcriptase (RT) encoding viruses, the hepadnaviruses. FV behave like retroviruses in that viral RNA is reverse-transcribed into DNA and then integrated into the host genome as provirus. However, reverse transcription occurs during budding and assembly, so that the genome is actually DNA, as in the hepadnaviruses (106, 174). And, unlike other retroviruses, which produce Gag-Pol fusion proteins, the Gag and Pol proteins of FV are produced as separate proteins, from separate RNA transcripts (170). In addition to a conventional retroviral LTR promoter, FV has been found to contain a unique internal promoter (IP) that regulates expression of Tas and Bet (99). Some features of FV are contrasted with those of primate lentiviruses, and specifically, the immunodeficiency viruses (Table 1.2). Other features of FV, including the genome and assembly pathway are reviewed in (84, 85, 87).

It is not understood why FV infection is nonpathogenic while infection by its closest relatives, the hepadnaviruses such as hepatitis B virus (HBV), or the complex retroviruses such as human immunodeficiency virus (HIV) and human T-cell Leukemia Virus (HTLV), cause pathologies that can lead to death (Table1.1). It may be that the features of FV that differ from those of orthoretroviruses and hepadnaviruses are critical to the nonpathogenic character of in vivo FV infection.

There are no reports of FV-induced pathology in any naturally infected host (97). Evolutionary analysis has determined that FV have co-speciated with NHP for an estimated 60 million years (155), making FV one of the oldest vertebrate RNA viral lineages. This unusually long period of co-speciation may have allowed FV to evolve a nonpathogenic and perhaps a commensal or even symbiotic relationship with its hosts. Interestingly, there is no human-specific FV, although the isolation of a FV from lymphoblastoid cells within a human nasopharyngeal carcinoma suggested otherwise (1). After further characterization and sequencing of the isolate over two decades later, it was revealed that the FV isolate was the SFV chimpanzee (SFVcpz) virus, later to be called prototype FV (PFV) (53). This was thought to be either zoonotic infection or a laboratory contaminant. Subsequent research confirmed the occurrence of zoonotic infections from NHP to humans, and the absence of human-specific FV and human-to-human transmission, reviewed in (50).

There are no well-substantiated reports of FV-induced pathology in zoonotically-infected hosts (97). Humans zoonotically infected with FV exhibit a persistent, lifelong infection and FV-specific antibodies in the absence of evident pathology, similar to infection in natural hosts. Before the identification of human populations with high prevalence of FV infections, a number of epidemiologic studies were performed to evaluate whether a variety of human diseases, including autoimmune disease and neurodegenerative disorders, were associated with FV infection, reviewed in (97). In many cases, an initial study found a disease association, but subsequent studies by different groups failed to confirm these findings. For example, one study reported a strong association between FV and Graves disease (77), but two follow-up papers from different research groups failed to support these findings (49, 167). Claims of disease associations with FV are still being made (152), yet no studies have identified FV as an etiologic agent. FV has been described as a "virus in search of a disease" (165). To date, there is no solid evidence for a direct pathologic effect of natural or zoonotic FV infection.

One paper has demonstrated a pathologic potential of FV, not in the context of FV infection, but in a transgenic mouse model. Findings published in the journal *Science* reported that transgenic (Tg) mice engineered to express the FV genome in a noninfectious form, under its own LTR promoter, succumbed to neurodegenerative disease in the brain and central nervous system (12). In contrast, experimental infection of mice with prototype foamy virus (there is no mouse-specific FV) results in an asymptomatic persistent infection, similar to that of natural hosts (21, 137). Therefore, it is likely that the pathology observed in the Tg mice was due to expression of FV in cell types that are not normally infected by FV. This is the only in vivo model that has ever indicated a pathologic potential of FV.

The apparent paradox of a cytopathic phenotype in vitro, and an innocuous one in vivo, leads to speculation about what viral–host dynamics may be responsible. Has foamy virus evolved a homeostasis with its host? Do the characteristics of FV that differ from those of lentiviruses or oncogenic retroviruses render it nonpathogenic? Does the host respond to FV infection by inducing innate and/or adaptive immune responses that limit viral cytopathicity in vivo? Or is in vitro cytopathicity itself an artifact?

FV Hosts and Transmission Modes

FV have been isolated from all primate species examined (7, 58, 64, 65, 91, 95, 124, 126, 150). The tree shown in Fig. 1.1 indicates evolutionary relationships between FV isolates that mirror the evolutionary relationships between the primate species themselves [discussed further in (20, 155)].

The prevalence of infection among NHP that are group-housed in primate centers is high and can reach >90% (9, 97, 140). The majority of animals seroconvert as juveniles, by the age of 2 years, suggesting a nonsexual route of transmission (personal communication, Michael Axthelm; (9)). Biting or licking has been postulated to be the mode of transmission (38, 87, 97), although this remains unproven.

Cross-species transmission to humans has been documented in populations in close contact with NHP including veterinarians, animal caretakers, bushmeat hunters, monkey-pet owners, and zookeepers (51, 66, 154, 166). FV isolated from human infections have originated from various chimpanzee subspecies, African green monkeys, mandrills, gorillas, and baboons (Fig. 1.1). Human transmission has also been documented from macaques (19).

Human cases are rare, ranging from 2% to 3% within at-risk populations (19, 51, 66, 97, 154). These infections are usually associated with a severe bite, which suggests that saliva is the means of transmission. No human-to-human transmission has been documented, suggesting that humans are dead-end hosts. However, very few human cases have been examined. It is unclear whether the apparent block in human-to-human transmission is caused by lack of significant viral replication in human hosts, behavioral differences between humans and NHP, or differences between NHP and humans in the site of initial infection. However, until the site of initial infection and the mode of transmission are proven for natural hosts, it is difficult to predict what differences account for the lack of human-to-human transmission.

FV Cellular Tropism

Persistent viruses often have transcriptional regulation that results in latency in some cell types and permissive infection in others. In latent FV infections, proviral DNA is present at a low copy number in the absence of viral RNA, whereas in a permissive infection viral DNA is present at higher levels, along with viral RNA.

Extensive studies with FV isolated from primate tissue cultures have revealed that FV tropism in vitro is not restricted to a particular species or cell type (54, 101). The viral receptor for FV has not been identified, but is thought to be ubiquitous since all vertebrate cell types available can be infected by FV (54). However, the outcome of infection can differ among classes of cell types (101). In cells of epithelial or fibroblast origin, infection yields a highly cytopathic phenotype like the foamy cultures observed in primary primate cells or tissue explants. In contrast, infection of a variety of leukocyte-derived cells results in a persistent or latent infection which can be shifted to a permissive, cytopathic infection if the cells are activated by engagement of antigen receptors or mitogens (98, 99, 173).

The FV promoters demonstrate a cell-type specificity that correlates with viral replication. Analysis of FV promoter activity revealed a bimodal transcription pattern with the LTR promoter active in permissive cell types while the internal promoter (IP) was more active in persistently infected cells (99). When persistently infected cells were activated by various stimuli, resulting in a permissive infection, the promoter use also switched (99). This study suggested a self-limiting infection in leukocyte-derived cells, governed by viral gene regulation.

Like the oncoviruses such as murine leukemia virus (MLV), but unlike the lentivirus, HIV, FV, has been found to require cell division for productive infection in vitro (8, 157). Trobridge and Russell (157) found that a partial cell cycle was required for efficient FV transduction. There has been some disagreement about whether FV can transduce nondividing cells (100, 127, 131). However, the consensus is that, while FV can infect quiescent cells, cell division is required for viral gene expression and replication (131, 157).

There have been few in vivo studies of the cellular tropism of FV. Those few have examined blood samples obtained from FV-infected NHP or zoonotically-infected humans. In samples from natural hosts, several peripheral blood mononuclear cell (PBMC) subsets, including CD4$^+$ T cells and monocytes, were found to harbor provirus (163). In human samples, however, the results were less conclusive. In two FV-infected humans, CD8$^+$ T cells were the only PBMC subset containing FV DNA (163). However, in another human, monocytes and B cells were positive for FV DNA, while T cells were negative (22, 163). FV isolated from the latter discordant subject had a deletion resulting in a defective Bet protein (22), which may account for the discordancy, as Bet may be involved in inhibiting innate immunity (discussed in 'Innate Immunity').

Further evaluation of tissues and organs have revealed widespread persistence, but the specific cell types in these tissues that harbor FV DNA have not been identified (37, 109). The finding that a latent infection in leukocytes could switch to a permissive one with activation in vitro suggests a model of FV spread within a host mediated by this migratory cell type. Since many leukocyte subsets migrate throughout host tissues, and become activated at a low level throughout host tissues, it may be that they are responsible for the observed spread of FV infection within a host.

Tissue and Cellular Specificity of Replication

While PBMC subsets have been shown to harbor provirus, there are no reports of FV RNA in the blood (37). This suggests that the blood is a site of persistence rather than of significant viral replication. Only one research group has looked further at the tissue-specificity of FV replication in naturally infected primate hosts. Falcone et al. studied African green monkeys, finding low levels of viral DNA in all tissues examined (37). In contrast, FV RNA, indicative of viral gene expression and replication, was found exclusively, albeit sporadically and at a low level, in the oral mucosa. This data suggested that FV was mostly latent, a characterization I found at odds with the high transmission rate of FV observed in NHP.

The cell type in the oral cavity that is permissive for FV replication in vivo is not yet known. Until this has been determined, distinguishing between potential theories regarding FV latency and replication within a host will be difficult or impossible. However, this finding of restricted viral replication leads to many further questions: What makes the oral cavity permissive for FV replication? What restricts FV replication elsewhere? Does the host's systemic immune system prevent FV from replicating in the blood, or elsewhere? Or can FV replicate only in tissue-specific environs, due to differential activation of FV promoters or other reasons?

Antiviral Immunity

Viral–host dynamics in persistent viral infections involve antiviral host immunity that preserves the host, along with viral evasion of host defenses that allow the virus to persist within the host. Many persistent viruses elicit host immune responses that limit viral replication and thus host pathology. Host immunity includes adaptive and innate immunity, both of which have well-established antiviral functions. Adaptive immunity acts via T cells and antibody-producing cells (B cells), which have significant antiviral activity. T and B cells are clonally expanded after antigen encounter, resulting in enrichment and priming of microbe-specific cells, which create an 'immunologic memory'. Innate immunity produces antiviral cytokines and other factors with viral inhibitory activity. The kinetics of innate immunity is not altered after repeated encounter with microbes, and thus, does not exhibit immunologic memory.

T cells can be broadly divided into two classes based on the presence of CD8 or CD4 surface molecules involved in antigen recognition. $CD8^+$ T cells are implicated in direct killing of virally infected cells. $CD4^+$ T cells are involved in producing antiviral cytokines, and in stimulating $CD8^+$ T cells and long-lived antibody-producing B cells. Antibodies can function by neutralizing or opsonizing free virus, or by clearing virally infected cells that have viral proteins expressed on their surface.

Persistent viruses are also associated with suppression of host adaptive and innate immunity. For example, hepatitis B virus (HBV) has been shown to induce a state of host 'immunologic tolerance', in which T cells specific to the virus are deleted from the host T cell population (3). Another persistent virus, the Epstein-Barr virus (EBV), suppresses the production of host antiviral cytokines (3).

A key question is whether FV replication in vivo is limited by the host immune system. Given that the oral mucosa supports viral replication, are there host immune system responses to infection at this permissive site? Perhaps there are immune responses controlling FV replication in peripheral tissues, but no significant mucosal immune responses in the oropharyngeal tissues.

Findings from recent studies suggest that FV has mechanisms to suppress host innate immunity (30, 79, 88, 128). One study reported low levels of mucosal antibodies in the saliva, along with long-lived plasma antibodies (26). I suggest, based on others' findings, and data presented here, in Chapters II, III, and IV, that a combination of mechanisms including suppression of immunity, low levels of mucosal antibodies, tissue-specific microenvironments, and the viral transcriptional regulation that yields latency in resting leukocytes, may contribute to limiting FV replication systemically while permitting it in the putative permissive site, the oral cavity.

Innate Immunity

Because of the long coevolution of FV and their hosts, FV provide an interesting model system to study viral adaptation to host innate immunity and, on the other hand, to identify host innate immune factors that target viruses. FV are sensitive to Type I interferons in vitro, but do not seem to significantly induce them in their target cells (39, 123, 130). This is consistent with the theory that FV have active mechanisms to downregulate host innate immune responses. Recently, the two FV nonstructural proteins Tas and Bet have been implicated in overcoming host innate intracellular immunity. Tas is a transcriptional activator required for FV transcription (121) and Bet, while not required for primate FV infection in vitro, has been proposed to regulate viral latency (43, 80, 98). Recent evidence suggests that Tas also plays a role in inhibiting an RNA silencing complex (79) and that Bet, like the HIV Vif protein (143, 144, 175), is active in counteracting apolipoprotein B-editing enzyme catalytic polypeptide 3 (APOBEC3) antiviral activity (88, 128)

Antiviral Antibodies

Serum antibodies against the FV Gag and Bet proteins, as well as virus neutralizing antibodies, have been associated with FV infection in the majority of hosts examined (47, 55, 57). In a mouse model of FV infection, serum antibodies against Bet were associated with limited viral dissemination (137). There have been no reports of experimental infection of a primate host with a FV, where viral spread, replication, and host immune responses to infection were followed. Therefore, information regarding immune responses to FV infection is available only for primates naturally infected with FV, for which the time of initial infection is often unknown.

To address whether mucosal immunity is elicited by FV infection, Cummins et al. (26) studied four humans who had acquired FV from chimpanzees, using samples obtained from the Centers for Disease Control, including Case 6 shown in Fig. 1.1 These humans, as well as nine naturally infected chimpanzees, were evaluated for FV-specific antibodies of the IgG and IgA isotypes in plasma, urine and saliva. IgA antibodies in mucosal secretions are generally considered to be a hallmark of mucosal immunity while IgA antibodies in the serum are, at best, weakly correlated with mucosal immunity (177). Interestingly, Cummins et al. found no detectable FV-specific IgA antibodies in the urine, sera, or saliva of any host, while high levels of IgG antibodies to the FV Gag and Bet proteins were evident at all sites (26). However, it is possible that antiviral IgA antibodies had been elicited early after FV infection, but were not detectable in this study. Samples were collected from humans 10–24 years after initial infection, and from the FV^+ chimpanzees with unknown times since initial infection (26). Thus, this study shows that FV infection does not stimulate long-lived mucosal immunity in naturally infected chimpanzees or in zoonotically infected humans. In contrast, FV-specific IgG is long-lived, indicating a systemic immune response to FV antigens. The authors suggested that the absence of mucosal IgA may explain permissive infection in the oral mucosa (26).

Antiviral T cells

Many cytolytic viruses are most effectively limited by antiviral cytokines produced by host T cells (3). T cells recognize cell-associated antigen, and since FV spreads from cell to cell in vitro, and perhaps also in vivo, this would result in the concentration of viral antigens within cells. This mechanism of viral spread would render T cells a central mediator of antiviral immunity against FV.

Early studies utilizing rabbits infected with PFV indicated transient, generalized immunosuppression induced by FV infection (56, 133). Host T cells were suppressed in their ability to proliferate in response to polyclonal mitogen stimuli for 5–30 days post infection (p.i.) with FV (56, 133). This has not been evaluated in natural hosts, but it is possible that FV infection suppresses host T cell activation to evade host immunity and perhaps promote its own dissemination. Further, this finding led to the question of whether FV infection is associated with opportunistic infections of other persistent viruses that reactivate with immunosuppression.

There have been no reports examining FV-specific T cells. These T cells would be involved in specifically recognizing FV-infected cells and limiting viral replication. Based on the absence of FV-specific mucosal IgA along with long-lived systemic IgG, it may be that FV-specific T cells are in low frequency in the oral cavity but enriched in the blood. Alternatively, the virus may have mechanisms that subdue T cell responses to itself so that it is never cleared from the host and thus, able to establish lifelong persistence.

Viral Replication in Immunosuppressed Hosts

One approach to studying immune control of viral replication is to evaluate pathologies or increases in viral replication after host immunosuppression. Only one report has examined FV infection in immunosuppressed hosts (9). This study reported a three-fold increase in the ability to culture FV from saliva of transiently myelo-suppressed baboons (9). However, other parameters such as viral DNA or RNA levels were not examined, nor were quantitative analyses done. There have been no other reports of FV replication or FV-induced pathology in the context of host immunosuppression.

FV as a Therapeutic Vector

FV vectors show some promise for the therapeutic delivery of genes. Positive indications include the lack of documented pathogenesis, lack of preexisting immunity in most human populations, and the absence of reported human-to-human transmission. Additionally, FV can infect most cell types in vitro (54), has a large genome size and thus, a large packaging capacity, and does not preferentially integrate into genes (158). Based on the ability of FV vectors to transduce hematopoetic cell progenitors (159), FV vectors may be suitable for delivery of genes such as small interfering RNAs (siRNAs) that suppress immunodeficiency virus replication (23, 25). Park et al. engineered the *M. mulatta* FV strain SFV-1 to express siRNAs targeting the simian immunodeficiency virus (SIV) rev/env genes (116). They successfully used this FV vector in vitro to downregulate SIV gene expression and decrease SIV titers in infected cells.

FV vectors are also attractive candidates for cancer gene-therapy because their replication is restricted to dividing cells (157). Heinkelein et al. developed FV vectors to deliver suicide genes to solid tumors, using an athymic mouse model (48). Suicide-gene therapy is a two-step process developed to target and eliminate solid tumors. In principle, suicide genes encode proteins that render prodrugs toxic and induce selective death of vector-transduced tumor cells in the presence of the prodrug (112). These authors found that, even in the absence of the prodrug, these FV vectors demonstrated significant oncolytic activity (48). Further examination revealed that the wild-type FV itself had oncolytic activity and significantly suppressed tumor growth. Additionally, as with natural FV infection in primates, FV DNA was widely disseminated in tissues, and persisted in these diverse tissues for the duration of the study. Thus, the data from athymic mice suggest that conventional T cells are not integral to wide-tissue dissemination of FV in the murine host. Despite their success in suppressing tumor growth with FV vectors, the authors warned of the potential risks of using FV vectors in humans. They proposed that FV adaptation to humans could result in viral infection taking on a new pathogenic character (48).

A concern about using retroviral vectors for gene therapy is the danger of integration into transcriptionally active genes, and specifically, into proto-oncogenes. However, a recent extensive study of FV integration sites in human cells by Trobridge et al., showed that FV have a low rate of integration into transcriptional active genes, and may be safer than other retroviral vectors (158).

Outstanding Issues

Despite advances in understanding the molecular biology of FV replication, little is known about foamy virus–host dynamics and why infection by this retrovirus is nonpathogenic. One critical question is: what cell type is permissive for FV replication in vivo? Another is: how does the virus enter and exit a host? Specifically, what is/are the mode(s) of transmission, and the site(s) of initial infection? And why is there in vitro cytopathicity, but in vivo a lack of pathology? Central questions are whether the host immune system limits the viral infection in vivo, and specifically, whether T cell or innate immunity is involved. Determining whether FV induces any pathology in immunosuppressed hosts is one approach to answering these questions.

Macaques infected with SIV are widely used as a model for AIDS, and these animals are coinfected with FV. Although it is known that persistently FV-infected T cells are more permissive in vitro to HIV infection than are FV-uninfected cells (136), studies to examine differences in disease progression and possible interactions between these two retroviruses have not yet been undertaken.

I have summarized our current knowledge regarding FV and primate immunodeficiency viruses (Table 1.2). It is clear that there are fundamental virologic and immunologic parameters of FV in vivo infection that are unknown. In this thesis, I address three outstanding areas of FV–host interactions, namely, host immune responses to viral infection, the tissue-specificity of viral replication, and the cellular tropism of viral replication. In Chapter II, I present data regarding $CD4^+$ T cell and antibody responses to FV infection. In Chapter III, I examine the tissue-specificity of viral replication in normal and SIV-immunosuppressed hosts, and in Chapter IV, I identify the cell type in which FV replicates in its natural host.

Table 1.1. The Retroviridae family and associated pathologies.

Subfamily	Genus	Some Associated Pathologies
Orthoretovirinae	*Alpharetrovirus*	Avian leukemias and sarcomas
	Betaretrovirus	Mouse mammary tumors Sheep lung carcinomas
	Gammaretrovirus	Mouse leukemias
	Deltaretrovirus	Human T cell leukemias
	Epsilonretrovirus	Fish sarcomas
	Lentivirus	Human immunodeficiencies
Spumaretrovirinae	*Spumavirus* (foamy virus)	None

Table 1.2. Comparison of foamy viruses and immunodeficiency viruses. The features of FV indicated by asterisks are those areas of study addressed by my research and described in subsequent chapters of this paper. ds, Double stranded; ss, Single stranded.

Feature	Foamy virus	Human and simian immunodeficiency viruses (HIV and SIV)
Genome	dsDNA	ssRNA
Virion	Immature capsid	Mature capsid
Promoter	LTR and internal promoter	LTR
Reverse transcription	During assembly and/or budding	Early in infection, after uncoating
Cellular tropism	* Unknown permissive cell type *in vivo*; Wide *in vitro* tropism and *in vivo* persistence	Macrophage, lymphocytes
Sites of replication	* Mostly unknown oral cavity?	Lymphoid compartments, mucosal tissues, blood
Limited by CD4$^+$ T cell immunity?	* Unknown	Yes
Cell division required for replication?	Yes	No
Integration site preference	Near transcription start sites	Within genes, throughout transcription units

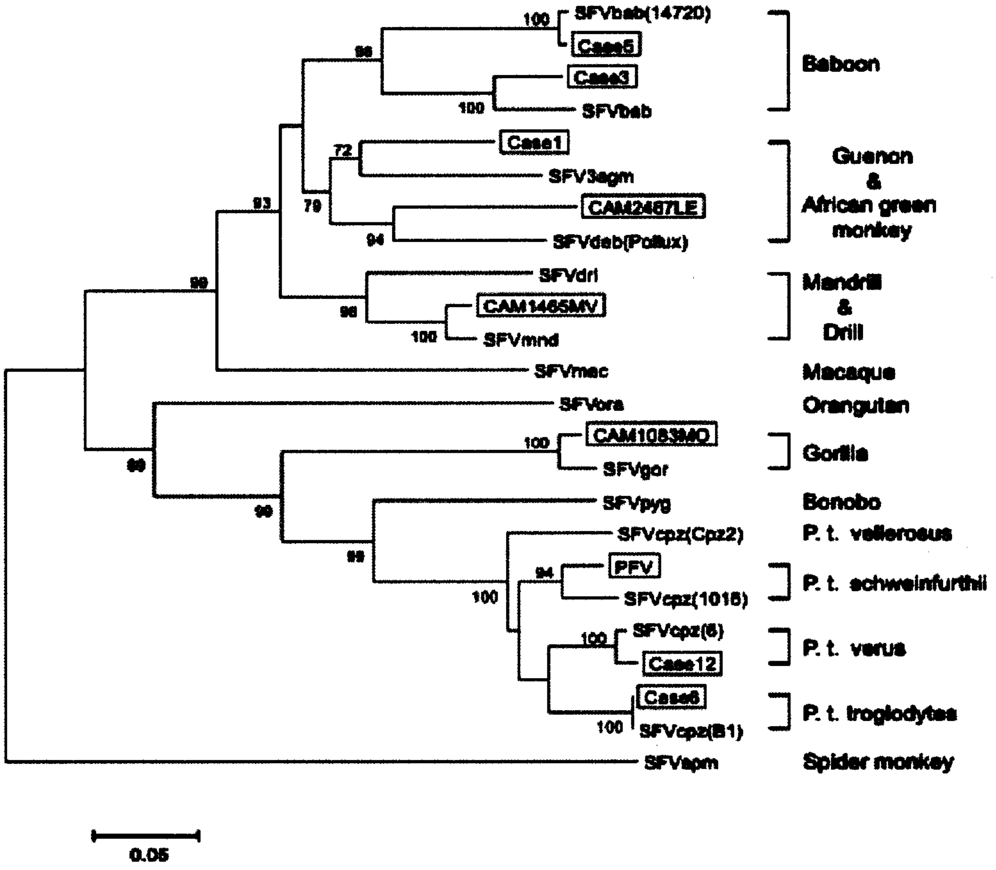


Figure 1.1. Inferred evolutionary relationships of foamy viruses.
Cases in humans resulting from cross-species transmission from multiple ape and monkey species are shown in boxes. For additional details on the FV sequences see references . Unrooted phylogenetic relationships of the 425-bp polymerase SFV sequences were determined using 2-Kimura distances in the neighbor-joining method implemented in the MEGA software package. Support for the branching order was determined by 1,000 bootstrap replicates; only values greater than 70% or more are shown. Branch lengths are proportional to the evolutionary distance (scale bar) between the taxa. The bar indicates 0.05 nucleotide substitutions per site. Figure provided by William Switzer and Walid Heneine. Taken from Fields Virology, 5th edition (D.M. Knipe and P.M. Howley, eds., Lippincott Williams & Wilkins, 2006) with permission. Adapted from (108).

Chapter II: Host Immune Responses to Foamy Virus Infection

Background

The persistence of virus in a host involves virus–host dynamics that result in the maintenance of host integrity and survival along with avoidance of viral clearance. Many persistent viruses elicit host immune responses, in particular T cell and antibody responses that limit viral replication and thus host pathology. Persistent viruses also have mechanisms to downregulate host immune responses in order to avoid absolute elimination from their host. FV is a persistent retrovirus that replicates minimally in the blood and peripheral tissues, and to a significant extent in the oral mucosa (Chapter III), but is nonpathogenic in its hosts. In vitro, however, FV is highly cytolytic to multiple cell types including epithelial cells and fibroblasts. This apparent dichotomy suggests host components not present in vitro are involved in limiting viral cytopathic effect (CPE) in vivo. Specifically, host adaptive immune responses to FV infection may be involved in significantly limiting FV replication and cytopathicity within a host.

The immunology of FV infection has been poorly studied compared to that of other retroviruses. T cell responses to infection by FV have not been evaluated, despite the significant evidence that virus-specific T cells promote host survival by controlling simian immunodeficiency virus (SIV) (103, 138), cytomegalovirus (CMV) (69, 70), and Epstein-Barr virus (EBV) (114). Additionally, only a few studies have examined the specific kinds and sites of antibodies produced after FV infection. There have been no reports of experimental FV infection where immune responses have been followed. Studies have been in the context of natural infection, often when time since initial infection is unknown, and in aged hosts, so that the dynamics of immune responses are not well understood. Most nonhuman primates both in primate colonies and in the wild, are known to become FV infected by young adulthood (M. Axthelm, personal communication) (9, 67) [reviewed in (97)].

In many viral infections, CD4$^+$ T cells are required for the generation and maintenance of CD8$^+$ T cell responses and potent antibody responses (2, 68, 93, 142). Little is known about FV-specific T cells. However, based on the presence of long-lived FV-specific antibodies in infected hosts (26), it is highly likely that FV-specific CD4$^+$ T cells are present to stimulate B cell production of these antibodies (24, 177). A critical issue is whether FV-specific T cells, which could inhibit FV replication systemically (i.e., in the blood and peripheral tissues), are present in the blood. Further, since the majority of viral infections occur via mucosal surfaces, and FV replicates in the oral mucosa, it is also of interest to know whether FV-specific T cell populations are present at mucosal sites. The presence of FV-specific CD4$^+$ T cells systemically or in mucosal sites has not been examined, so these remain outstanding issues of interest.

Cytolytic viruses are thought to be controlled most effectively by dominant antiviral cytokines secreted by CD4$^+$ T cells (3, 87). However, it is unknown whether FV infection elicits host T cell secretion of these inhibitory cytokines.

FV-induced CPE and infection of fibroblast-derived cell lines are known to be inhibited by factors secreted by polyclonally activated peripheral blood lymphocytes (PBL), including interferon-γ (IFN-γ) (39). While it has not been determined whether FV infection is sensitive to tumor necrosis factor-α (TNF-α), this cytokine is known to have general antiviral activity (2) and its production is associated with multiple dominant subpopulations of $CD4^+$ T cells, both the T helper 1 (Th1) and T helper 2 (Th2) T cells (141).

Antiviral antibodies can control viral infection by neutralizing, or clearing, free virus (a process known as opsonization), or by eliminating virally infected cells (3). Until ~6 months of age, NHP harbor maternal antibodies that are FV-specific, and during this time they do not acquire FV infection, suggesting that maternal antibodies may protect them from FV infection (9). However, the lack of infection might be due simply to the absence of behavior related to the mode of transmission, since a number of studies indicate that juveniles become infected with FV at about 2–3 years of age (personal communication, M. Axthelm) (9, 67). In the majority of hosts examined, serum antibodies against the FV Gag and (nonstructural) Bet proteins have been associated with FV infection (47, 55, 57). Antibodies against FV Env and Pol (47) and neutralizing antibodies, with titers as high as 1/640 (108), are found in some hosts (57, 65, 67). However, the importance of FV-neutralizing antibodies in the control of FV spread within a host is questionable. In vitro infectivity is through cell-associated virus (87), which may reflect an in vivo mechanism to escape from neutralizing antibodies.

However, FV-infected hosts do appear to sustain long-lived IgG antibodies specific to FV (26).

Whether mucosal antibodies are present in saliva is of importance because the oral mucosa is the site of FV replication, and because the majority of all viral infections occur via mucosal surfaces. IgA antibodies in mucosal secretions are generally considered to be a hallmark of mucosal immunity (78, 177), and secretory IgA (sIgA) has been proved important in limiting viral entry at mucosal sites (96). There is recent evidence that sustained FV-specific IgG but not IgA antibodies are evident in infected chimpanzees and zoonotically infected humans, and that FV-specific IgA antibodies are absent from the sera and saliva of all infected hosts (26). When FV-specific IgG was evident in saliva, its levels and antigen specificity correlated to the IgG in the blood, suggesting that the IgG in the saliva had transudated (passively transferred) into the oral secretions, and had not been locally produced. This absence of mucosal antibodies may be a co-evolved feature of viral–host interaction that renders viral replication permissive in the oral mucosa. Whether long-lived systemic IgG antibodies affect viral replication in the blood is unresolved. Further, the dynamics of antibody production after initial infection remains unknown, since these data were collected from subjects where the time since initial infection was at least >10 yrs. post infection.

I utilized a large cohort of rhesus macaques (RM) to study antibody responses and $CD4^+$ T cell responses to natural FV infection. In order to examine $CD4^+$ T cells specific to FV antigens, I developed an assay to detect $CD4^+$T cells that had been previously expanded in their host in response to FV infection, using an intracellular cytokine (ICC) staining assay that detects memory (previously activated) T cells, but not naïve T cells. I evaluated TNF-α secreting $CD4^+$ T cells, since production of TNF-α is associated with multiple $CD4^+$ T cell subpopulations and has been utilized to enumerate other virus-specific T cells in RM (120). I also examined IFN-γ secreting CD4 T cells. I measured memory $CD4^+$ T cell responses most extensively in the blood, but also in a mucosal site, the lung, by studying bronchoalveolar lymphocytes (BAL). I also characterized antibodies produced in response to FV infection, by evaluating FV Gag-specific IgG and IgA in the sera and saliva, and FV Env-specific IgG antibodies in the sera, using highly quantitative enzyme-linked immunosorbent assay (ELISA)-based methods that I developed.

Methods

Animals

All animals used in these studies were rhesus macaques (*Macaca mulatta*) of Indian origin, bred and housed at the Oregon National Primate Research Center. All studies were conducted in accordance with the standards of the Center's Animal Use and Care Committee and the approved protocols of the *Guide for the Care and Use of Laboratory Animals* (60). All animals were in good health, of either sex, and ranged in age from 2.8 to 7.9 yrs. when PBMC and plasma samples were collected. Saliva samples were taken from animals ranging in ages from 3.7 to 4.8 yrs. The RM were naturally infected with simian foamy virus (SFV) through interaction with SFV-infected animals in the facility, but time since infection with FV was unknown for any individual animal. Specific-pathogen-free (SPF) animals were separately housed and maintained as FV-free. Both colony and SPF RM were identified as infected with SFV by screening for the presence of plasma antibodies against the SFV (SFV) Gag antigen, as detected by a quantitative ELISA (described below).

Isolation of PBMC, Plasma, BAL, and Saliva

PBMC was isolated by standard Ficoll-Hypaque density gradient centrifugation of whole blood. Briefly, whole blood was collected in sodium citrate and plasma was taken after centrifugation at 1500 rpm for 15 min. Histopaque (Sigma) was used to separate mononuclear cells from other cell types. BAL cells, a source of T cells from the lung, were isolated as previously described by Pitcher et al (120). Briefly, the lung wash was centrifuged at 900 rpm for 10 min, the supernatant removed, and the cells resuspended in complete RPMI-1640 supplemented with 10% Fetal Calf Serum (R-10; Cellgro). Three washes were performed before the cells were resuspended in medium for use in assays. Saliva was obtained from buccal swabs collected by rubbing polyester-tipped swabs (Fisher) on the cheeks, tongue and inside of the throat. Swabs were placed in 1 ml of HBSS and vortexed, and cellular material was pelleted by centrifugation at 1000 rpm for 10 min. The supernatant was taken, sodium azide was added to a final concentration of 0.01%, and the saliva fraction was stored at –80°C until further use.

Antigen Preparation

To stimulate FV-specific memory CD4$^+$ T cells, a SFV cell-free virus lysate was prepared. The pSFV-1 viral clone (29) was kindly provided by A. Mergia (University of Florida). Baby hamster kidney cells (BHK) cells (ATTC# CCL-10) were infected with SFVmac and harvested when the FV-induced CPE affected 80–90% of the culture. The cells were harvested by scraping with a rubber scraper and subjected to three freeze–thaw cycles and sonication to release intracellular virus. The lysate was centrifuged at 1000 rpm for 10 min to remove cell debris. The clarified supernatant was collected and used as the viral antigen. The virus titer of the preparation was determined by the simian foamy virus-activated β-gal (SFAB) assay, utilizing an FV indicator cell line, as previously described by Jones-Engel et al. (67). A control lysate from uninfected BHK cells was prepared with the same protocol as described for the SFV-infected cells. In order to determine the optimal concentration of control lysate for measuring FV-specific memory CD4$^+$ T cell responses, dilutions of the lysate ranging from 1:10 to 1:100 were tested with PBMC from a FV$^+$ RM that showed significant reactivity at a 1:10 dilution, and from a FV$^-$ RM that showed no SFV-specific reactivity. After ICC was performed, as described below, the optimal concentration was determined to be 1:20 and this dilution was used for all assays. A RhCMV cell-free viral lysate (kindly provided by M. Axthelm and L. Picker, OHSU, Vaccine & Gene Therapy Institute) was used to stimulate CMV-specific memory T cells and was prepared as previously described (120). Briefly, the RhCMV lysate was prepared the same way as the SFV lysate (described above), except that the RhCMV lysate was further concentrated by performing a high-speed centrifugation to pellet the virus, and an ammonium sulfate precipitation to concentrate the antigens.

To identify FV Gag-specific antibodies by ELISA, the N terminus of the SFVmac Gag protein (amino acids 1 to 193) was produced as a glutathione *S*-transferase (GST) fusion protein by cloning bases 1736 to 2318 of pSFV-1 into the pGEX-2X vector (Promega). The fusion protein or GST alone was expressed in the *Escherichia coli* strain PLys and extracted by standard methods. In order to determine the optimal concentration of antigen where antigen was in excess, and plasma dilutions optimal for measuring the variation in FV Gag antibodies, the SFVmac Gag1–193-GST antigen was titrated by performing a matrix of two-fold dilutions, ranging from 2 to 0.005 μg/ml, and dilutions of serum samples ranging from 1:50 to 1:800 in an ELISA (described further below). The optimal concentration was considered to be the lowest concentration of antigen where saturation by antibodies in serum was evident. Using this assay, the optimal concentration of antigen was determined to be 0.5 μg/ml for the FV Gag ELISA. To identify FV Env-specific antibodies by ELISA, the surface (SU) protein of PFV was produced as a GST fusion protein by cloning bases 6988 to 7412 of PFV into the pGEX-2X vector (34). The FV SU antigen was used in a matrix at 128–0.25 μg/ml to determine the optimal concentration for use in a quantitative ELISA. The optimal concentration was determined to be 32 μg/ml.

Intracellular cytokine staining for FV-specific memory CD4$^+$ T cells

Intracellular cytokine (ICC) staining was used to enumerate FV-specific CD4$^+$ T cells, following a protocol similar to that of Pitcher et al. (120) and Waldrop et al. (164). First, PBMC were incubated at 10^6 cells/ml in R10 with appropriate antigen and CD28 and CD49d co-stimulatory antibodies (Pharmingen), at 0.5 μl per reaction. PBMC from each RM was stimulated with each of the following antigens: SFV cell-free lysate, control uninfected cell-free lysate, CMV cell-free lysate, staphylococcal enterotoxin B (SEB) at 0.2 μg/ml (Toxin Technology), costimulation only, and medium alone. After 1 hr incubation at 37°C in 5% CO_2, Brefeldin A (BFA) (Sigma) at 10 μg/ml was added to inhibit cytokine secretion, and then the cells were cultured for an additional 5 hrs. Cells in capped tubes were placed at 4°C and processed within 24 hours. The cells were pelleted, washed twice in fluorescence-activated cell sorter (FACS) buffer (Phosphate Buffered Saline (PBS) with 0.1% BSA), and then surface stained by a

30-min incubation at room temperature in the dark with directly-fluorochrome-conjugated antibodies to CD3 (clone SP34), and CD4 (clone L200) or CD8β (clone 2ST8.5h7), all obtained from BD Biosciences. This incubation was followed by two washes in FACS buffer. Subsequently, the cells were permeabilized with fixation/permeabilization solution (BD Biosciences) at 2X concentration for 10 min, and washed twice in FACS buffer. Cells were then intracellularly stained by incubation for 20 min at room temperature in the dark with directly conjugated antibodies to CD69 (clone L78), to identify activated cells, and to cytokines, either TNF-α (clone 11) or IFN-γ (clone B27), all obtained from BD Biosciences. Cells were washed twice and resuspended in FACS buffer with 150μl of 1% paraformaldehyde. The antibodies were conjugated with one of the following fluorochromes: fluorescein (FITC), phycoerythrin (PE) Texas Red; Texas Red, allophycocyanin (APC); APC-Cy7; or Am-Cyan. A FACScan or FACSCalibur was used for 4-parameter flow cytometry and Paint-a-gate software (BD Biosciences) was used to determine what percentage of $CD4^+$, $CD3^+$ T cells were antigen-specific. Criteria for delineating the antigen-specific populations by CD69 and cytokine expression have been previously reported in Pitcher et al. (120). For each sample, at least 100,000 events were collected. To quantify the FV-specific population, the frequency observed from stimulation with uninfected cell lysate was subtracted from the frequency of those observed with SFV-infected cell lysate. When samples were not limited, they were tested in duplicate, and the mean was reported. The same antigen stimulation and ICC protocol described for PBMC were followed for BAL.

Quantitative ELISA for FV Gag-specific IgG and IgA

The ELISA conditions were similar to those described by Jayaraman et al. (14) for SIV antigens. Briefly, 96-well Immunosorp plates (Nalge Nunc) were coated with 50 ng/well of either the SFVmac Gag1–193-GST antigen or the GST control antigen alone, which had been diluted to 0.5 μg/ml in a carbonate-bicarbonate buffer (10mM Na_2CO_3, 40mM $NaHCO_3$, pH 9.6), and the plates were incubated overnight at 4°C. The antigen-binding buffer was removed, and the plates were incubated with blocking buffer (5% nonfat dry milk in PBS) for 1 hr at room temperature. The blocking solution was removed, and heat-inactivated plasma was serially diluted in twofold dilutions in blocking buffer that ranged from 1:100 to 1:51,200, and added to the plate at 100 μl/well. The wells were washed three times in wash buffer (0.1% Triton X-100 in PBS). Then the wash buffer was removed, and either the secondary anti-monkey IgG HRP (Cappel) or IgA HRP (Alpha Diagnostics) was added at 1:5000 or 1:1000, respectively. The secondary antibody was removed and the plate was washed five times in wash buffer, and in the last wash, left in wash buffer for 5 min. The wash buffer was removed and TMB substrate (Sigma) was added at 100 μl/well and incubated for 8 min. To stop the reaction, 100 μl of 1N H_2SO_4 was added. The absorbance at

450/405 nm was read by using an EL808 Ultra Microplate Reader (Bio-tek Instruments, Inc.) and KC4 software, version 3.02 (Bio-tek Instruments, Inc.). Midpoint titers were obtained by determining the reciprocal of the plasma dilution at half of the maximum optical density $[(OD_{max})/2]$. Every individual assay included standard plasma derived from an RM with high levels of FV Gag antibodies, in order to normalize for interassay variability, and all titers were normalized to this plasma standard. Additionally, all assays included plasma from an RM previously identified as FV^-. In every assay, this serum gave a value that was at or below background. When possible, each plasma sample was tested twice. For testing saliva, the same protocol was followed except that three-fold dilutions, ranging from 1:3 to 1:243, were performed. Background responses were defined as those above the mean value of the sera or saliva reactivity of GST alone, ±2 standard deviations.

Validation of the anti-IgA antibody

In order to confirm that the anti-IgA antibody was specific to IgA, and did not crossreact with IgG, I tested it in an ELISA for SIVgp130, utilizing macaque monoclonal IgG antibodies recognizing this antigen. Briefly, the SIV gp130 antigen (kindly provided by N. Haigwood, SBRI), at 2 μg/ml, was used to coat the wells of ELISA plates. The macaque monoclonal IgG antibodies E31 and B23 (kindly provided by J. Overbaugh, FHCRC) were used at 25 μg/ml, 12.5 μg/ml, and 6.25 μg/ml, and the anti-IgG and IgA antibodies were used in the same concentration as described above for the FV ELISA. Subsequent steps were the same as those followed for the FV Gag ELISA.

Quantitative ELISA for FV SU Env-specific IgG

The protocol developed for FV Gag IgG antibodies was followed, with the following changes. The SU-GST antigen was used to coat ELISA plates at 32 μg/ml, with 100 μl added per well. Plasma was added in two-fold dilutions, from 1:25 to 1:12,800.

Statistical Analyses

Linear regression and r^2 values were derived using Microsoft's Excel® Software.

Results

FV-specific T cells

The intracellular cytokine (ICC) staining assay is generally considered to be an 'antigen recall' assay that measures the presence of memory T cells for a specific antigen. I developed an ICC staining assay to measure FV-specific $CD4^+$ T cells. I found that $CD4^+$, but not $CD8^+$ T cells, were stimulated by the FV viral lysate that I utilized. The stimulation of $CD4^+$ but not $CD8^+$ T cells has been observed for other similar whole-virus antigens (personal communication, Louis Picker), and is related to the efficiency and time course of antigen processing and presentation in the antigen-stimulated cell culture.

FV-specific $CD4^+$ T cells in peripheral blood were quantified in thirty RM, six of which were specific pathogen-free (SPF) and FV^- (as determined by the absence of FV-specific antibodies). Figure 2.1 shows representative FACS plots of PBMC from one FV^+ and one FV^- RM stimulated with a panel of antigens. Each graph shows the percent of total $CD4^+$ T cells that are antigen-specific, as measured by expression of TNF-α and CD69, an early T cell activation marker (153). A discrete FV-specific $CD4^+$ T cell population of 0.13% is evident in PBMC from the FV^+ but not the FV^- RM. Further, this $CD4^+$ T cell population is specific to PBMC stimulated with the SFV antigen and absent from those stimulated with the control, the uninfected-cell lysate. The FV^- RM (which had been maintained as CMV^-) showed, as expected, no T cell response to the CMV antigen, while the FV^+ RM, which was also CMV^+, showed a 2.31% CMV-specific $CD4^+$ T cell population. As expected, both FV^+ and FV^- PBMC responded to SEB, a polyclonal $CD4^+$ T cell activator. After identifying a FV^+ RM with significant FV-specific $CD4^+$ T cells, I performed a titration of the viral antigen to determine the optimal antigen concentration, i.e., where responses were maximal and not evident in the same concentration of the negative control antigen, to use for subsequent studies. After testing at dilutions ranging from 1:5 to 1:100, I found that the SFV antigen was optimal at a 1:20 dilution. All further studies utilized viral and control antigen at this dilution.

PBMC from each of the RM in the cohort was evaluated with this panel of antigens. The FV-specific $CD4^+$ T cell populations are displayed in Figure 2.2. The antigen-specific populations appear discrete, and based on the absent or low responses from SPF, FV^- RM, appear to reflect real $CD4^+$ T cell-effector populations. Responses are diverse, with antigen-specific T cells comprising 0.02–0.23% of peripheral blood $CD4^+$ T cells (Figure 2.2). Overall, FV-specific $CD4^+$ T cell populations comprised 0.23–0.10% of total $CD4^+$ T cells in one third of the FV^+ animals (8/24); 0.1–0.03% in another third (8/24), and 0.02%–undetectable in the other third (8/24). The maximal response observed in SPF, FV^- PBMC was 0.02%, and this was observed in only two of the six FV^- RM, while the other four had undetectable responses to the SFV antigen. As expected, all of the FV^+, CMV^+ RM displayed $CD4^+$ T cell responses to the CMV antigen, and the FV^-, CMV^- RM had no detectable CMV responses, yet both groups responded to the SEB polyclonal $CD4^+$ T cell activator (data not shown).

Since a previous study showed that FV replication was sensitive to IFN-γ (39), I was interested to know the frequencies of FV-specific $CD4^+$ T cells that produced IFN-γ^+. Initially, I quantified FV-specific IFN-γ^+, $CD4^+$ T cells in eight FV^+ RM and two FV^- RM to determine how this population compared to the TNF-

α^+ population. I found, in general, weaker FV-specific responses, from 0.01% to 0.08%, with six of the eight RM having responses above those seen in the FV^- group. The FV-specific IFN-γ^+, $CD4^+$ T cell frequencies did not significantly correlate to the TNF-α FV-specific $CD4^+$ T cell frequencies ($r^2 = 0.4103$) (data not shown).

In order to understand how $CD4^+$ T cell responses to FV infection are regulated, I was interested to know whether time post infection (p.i.) or responses to CMV were related to the potency of the $CD4^+$ T cell responses to FV. Since I did not know the exact time of initial FV infection, I used age of the animal as a marker for time p.i., since most of FV infection in NHP occurs by the age of 2–3 yrs. I determined that there was no correlation between age of the RM and the size of T cell populations ($r^2 = 0.04$). Nor was there a correlation between the sizes of the CMV- and SFV-specific $CD4^+$ T cell populations ($r^2 = 0.0015$) (data not shown).

Antiviral T cells from BAL are considered to be a good measure of mucosal $CD4^+$ T cell response (119). While obtaining BAL involves a more invasive procedure than obtaining blood, I was able to obtain BAL from three FV^+ RM and one FV^-, SPF RM. I evaluated FV-specific $CD4^+$ T cells, using the ICC assay, in both BAL and blood taken at the same time. I found that in all three FV^+ RM, FV-specific $CD4^+$ T cell responses were significantly enriched in the BAL compared to the blood. The BAL had approximately 7 to 60 times greater proportions of FV-specific CD4 T cells than the blood of the same RM (Table

2.1). BAL FV-specific responses ranged from 0.65 to 4.6%, and of those in PBMC, from undetectable to 0.19% of total $CD4^+$ T cells. As expected, there were no FV-specific $CD4^+$ T cells detected in BAL or PBMC from the FV^-, SPF RM. These preliminary data suggest that FV-specific $CD4^+$ T cells are enriched in mucosal sites, which are where viral replication is detected (further discussed in Chapters III and IV) and, in general, where environmental antigens such as microbes are encountered.

FV-specific Antibodies

I developed a highly quantitative FV Gag ELISA (described in Methods, above) specifically to detect FV-Gag antibodies of the IgG isotype, in order to study the variation in an large cohort of naturally FV-infected RM. Titration curves, used to derive midpoint titers, are shown in Figure 2.3 for several representative RM (five FV^+ and one FV^- RM). These curves are consistent with high avidity antibody. I evaluated FV-Gag IgG titers from plasma samples obtained from 34 FV^+ and 7 FV^-, SPF RM (Figure 2.4). The titers ranged from 356 to 8530 in the FV^+ RM, yielding approximately a 24-fold maximal difference in titer within the population. All SPF RM had undetectable levels of FV-specific antibodies. I theorized that the Gag-specific antibody titer is altered with time post infection, and that age could therefore stand as a rough measure of time post infection. However, the titer did not correlate with age ($r^2 = 0.0011$), suggesting either that age is not a good predictor of time since acquisition of FV infection, and/or that genetic heterogeneity of the population and other factors are involved in determining variability in FV Gag IgG levels.

I also tested saliva collected from nine FV^+ and one FV^- RM. I found FV Gag IgG antibodies in saliva from eight of the nine FV^+ animals but not from the FV^- animal. While eight of the saliva samples had significant antibody levels, there was a large amount of variation. For one RM, $OD_{max} = 0.6$, while for the other animals, OD_{max} ranged from 0.124 to 0.06 (data not shown). Thus, I can conclude that FV-specific IgG is present in the oral cavity, the site of FV replication, although levels are variable between hosts. However, antibodies of the IgG isotype could be indicative of transudated (passively transferred) sera antibodies rather than *de novo* mucosal antibody production.

Next, I tested for FV Gag IgA antibodies in thirteen FV^+ and one FV^- RM, and was able to determine midpoint titers for 12 of these (Figure 5). Strikingly, two of the RM demonstrated high levels of FV Gag IgA; these were two animals of relatively young age (both 3.26 yrs), which had been housed together. However, another RM (age 3.25 yrs) in the same housing location did not have high IgA levels. In the other ten FV^+ animals, IgA was detectable, but at relatively low levels. Since midpoint titers were determined in separate assays for IgG and IgA, similar titers do not indicate the same antibody concentration, and titer comparisons between IgG and IgA do not reflect actual concentration differences. Examination of IgA titers reveals an approximately 19-fold difference in FV Gag

IgA levels within this group of RM. I also evaluated saliva and did not detect FV Gag-specific IgA.

One potential caveat regarding the reliability of the FV Gag IgA ELISA, is that the FV Gag IgG may be in a sufficiently high concentration as to mask the Gag antigen from the FV Gag IgA, predicted to be in lower abundance. However, my results argue against this possibility. First, I developed the IgG assay with antigen in excess, and I use this same antigen concentration to test the IgA levels. Next, it would be expected that high levels of FV Gag IgG would be inversely proportional to the FV Gag IgA levels if there is masking, but this was not the case. The two RM with relatively high levels of IgA have an approximately 3-fold difference in FV Gag IgG titers. The 3.26-yr-old RM with the higher FV Gag IgG has an IgG titer comparable to another RM with an approximately 9-fold lower FV Gag IgA titer. Therefore, I do not think that this is a major consideration.

I also wanted to confirm that our IgA isotype-specific antibody was not cross-reactive with any other antibody isotypes, particularly to IgG. If there were crossreactivity, then we would expect to observe a correlation between levels of FV Gag IgG and IgA within each RM, which we did not observe. To test the specificity of the anti-IgA secondary antibody, I utilized RM monoclonal IgG antibodies E31 and B23, reactive to SIVmac239 gp130 antigen, and an ELISA developed for the SIVmac 239 gp130 antigen (61). The anti-IgA and anti-IgG secondary antibodies used in the FV Gag ELISA were also used as secondary antibodies in the SIVgp130 ELISA. I found that the anti-IgA antibody did not significantly react with the RM IgG monoclonal antibodies while the anti-IgG did react, as expected (data not shown). Thus, I conclude that I am observing real differences in FV-Gag IgA titer amongst these study subjects.

I examined eight of these FV^+ and two FV^- RM for IgG antibodies to the SU domain of the FV (Env) envelope protein, likely to contain epitopes important in virus neutralization. I found significant reactivity in some of the FV^+ RM, with approximately 10-fold differences between animals for which midpoint titers were obtained (Figure 2.6). However, I was unable to obtain midpoint titers for five of the RM because maximum OD was less than the $OD_{max}/2$. Interestingly, the FV SU Envelope IgG titers roughly correlated with the levels of FV Gag IgA antibodies ($r^2 = 0.82$). One possibility is that FV SU Envelope IgG and FV Gag IgA antibodies may be produced only after initial infection, and wane with time.

Relationship between FV-specific T cells and Antibody Responses

It is thought that virus-specific $CD4^+$ T cells play a role in stimulating the production of long-lived antiviral antibodies (24). Thus, FV-specific $CD4^+$ T cells and antibodies may follow interdependent regulation. If so, then their frequencies would be expected to correlate. Therefore, I was interested to determine whether there is a correlation between the frequency of FV-specific $CD4^+$ T cells and FV-specific antibody titers. For 23 FV^+ RM and 6 FV^- SPF RM in the cohort, both the FV-specific $CD4^+$ T cell frequencies in the blood and the IgG FV Gag in the sera were determined. Figure 2.7 presents both parameters for each RM. The magnitude of the FV-specific T cell populations did not significantly correlate to FV Gag IgG antibody titer (r^2= 0.185). IFN-γ secreting FV-specific CD4$^+$T cell frequencies had a negative correlation to FV Gag IgG antibody titer, but this correlation was not significant (r^2 = 0.211). Thus, FV-specific CD4$^+$ T cells in the blood were not predictors of FV Gag sera IgG titer; these two immunologic parameters appear to have distinct and independent regulation.

Discussion

In this research, I characterized and quantified memory CD4$^+$ T cells and antibodies specific to FV, in a large cohort of RM. The results of these studies are summarized in Table 2.2. The relatively low frequencies of FV-specific CD4$^+$ T cells in the blood —between 0.03% and 0.23% of total CD4$^+$ T cells—suggest that T cells are not primarily responsible for limiting FV replication in the blood. However, the enrichment of FV-specific CD4$^+$ T cells in the lung compared to the blood suggests that CD4$^+$ T cells could limit viral replication in mucosal tissues. I found significant levels of FV-specific IgG antibodies in the blood, and therefore it may be that antibodies limit FV replication systemically. FV SU (Env)-specific sera IgG and levels of FV-specific sera IgA were the only immunologic correlates determined by my studies, which may indicate that both are produced with initial FV infection. My preliminary data indicate the presence of FV Gag-specific IgG but not IgA in saliva, were consistent with previous findings (26). This lack of significant IgA in the saliva may be a mechanism that permits viral replication in the oral mucosa.

The FV-specific $CD4^+$ T cell frequencies are lower than those found with some other persistent viral infections, such as RhCMV, where CMV-specific $CD4^+$ T cells account for 0.16–5.8% of $CD4^+$ T cells in the blood (69). In EBV-infected humans, virus-specific memory $CD4^+$ T cells were found at frequencies of 0.05–1.26% in PBMC (with a mean of 0.33%) (5). Table 2.2 summarizes immune responses to other persistent viruses and compares these to what I have learned about FV from my studies.

While the higher frequencies of RhCMV or EBV-specific $CD4^+$ T cells compared to FV-specific $CD4^+$ T cells may be a result of differences in the assays, for example, in antigen preparations, the differences may be due to higher levels of RhCMV or EBV replication compared to that of FV. Latent RhCMV and EBV often reactivate, whereas FV is thought to be mostly latent. However, my further studies described in Chapters III and IV suggest that FV is not latent, and therefore this is not likely to be the explanation. Differences in localization of viral antigen and sites of viral replication between CMV or EBV, and FV may explain the greater enrichment of CMV- or EBV-specific $CD4^+$ T cells in the blood.

The mucosal enrichment of viral-specific $CD4^+$ T cells has been observed for CMV-specific $CD4^+$ T cells (120). CMV replicates at mucosal sites, as does FV, which replicates in oropharyngeal tissues, including the lung of some hosts (further described in Chapter III). Since viral antigen would be enriched in oral mucosal sites, we would expect viral-specific T cells to localize and accumulate there as well. However, this finding raises the question, "if FV-specific T cells are enriched in these sites of replication, and they are inhibitory, then how can viral replication be permissive there?"

I found a range of FV Gag IgG titers as well as IgA titers in the sera of FV-infected NHP. This contrasts with another report in which sera IgA could not be detected in chimpanzees and humans >10 yr p.i. (26). I surmise that the greater time since infection, the use of a less sensitive method (Western blot), and different host species used in the previous research are responsible for our different observations.

Cell-to-cell mediated viral spread has been observed for FV in vitro, and therefore, may also occur in vivo. If so, then the viral envelope (Env) antigen may be sequestered from the host immune system during virus spread, and most accessible to the host immune system during primary infection, when extracellular virions are most likely to be present. Thus, Env-specific antibodies would be produced preferentially with initial infection. Further, transient EBV-specific sera IgA has been associated with initial EBV infection (78, 125). Therefore, IgA production may be a host response common to both primary viral infections. Thus, further examination of FV Env-specific antibodies and FV-specific IgA is warranted.

It may be that the site of viral infection differs from the site of viral replication, and that different types of immune responses are induced at these disparate sites. This would likely result in an apparent lack of correlation between immune parameters that are initiated during different phases of infection. If initial FV infection occurred at the skin, for example via a bite, one set of immune responses would be induced, while FV replication in the oral mucosa would stimulate a different set of responses. For example, short-lived FV SU IgG and FV Gag IgA, and sustained FV Gag IgG, may be produced in response to initial infection, while relatively low frequencies of peripheral FV-specific T cells, relatively higher frequencies of mucosal FV-specific CD4$^+$ T cells, and little to no FV-specific salivary IgA are produced in response to viral replication. These ideas are considered and discussed further in Chapter V. However, all of these outstanding issues regarding viral–host dynamics after infection are best addressed by experimental infection, where immune responses are followed in individual hosts (discussed further in Chapter V).

In my studies, I found that age did not significantly correlate with immune parameters. Thus, it could be that FV reinfection occurs throughout a lifetime and confounds an analysis using age as a surrogate for time post infection. It is not known whether animals are reinfected with FV; this should be evaluated. Also, my studies did not extend to quantifying FV-specific CD8$^+$ T cells, which are known to mediate direct infected-cell killing, and other antiviral functions. Therefore, future studies should include analysis of this T cell population.

Consistent with the hypothesis that FV-specific antibodies limit viral replication, was the lack of FV-specific IgA in saliva, as FV replication is restricted to the oropharyngeal-associated tissues, as described further (Chapters III and IV). It would be of interest to determine whether FV-specific IgA is present in other mucosal sites where there is no detectable FV replication. Similarly, with HIV and SIV infection, there is an absence or low level of HIV- or SIV-specific IgA at mucosal sites (102, 134).

In contrast to a model that proposes that the host immune system, specifically the FV-specific T cells, inhibits viral replication systemically, the relatively low frequencies of FV-specific T cells in the blood suggest that T cells are not primarily responsible for limiting FV replication in the blood. Therefore, I think it is likely that other factors, such as viral transcription regulation, are involved. Perhaps the combination of homogeneous cell types and the absence of highly activated mononuclear cells in the blood result in uninduced viral transcription and viral latency. In vitro, leukocytes are persistently infected with FV, but with activation, produce virus that spreads to co-cultured permissive cell types, such as fibroblasts or epithelial cells. In vivo, PBMC often have a less activated phenotype than mononuclear cells in tissues. Mononuclear cells in the blood are in a relatively quiescent state, while those in peripheral tissues would be more activated, and thus, more permissive for FV replication. Further, infected leukocytes in the blood would have less extensive contact with highly permissive cell types such as fibroblasts or epithelial cells than would leukocytes in the mucosal tissues or in an in vitro co-culture.

Experiments described in this chapter have expanded our knowledge regarding host immune responses to FV infection. However, immunosuppression studies are needed to discover whether viral replication is limited by the host immune system. Only one study has evaluated immunosuppressed NHP hosts for alterations in viral replication and suggested that cells suppressed with myleosuppressive drugs are involved in limiting viral replication in the oral cavity (9). My analysis in Chapter III extends the immunologic studies described in this chapter by examining the tissue sites and levels of FV replication, in both normal and immunosuppressed hosts, in order to understand whether the host immune system limits viral replication.

Table 2.1. Proportion of total CD4$^+$ cells found to be FV-specific in PBMC and BAL.

Results from four rhesus macaques are shown. UD, Undetectable. Peripheral blood mononuclear cells, PBMC; bronchoalveolar lavage lymphocytes, BAL.

RM	PBMC	BAL
FV$^-$	UD	UD
FV$^+$1	0.12	4.60
FV$^+$2	UD	0.65
FV$^+$3	0.19	1.26

Table 2.2. T cell and antibody responses to FV and other persistent viruses. The parameters determined by the research elaborated in this chapter are shown in boldface.

Virus	**Virus-specific T cells**	**Antibodies**	**Limited by Host Immunity?**
EBV	$CD4^+$ T cells 0.05–1.26% of PBMC(5)	Transient IgA(125) Neutralizing IgG Titers differ between individuals but difference is stable over time (125)	yes
CMV	RhCMV $CD4^+$ 0.16–5.8% of PBMC (69)	Neutralizing, not well characterized Critical role in protection (117)	yes
FV	**$CD4^+$ 0.03–0.23% of PBMC; 0.65–4.6% of BAL**	**Gag sera IgG** **Gag salivary IgG, not salivary IgA** **Sera IgA and SU IgG in some hosts** Neutralizing Role in Infection?	unknown

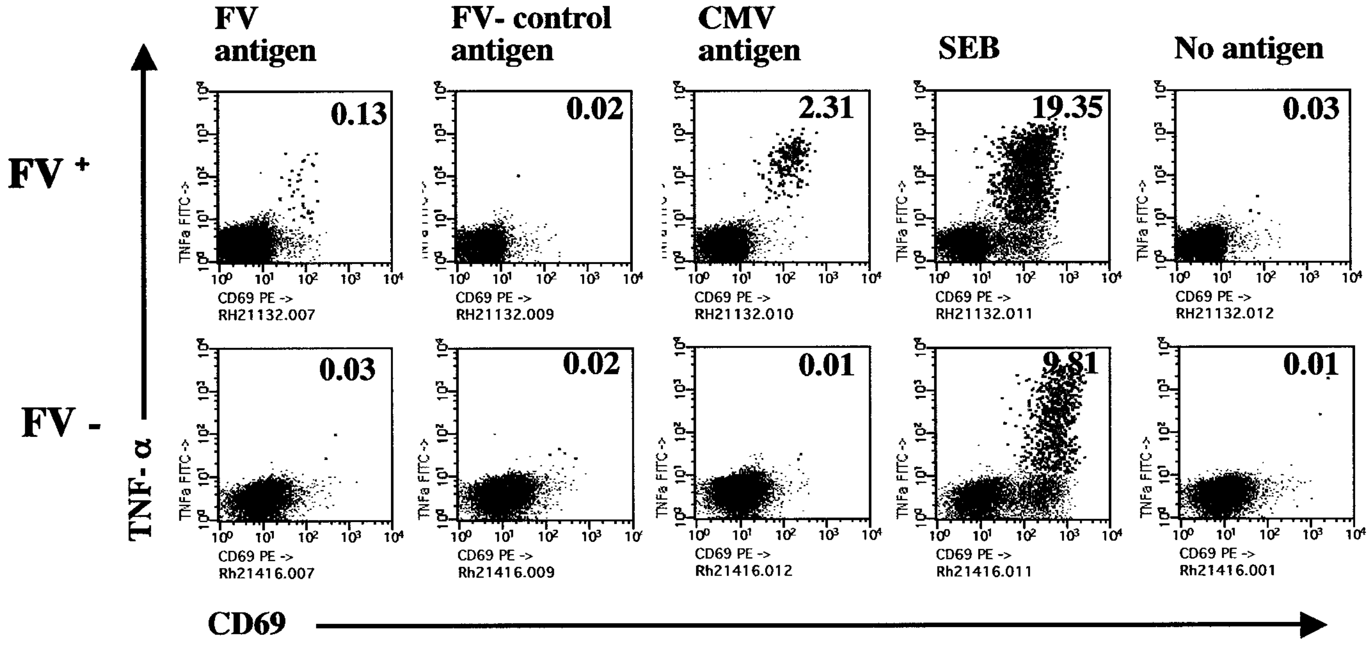


Figure 2.1. FV-specific CD4+ T cells in FV-infected and FV-uninfected rhesus macaques.
Representative FACS plots show CD4+ T cell populations activated by specific antigens, using PBMC isolated from one FV+ and one FV- (SPF) rhesus macaque. Cells are gated on CD3+ CD4+ small lymphocytes, and confirmed as memory T cells by staining for TNF-α and CD69, an early activation marker. The numbers in the FACS plots indicate antigen-specific CD4+ T cells as a percentage of total CD4+ T cells in PBMC. The antigen-specific populations are highlighted in

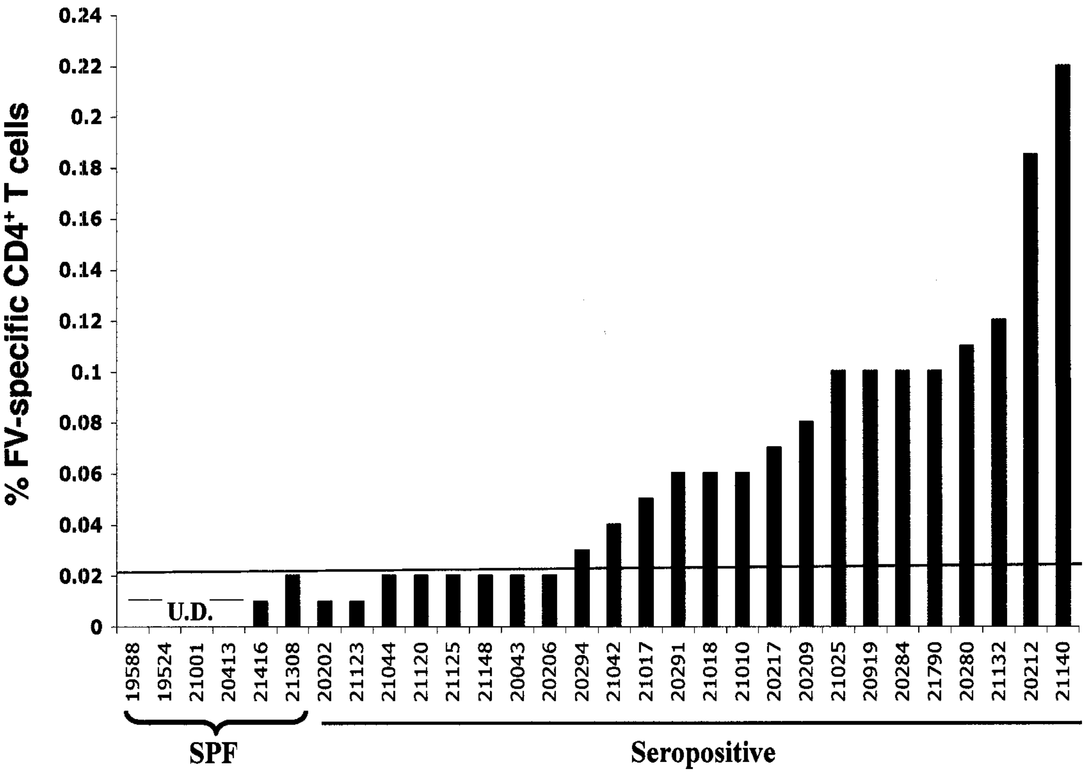


Figure 2.2. FV-specific CD4$^+$ T cells in PBMC from rhesus macaques. Bars represent the percentages of CD4$^+$ T cells that were FV-specific as determined by intracellular cytokine staining and FACS analysis. The x-axis shows the animal number. The red line indicates the highest response measured in SPF, FV- macaques and was considered background. n=30. UD, undetectable

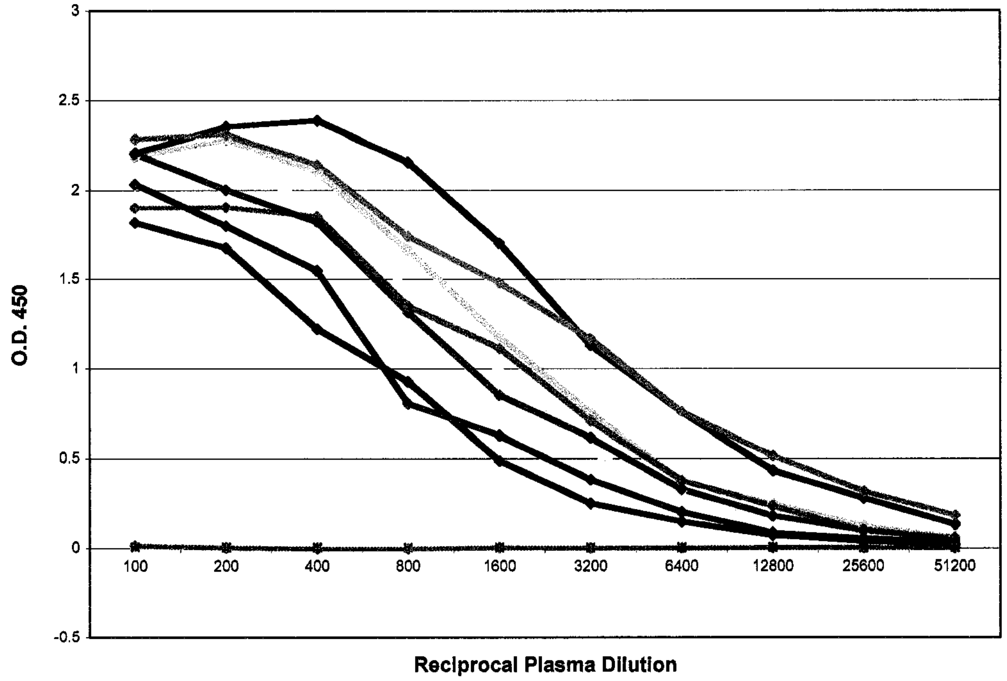


Figure 2.3. FV Gag-specific IgG titration.

A quantitative ELISA was performed and the results are shown. Two-fold dilutions were performed to titrate FV Gag IgG in the plasma of FV+ and FV-RM. The eight representative curves were obtained by the titration of plasma from FV+ RM, and were used to derive midpoint titers. The purple line shows results from an SPF, FV- RM.

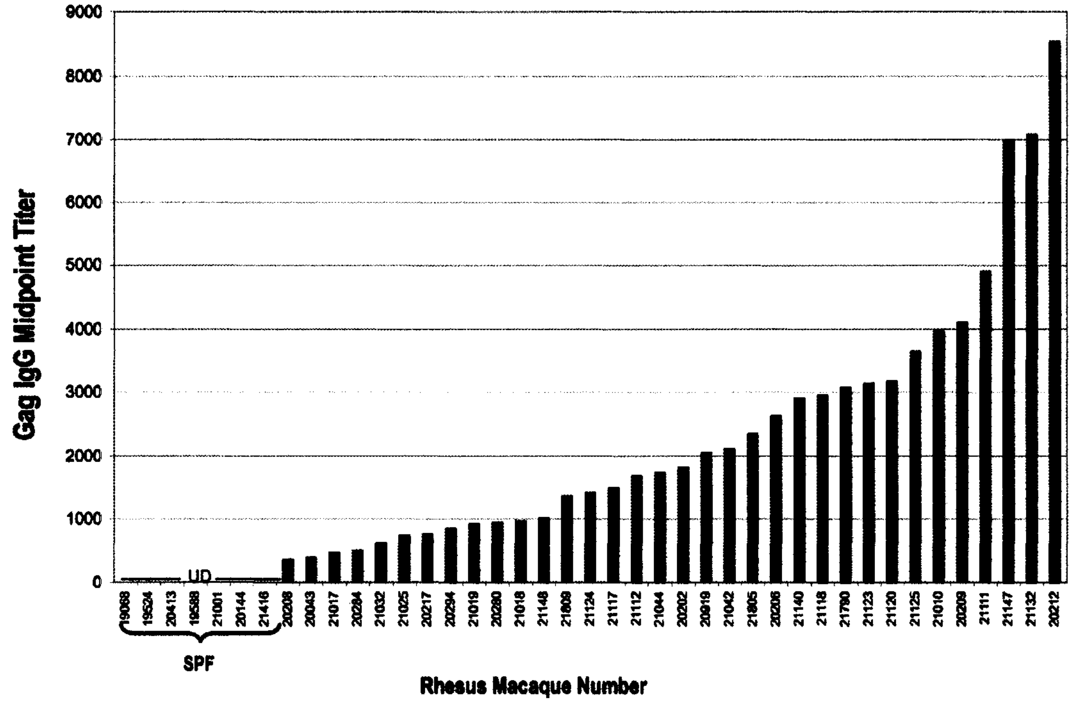


Figure 2.4. FV Gag-specific IgG in rhesus macaques.
Each bar represents the midpoint titer determined for plasma from an individual rhesus macaque. All seven SPF rhesus macaques had undetectable levels of antibodies. UD, undetectable.

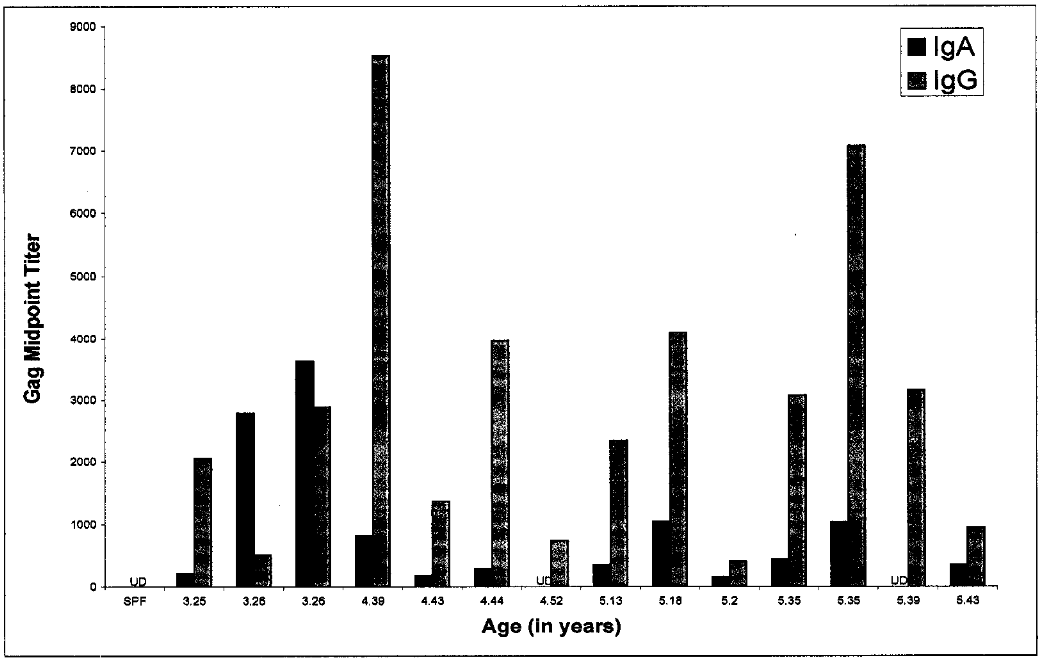


Figure 2.5. Relationship between age and levels of FV Gag-specific IgG and IgA.

Midpoint titers were determined for each RM plasma sample. UD, undetectable.

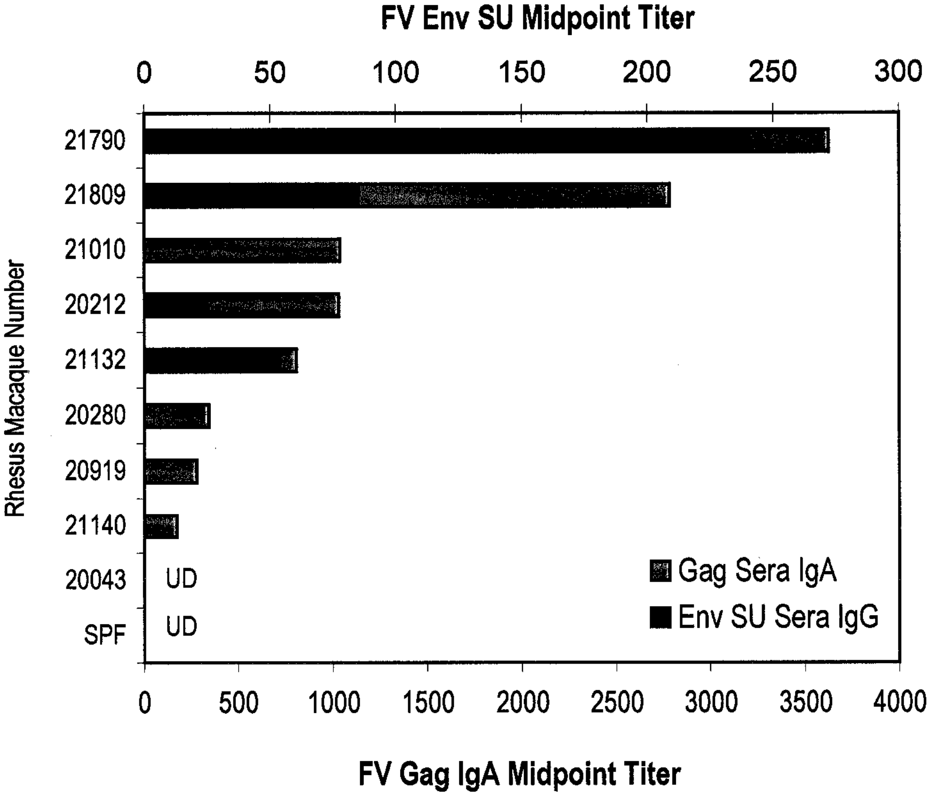


Figure 2.6. FV Env SU-specific IgG and FV Gag-specific IgA.
The bars show the Env SU midpoint titer (in black), and the Gag IgA midpoint titer (in grey) for each RM. Where no black bar is shown, the SU IgG titer was below the midpoint, unless noted as UD, undetectable.

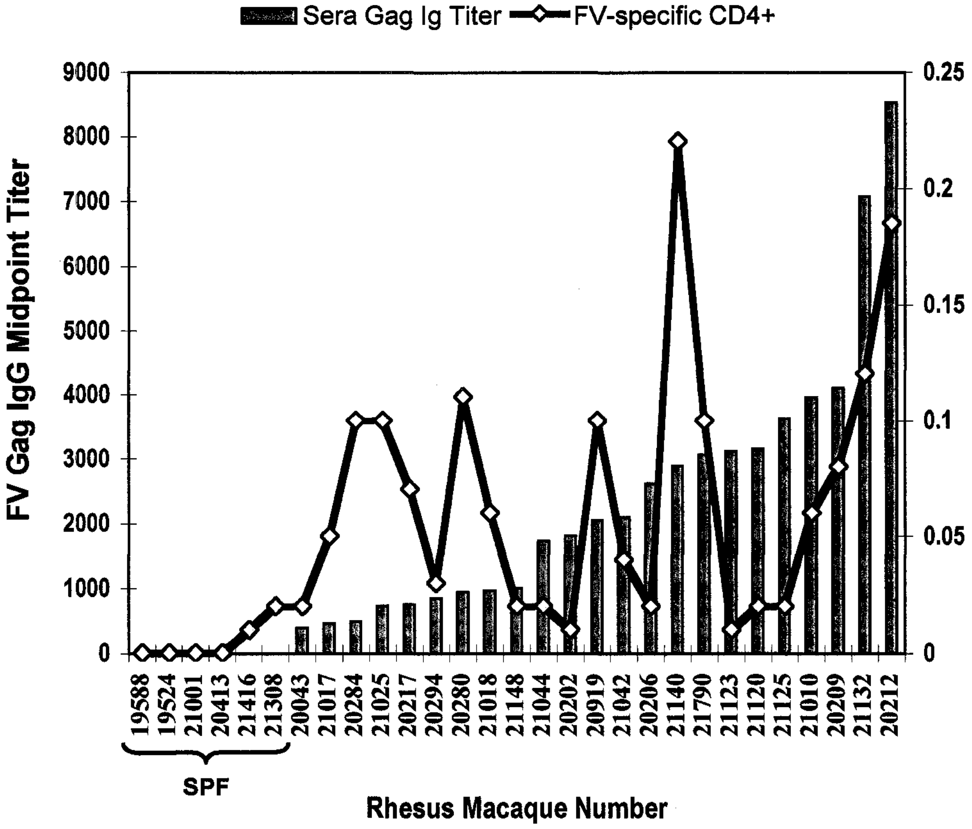


Figure 2.7. FV Gag-specific IgG and FV-specific CD4$^+$ T cells.
FV Gag-specific plasma IgG titers are represented in grey columns, and each point on the line denotes the percentage FV-specific T cells from the same RM. Animal numbers are listed on the x-axis; the first six animals shown are SPF animals.

Chapter III: The Tissue-specificity of Foamy Virus Infection in Normal and Immunosuppressed Hosts

Introduction

If FV infection were controlled by host immunity, then this would explain the dichotomous nature of FV infection in vitro and in vivo. Immune control has been evaluated for other viral infections by comparing the sites and levels of viral replication in normal and immunosupppressed hosts. However, since the tissue-specificity and levels of foamy virus replication in immunocompetent hosts have been only cursorily examined, these needed to first be established.

Previous studies of naturally infected NHP have characterized FV infection as primarily latent. In one study of naturally infected African green monkeys, a very low copy number of proviral DNA was detected in most tissues (37). FV RNA, indicative of viral gene expression and replication, was found exclusively, albeit sporadically and at a low level, in the oral mucosa (37). Yet FV has a high transmission rate, which is at odds with this characterization of FV infection as mostly latent. Therefore, I undertook studies to reexamine the levels and tissue-specificity of FV replication utilizing naturally infected rhesus macaques.

In macaques, immunosuppression by simian immunodeficiency virus (SIV) infection has been used to examine immune control of viruses including cytomegalovirus (CMV) (69, 70), and other herpes viruses. Depletion of $CD4^+$ T cells after infection with HIV or SIV is most pronounced at mucosal surfaces and occurs rapidly, within 10–17 days after infection, well before CD4$^+$ T cell loss is observed in blood [reviewed in (33)]. There is a selective loss of memory CD4$^+$ T cells (cells that recognize previously encountered antigens) from multiple tissues (94). In the gastrointestinal-associated lymphoid tissue (GALT) of the colon (81) and small intestine (160), the lung (119), and the vagina (161), 60–80% of memory T cells are depleted early after infection. The culmination of the subsequent chronic phase of immunodeficiency virus infection is associated with susceptibility to opportunistic infections.

Rhesus macaques (RM) infected with SIV are used as a model for AIDS. As most RM are naturally infected with simian foamy virus (SFV), I was able to study the effect of SIV-induced immunosuppression on FV levels using animals enrolled in ongoing SIV studies. I developed sensitive and quantitative polymerase chain reaction (PCR) assays for analysis of both RNA and DNA sequences and used these to examine the tissue specificity of FV infection (DNA) and replication (RNA) in rhesus macaques. These were compared to the patterns of FV replication in late stages of SIV infection when mucosal tissues were severely depleted of CD4$^+$ T cells. My results indicate that FV replication is readily detectable in oral swabs and several oropharyngeal tissues in both SIV$^-$ and SIV$^+$ animals. Most interestingly, SIV-induced immunosuppression expands the sites of FV replication to include the small intestinal jejunum, a site of CD4$^+$ T cell depletion, but not to other mucosal sites that are significantly depleted of CD4$^+$ T cells.

Methods

Animals and Virus

All animals used in these studies were rhesus macaques (*Macaca mulatta*) of Indian origin bred and housed at the Oregon National Primate Research Center. All studies were conducted in accordance with the standards of the Center's Animal Use and Care Committee and the "NIH Guide for the Care and Use of Laboratory Animals" approved protocols (60). Animals were euthanized in accordance with the Panel on Euthanasia of the American Veterinary Medical Association (115). The RM were naturally infected with SFV through interaction with SFV-infected animals in the facility, but the time since infection with FV was unknown for any individual animal. Both colony and specific pathogen-free (SPF) RM were identified as SFV^+ or SFV^- by screening for the presence of serum antibodies against the SFVmac Gag antigen, as detected by a quantitative ELISA assay (described in Chapter 2). Animals were infected with either SIVmac239, a CCR5 tropic virus, or SIVmac155T3, a CXCR4 tropic virus, as described by Picker et al. (119). SIV plasma loads were determined as previously described (83). Animals were considered immunosuppressed on the basis of presence of simian acquired immune deficiency syndrome (SAIDS)-related pathology. In all cases, there was >90% $CD4^+$ T cell depletion from mucosal sites, as measured by FACS phenotypic analysis of lymphocytes obtained by bronchoalveolar lavage (BAL), and from the jejunum (described further below). Absolute blood $CD4^+$ T cell counts were determined by obtaining total lymphocyte counts from whole blood and measuring the percentage of $CD4^+$ T cells in peripheral blood mononuclear cells (PBMC) (described below). Necropsy tissues were obtained from SIV immunosuppressed SFV^+ animals, and from normal SFV^+ animals that had been sacrificed for reasons unrelated to viral infections. The SIV-infected cohort was all male, with an average age of 5.1yrs; the SIV^- cohort consisted of 3 males and 2 females, with an average age of 3.2 yrs. Additional animals were used to obtain PBMC, blood, and buccal swabs. In these cases, all SIV^- animals were in good health, and SIV^+ animals were >70 days post infection (d.p.i) with pathogenic SIV.

Sample Collection

A set of tissues for each macaque was obtained at necropsy and sections were preserved in RNA stabilization solution, RNAlater (Ambion), according to the manufacturer's instructions, and stored at –20°C until nucleic acid isolation. Buccal swabs, composed of saliva and cellular material obtained by scraping the tongue, cheek, and gums, were collected with a polyester fiber-tipped swab (Fisher). PBMC were isolated by standard density gradient centrifugation of whole blood. Bronchoalveolar lavage (BAL) cells, a source of T cells from the lung, and jejunal lamina propria lymphocytes were isolated as described by Pitcher et al. (120).

Analysis of CD4$^+$ T cells

In order to determine the percentages of CD4$^+$ T cells in tissues and PBMC, bronchoalveolar lymphocytes, jejunal lamina propria lymphocytes and PBMC were phenotyped by flow cytometry. The lymphocyte preparations were stained with antibodies to the lymphocyte-specific marker CD3; and to the CD4 and/or CD8 T cell subset-specific molecules. The specific mAb clones used were α-CD3 (SP34), α-CD4 (L200), and α-CD8β (2ST8.5h7) that had been conjugated to one of the following fluorochromes: Pacific Blue, FITC, phycoerythrin (PE) - Texas Red; Texas Red, allophycocyanin (APC); APC-Cy7; or Am-Cyan (all obtained from BD Biosciences, Pharmingen, San Diego). Data were collected using a three-laser BD LSR flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego) and analyzed with FlowJo v. 6.1 (TreeStar, San Carlos, CA).

FV gag ELISA

The FV Gag IgG ELISA was performed as described in detail in Chapter II.

Isolation of DNA and RNA from PBMC, buccal swabs and tissues

Buccal swabs were placed directly into RLT buffer (Qiagen) containing 1% beta-mercaptoethanol (BME) (Sigma). The tube was briefly vortexed, the swab was removed from the solution, and the solution was then frozen at –80°C.

The swab solution was thawed at 37°C for 15 min immediately prior to RNA extraction. Total RNA was extracted according to standard protocol using the RNeasy Mini Kit (Qiagen). Tissues preserved in RNA stabilization solution were removed from the solution, minced and placed in either RLT buffer (Qiagen) with 1% BME for RNA extraction, or in ATL buffer (Qiagen) for DNA extraction. For DNA and RNA isolation, tissues were homogenized in the presence of 2.5 mm zirconia beads (Biospec, Inc.). Homogenization was performed using a Mini Bead Beater-8 (Biospec, Inc.) for 3 homogenization cycles at 3 min per cycle. Tissue homogenates were then removed from the beads. Total DNA and RNA were extracted from the tissue homogenates as well as from PBMC using the DNA Mini (Qiagen) and RNeasy Mini Kits (Qiagen), respectively. DNA and RNA were quantified by spectrophotometry. DNA preparations were treated with RNAse A according to the manufacturer's instructions (Qiagen). RNA preparations were treated with RNase-free DNase (Promega) at 1u/μg for 2 hours at 37°C, and then for 15 min at 65°C to heat-inactivate the enzyme.

Primary foamy virus isolation and sequencing of the FV gag gene

PBMC isolated from eight FV^+ rhesus macaques were co-cultured with telomerized fibroblasts (TF) cells, a SFV^- rhesus macaque fibroblast cell line generated by expression of the human telomerase protein in neonatal RM fibroblasts (74). The PBMC were treated with IL-2 as previously described (139), prior to co-culture. Infected TF cells were maintained until significant cytopathic effects (CPE) were observed. The cells were used to obtain virus as described by Yu et al. (170). Cell-free viral preps were used to infect fresh TF cells, until 80–90% CPE was observed. Cells were then lysed, and genomic DNA was isolated using the DNA Mini Kit (Qiagen). The complete 1.9 kb *gag* gene was PCR amplified from DNA with primers ATGF1 and R1, and primary isolates were sequenced using eight primers spanning the *gag* gene. The sequencing primers were as follows: ATGF1, ATGGCAGCAATAGAAGGTGATTTAG; R1, GTTCCCTT-GATTTCCGCTTCCAGAG; 500F3, CCAGTTGCAGGTACTCCACCTCC; 1500R3, GGCTTTGTCCCC-TGGCGTTCAAGC; 1600F4, GGAAGAGGTGCTCGGGGC; 200R4, GCTTCTAGAG-TAAACATGACTGCTGGATTAGC; 1316F, CTGGACAA-GCTGTAGTTACTGCTAT-GC; 656R, GGTGAAGAAGCAGCAGCAGGTTG. A contiguous *gag* sequence for each isolate was generated with Sequencher version 4.5 (Applied Biosystems), and the complete *gag* nucleic acid and predicted protein sequences were aligned using Clustal X, version 1.83.

Quantification of FV DNA and RNA

Quantitative PCR assays for DNA (qPCR) and RT-PCR for RNA (qRT-PCR) were performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Primers and probes were designed to detect an 80bp highly-conserved nucleic acid binding region of the *gag* gene of the primary isolates (149, 171). This region was completely conserved among the eight primary isolates whose sequences were obtained. The primers used were *gag*1759F, ACGAACATCTGGTGCGGG and *gag*1835R, CTGCGTTTCCACCAGCTGA, and the probe, *gag*1789 6-carboxyfluorescein (FAM)-AGGAAGAGGGAACCAAAACCGAAACCA-6-carboxyltetramethylrhodamine (TAMRA). The qPCR conditions used were as follows: 95°C for 1 min; 95°C for 15 sec; 60°C for 1 min, for 45 cycles. The qRT-PCR conditions used were as follows: 48°C for 30 min; 95°C for 10 min; 95°C for 15 sec, 60°C for 1 min for, 40 cycles.

To standardize the qPCR, a 125bp region that included the PCR target sequence from one primary isolate was cloned into a PCR cloning vector, TOPO-TA (Invitrogen). Known amounts of the target sequence (from 10^1 to 10^7 copies) were added to 500 ng of human genomic DNA that had been quantified by the manufacturer (Promega) to generate DNA standard curves in every individual assay. In addition to a standard curve, each PCR run included a buffer-only and a SFV-negative control. DNA derived from PBMC or tissue samples was used at 500 ng (10^5 cell equivalents) per PCR reaction. The lower limit of detection of the qPCR was 10 copies per 10^5 cell equivalents.

A *c-myc* cellular DNA qPCR was done for each sample in every individual assay to normalize for cellular DNA content (*c-myc*F1, GCCCCTCAACGTTAGCTTCA, *c-myc*R1, CGCAGTAGAAATACGGCTGCA, probe, FAM-CAACAGGAACTATGACC-TCGACTACGACTCG-TAMRA). All assays included a *c-myc* standard curve generated by adding ten-fold dilutions of human genomic DNA to the PCR reactions, ranging from 10^1 to 10^5 copies.

Each PBMC DNA sample was tested in duplicate, in two or more independent PCR assays. Each tissue DNA sample was tested in duplicate, in three independent assays, and standard deviations were calculated. In the qPCR assays >90% of the samples tested in duplicate had <2-fold variability, while the maximum variability was 4.4-fold. All qPCR standard curves had correlation coefficients of ≥ 0.95.

To generate standards for the qRT-PCR, the PCR target sequence was subcloned from the TOPO-TA vector into the pNEB vector (New England Biolabs) with a T7 promoter inserted upstream of the cloning site so that RNA could be transcribed using T7 RNA polymerase. In vitro transcription was done using the Riboprobe Combination System according to standard protocols (Promega). Ten-fold dilutions (ranging from 10^1 to 10^7 copies) of the viral RNA sequence were added to 50 ng (10^4 cell equivalents) of RNA isolated from TF cells. Control reactions lacking reverse transcriptase were included in order to insure that there was no DNA contamination in the RNA preparations, and SFV negative RNA was used as a control in every individual assay. The lower limit of detection of the qRT-PCR was 50 copies per 10^4 cell equivalents.

RNA input was normalized by spectrophotometry as well as by a qRT-PCR for 18S RNA that was performed in every assay using a commercially available primer-probe set (Applied Biosystems). An 18S standard curve was generated in every assay by adding ten-fold dilutions (ranging from 10^1 to 10^4 cell equivalents) of RNA, quantified using spectrophotometry, to the 18S qRT-PCR reactions.

Buccal swab RNA was tested in duplicate, in two independent assays. RNA from tissues was tested in duplicate, in three independent assays, and standard deviations were calculated. Animals previously identified as SFV- were negative for SFV sequences by qRT-PCR. In the FV *gag* qRT-PCR assays, we found maximum variation in 12 replicates to be 1.5-fold, and for the 18S qRT-PCR, for samples run in 24 replicates, to be 1.7-fold. The correlation coefficient of all qRT-PCR standard curves was ≥ 0.95.

Statistical Analyses

Between-group differences in FV RNA levels were analyzed using the Mann-Whitney U test, with the exception of FV RNA levels in the tongue and DNA levels in the jejunum, which were evaluated using log-transformed data and an independent samples *t*-test. Prism v. 4 (Graph Pad Software) was used to perform statistical analyses.

Results

Sequence analysis reveals ≥ 90% sequence homology between primary isolates

I isolated SFV from eight RM that were housed at the Oregon National Primate Research Center, and performed sequence analysis as described in Methods. The *gag* gene sequences of the eight isolates were deposited in GenBank under the accession numbers DQ120930 through DQ120937. At the nucleic acid level, three of the eight viral *gag* genes were identical, while the remaining five were 90–98% homologous, as shown in a percent identity matrix (Figure 3.1A). All of the primary isolates were 84–85% identical to the *gag* gene of SFVmac (GenBank Accession # X58484). I utilized the sequence data to identify a region of the primary isolates that was identical. This region was contained within a glycine-arginine rich region, known as a GR box, previously identified as a highly conserved nucleic acid binding domain within the 3' region of the FV *gag* gene. We designed a probe and primers (Figure 3.1B) for quantitative PCR and RT-PCR that would amplify this region in the primary isolates, but not the lab-adapted SFVmac.

FV DNA is present at low copy number in PBMC and tissues from normal and immunosuppressed animals

I measured foamy viral DNA in PBMC from 11 SIV$^-$, immunocompetent RM by qPCR and found from 19 to 223 FV DNA copies per 10^5 cell equivalents of DNA (mean = 98) (Figure 3.2). PBMC obtained from 5 SIV-infected FV$^+$ RM had levels ranging from 38 to 141 FV DNA copies (mean = 83) (Figure 3.2). Thus, there are no significant differences in PBMC FV DNA levels between SIV-uninfected and SIV-infected macaques. Animals were screened for FV antibodies using a FV Gag ELISA assay, and, as expected, all seronegative animals were negative for FV DNA sequences (data not shown).

Next, I surveyed necropsy tissues from SIV-infected RM for FV DNA (Table 3.1). In the SIV$^+$ RM, severe CD4$^+$ T cell depletion was evident in mucosal sites such as the lung, as measured using bronchoalveolar lavage (BAL) samples. CD4$^+$ T cell depletion was >90% in the SIV-infected RM compared to immunocompetent animals, where BAL CD4$^+$ T cells are typically 25–60% of total lymphocytes (119). CD4$^+$ T cells in the peripheral blood were not as depleted, with the exception of RM SIV$^+$4, which had been infected with SIV for the longest time. CD4$^+$ T cell levels in the blood of normal RM range between ~600 and 2000 per μl of blood, with a mean of 1330 (135), whereas we found CD4$^+$ T cell levels of 70–415, with a mean of 323. All the SIV-infected RM were severely immunosuppressed based on the presence of at least one SAIDS-defining opportunistic infection (Table 3.1).

FV DNA was present in the majority of tissues examined from normal animals, similar to a previous finding in African green monkeys (37). We found levels ranging from 50 copies per 10^5 cell equivalents of DNA in the parotid salivary gland, to 10^4 copies per 10^5 cell equivalents in the tonsil and tongue. In immunosuppressed RM, we saw a similar distribution of FV DNA. In this study we did not specifically isolate high molecular-weight chromosomal DNA. Therefore, DNA extracted from infected cells might contain both integrated proviral DNA and viral genomic DNA. Our findings indicate that FV DNA, either in a proviral or genomic form, is present in PBMC and diverse tissues in both immunocompetent and immunosuppressed RM.

FV RNA is consistently detected at high levels in the oral cavity

The difficulty in detecting infectious virus in FV-infected animals suggests that most tissues harbor only latent provirus. In order to determine which, if any, of the tissues containing FV DNA are permissive for FV replication, we used qRT-PCR for viral *gag* RNA expression as an indication of viral replication. Since we used primers in the *gag* gene, our measurements include both genomic RNA and *gag* transcripts. Blood, buccal swabs and some necropsy tissues of normal and SIV-infected RM were examined. FV RNA was not detected in PBMC from nine immunocompetent and nine SIV-infected RM. This finding is not unexpected (163), and confirms that blood is a site of FV persistence but not of replication.

A previous study showed that FV infection of NHP leads to only very limited replication, even in the oral mucosa, which is not consistent with the observed efficient transmission (37). To determine levels of FV RNA in the oral cavity of the RM, I used oral swabs, a non-invasive, accessible source of saliva and cellular material. We evaluated 12 SIV$^-$ and 12 SIV$^+$ FV seropositive RM and found FV RNA in all of the FV$^+$ animals tested, but not in the animals which were FV$^-$ by ELISA (data not shown). Viral RNA levels in the oral cavity ranged from 1.4 x 10^4 to 4.7 x 10^8 copies per 10^4 cell equivalents of RNA (Figure 3.3). Such consistent detection of high levels of FV RNA in the oral cavity is consistent with the high rate of FV transmission amongst NHP.

If CD4$^+$ T cell-dependent mechanisms limit FV replication, then viral RNA levels should increase following SIV-induced immunosuppression. However, FV load in the SIV$^-$ population (mean = 7.1 x 10^7) was more than an order of magnitude greater than that of the SIV$^+$ population (mean = 4.3 x 10^6) (Figure 3.3). Thus, there is a statistically significant trend toward lower RNA levels in immunosuppressed animals ($p = 0.088$; Mann-Whitney U test), which is not consistent with a role for CD4$^+$ T cells in limiting viral replication in the oral cavity. However, other factors may be confounding our interpretation; for example, CD4$^+$ T cells could be permissive for FV replication, or they could spread FV to other cell types that are permissive for viral replication.

Multiple oropharyngeal tissues are permissive for FV replication in both normal and SIV-immunosuppressed hosts

We screened a large number of tissues obtained at necropsy from five SIV$^-$ RM and from the four SIV$^+$ immunocompromised RM described in Table 1. In samples from the SIV$^-$ animals, FV RNA was consistently found in multiple oropharyngeal-associated tissues such as the pharyngeal epithelium, tongue, and tonsils (Figure 3.4, Table 3.2). All five SIV$^-$ animals had detectable FV RNA in at least one of the oral tissues, although there was variability in the specific site of viral replication. The tongue was positive in all of the animals (Figure 3.4A), and the buccal epithelium (Figure 3.4 B), pharyngeal epithelium (Figure 3.4C), and tonsil (Figure 3.4D) in most of the animals. Only 1 animal had detectable RNA in the lung (Figure 3.4E). Interestingly, FV RNA was undetectable in both the parotid and submaxillary salivary glands. This indicates that FV enters saliva distally from the site of its secretion. All other tissues examined (Table 3.2) had undetectable levels of FV RNA.

A similar pattern was seen in the four SIV-immunosuppressed animals (Figure 3.5). In these animals, the buccal epithelium (Fig. 3.5A) and tongue (Figure 3.5B) were most consistently permissive, with two animals having RNA in the pharyngeal epithelium (Figure 3.5C) and lung (Figure 3.5E) and only one having FV RNA in the tonsil (Figure 3.5D). All of the SIV$^+$ immunosuppressed RM had FV RNA in at least one oropharyngeal tissue. Comparing FV RNA levels in oropharyngeal-associated tissues from SIV$^-$ and SIV$^+$ animals, we observed a statistically significant trend towards lower FV RNA loads in the SIV$^+$ RM. This trend was most evident in the tongue ($p = 0.13$; independent samples *t*-test; data not shown).

FV replication is extended to the small intestine with SIV induced immunosuppression

A striking finding was that the small intestine from all four of the SIV$^+$ RM contained detectable levels of FV RNA —between 50 and 4.3 x 10^5 FV RNA copies per 10^4 cell equivalents)—in contrast to small intestine from all of the five immunocompetent RM, where FV RNA was below the level of detection (<50 copies) (Figure 3.6). This difference is statistically significant (p=0.016; Mann-Whitney U test).

The jejunum samples I analyzed came from the middle region of the jejunum (jejunum 2). To determine whether FV RNA is absent from all regions of the jejunum in normal animals, I examined all regions (jejunum 1, 2 and 3) obtained from one SIV$^-$ animal, and detected no FV RNA in any region (data not shown). I evaluated CD4$^+$ T cells in the jejunum of two of the SIV$^+$ RM, and found 0.28% and 1.23% CD4$^+$ T cells (Table 3.1), in contrast to 12% in a normal young adult animal. Other researchers have reported normal levels of 5%–45% in juvenile RM, and 30%–50% in mature adult RM (162). Therefore, these SIV$^+$ animals are significantly depleted of CD4$^+$ T cells in the jejunum, with ≤10% of normal levels. Additionally, I found detectable FV RNA, 1.8 x 10^3 copies per 10^4

cell equivalents, in the mesenteric lymph node (MLN) of one SIV^+ animal (data not shown). Interestingly, this was observed in the RM with the lowest level of $CD4^+$ T cells, SIV^+4 (Table 3.1).

Discussion

I have performed the first systematic and quantitative evaluation of the tissue-specificity of FV replication in non-human primates and have found that FV infection is characterized by high levels of viral RNA (up to 10^4 copies/cell) in the oral cavity. These results are consistent with the reported high rate of FV transmission between natural hosts, and the frequent acquisition of FV by humans through monkey bites. However, the actual number of virions that this number represents is unknown, since the FV RNA copy number we determined includes both *gag* mRNAs and viral genomic RNA. I found that the tongue and oral secretions were most consistently permissive for FV replication, with oral swabs containing superficial cells and saliva having the highest levels. The lack of FV RNA in the salivary glands is consistent with FV entering saliva from infected cells in the oral cavity. Tissue culture studies have shown that FV are highly cell associated and that high levels of replication invariably leads to cell death (172, 173). Extrapolating from in vitro studies to the in vivo situation leads us to hypothesize that in vivo, FV also kills its target cells, which are shed into saliva in the oral cavity. Since replication is not associated with pathogenesis in vivo, it is reasonable that the permissive cells, containing cell-associated virus, have a naturally high turnover rate and even when not infected are sloughed off into saliva.

Contrary to expectation, I saw a small decrease in FV RNA levels in the oropharyngeal tissues in the late stages of SIV immunosuppression; this finding is inconsistent with T cell immune control of FV infection in these tissues. This decrease could be caused by SIV induction of innate immunity, such as Type I interferon (IFN) production (4, 11), or adaptive immunity, such as production of IFN-γ. FV infection does not normally induce IFN production, although FV are sensitive to IFN-α, -β, and -γ (39, 123). Alternatively, this decrease could be due to SIV-induced loss of a cell type that normally supports FV replication. Primate FV infects all cell types in vitro and is most cytopathic in cells of epithelial and fibroblast origin. However, in leukocyte-derived human cell lines it is generally persistent, producing very low levels of virus. For example, the titer of FV produced by clonal lines of infected Jurkat T cells is less than 10^2 infectious virions in 2 X10^6 cells (99) and because so few cells are productively infected, no cytopathic effects are seen. The small amount of virus detected is likely to be produced after spontaneous activation of very small numbers of cells. Therefore, it is possible that in vivo CD4$^+$ T cells, or another cell type lost after SIV infection, are persistently infected with FV, and activation of a subset leads to the levels of viral RNA seen. Until the permissive cell type in vivo is known (described in Chapter IV), detailed understanding of the mechanism is impossible.

Our most striking finding is the extension of FV replication to the jejunum of SIV-immunosuppressed animals. For two SIV$^+$ animals examined, the level of CD4$^+$ T cells in the jejunal lamina propria was significantly depressed, less than 10% of those observed in normal RM (Table 1). However, the small intestine is only one of many mucosal tissues that are severely depleted of CD4$^+$ T cells after SIV infection. For example, although significant CD4$^+$ T cell depletion has been demonstrated in the colon of SIV immunosuppressed RM (81), we did not observe FV RNA in this tissue. Thus, our results are not consistent with general control of FV replication by CD4$^+$ T cells, but suggest that SIV-induced changes that are unique to the small intestine account for the extended tissue tropism of FV replication.

A question of interest is whether there is increased migration of FV-infected cells to the jejunum from permissive tissues after SIV infection, which would be consistent with the lowered FV RNA levels we find in the oral mucosa. One way to address this is by measuring FV DNA levels in the jejunum. We did quantify FV DNA loads in the jejunum of the SIV$^-$ and SIV$^+$ cohorts, and found significantly higher levels of FV DNA in the SIV-infected macaques. However, because the functional genomes in virions are DNA (174), we cannot distinguish proviral DNA from viral genomic DNA. Therefore, higher DNA levels in the SIV$^+$ jejunum may result from increased viral transcription and replication and not necessarily from the accumulation of latently infected cells.

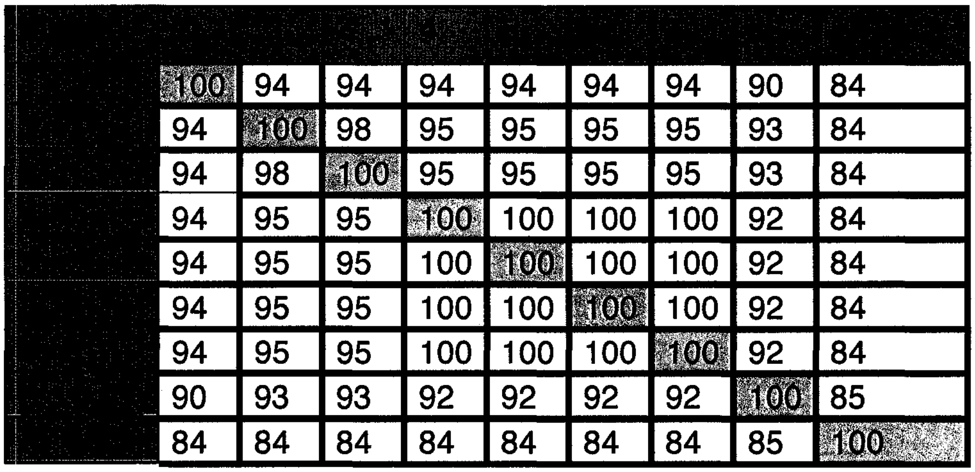
SIV infection is known to induce dysregulation of intestinal cytokines and growth factors, inducing hyperinflammation, maladsorption, and changes in the mucosal barrier in the small intestine (41). This altered tissue microenvironment could lead to induction of latent proviruses and drive FV replication at this ectopic site. While the Tas transactivator protein of PFV (prototype FV of chimpanzee origin) has been shown to transactivate genes under the control of the HIV-1 LTR (71), transactivation of the FV LTR by HIV Tat has not been examined. Cytomegalovirus (CMV), which establishes an opportunistic infection associated with AIDS-related immunosuppression, is present in the intestine of HIV-infected people and induces expression of latent HIV genomes in this tissue (146). Recently, rhesus macaque CMV (RhCMV) has been described in the gastrointestinal tract of SIV-infected macaques (59), and all of the SIV-immunosuppressed RM in our study had a RhCMV-induced pathology (Table 1). Thus, it is possible that RhCMV is involved in transactivation of latent FV proviruses in the intestine.

A current prominent model of HIV and SIV pathogenesis proposes that, subsequent to massive depletion of mucosal memory $CD4^+$ T cells, there is chronic immune activation, and opportunistic agents may contribute to this state (15, 16). With immunosuppression, persistent viruses such as CMV are transformed into opportunistic pathogens. Foamy virus, while apparently apathogenic in immunocompetent hosts, has now been shown to expand its replication to include the small intestine in immunosuppressed hosts. In this context, it is interesting to note that 'generalized giant cell disease' was observed in a number of macaques infected with SIV (82). Giant cells were seen with multiple nucleii in various tissues, including the small intestine. These were attributed to macrophages, but also resemble FV-induced syncytia, although it is not known whether these cells are producing FV. It is possible that FV plays a role in the pathology in the gut seen during progression to simian AIDS, and contributes to dysregulation of the immune system.

Finally, it is interesting to note that, in vitro, T cells that are persistently infected with FV are more permissive to HIV infection than uninfected cells (136). The differences in disease progression after SIV infection of FV^+ and FV^- animals have not yet been evaluated. Because monkeys infected with SIV and SHIV (SIV–HIV hybrid viruses) are widely used as a model for pathogenesis of HIV, and a majority of these animals are also infected with FV, the contribution of foamy viruses to simian AIDS needs to be more carefully examined.

Thus far, there is no evidence for human-to-human transmission, although only small number of cases have been examined (52). It remains an outstanding question whether this lack of transmission is due to differences in patterns of FV replication in the natural host compared to zoonotically infected humans, or to behavioral differences. It is not known whether foamy virus replication is as robust in the oral mucosa of human hosts. There are no reports of FV infection in immunocompromised people, but the status of FV in such individuals would be of great interest.

A.



100	94	94	94	94	94	94	90	84
94	100	98	95	95	95	95	93	84
94	98	100	95	95	95	95	93	84
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94	95	95	100	100	100	100	92	84
94	95	95	100	100	100	100	92	84
94	95	95	100	100	100	100	92	84
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84	84	84	84	84	84	84	85	100

B.

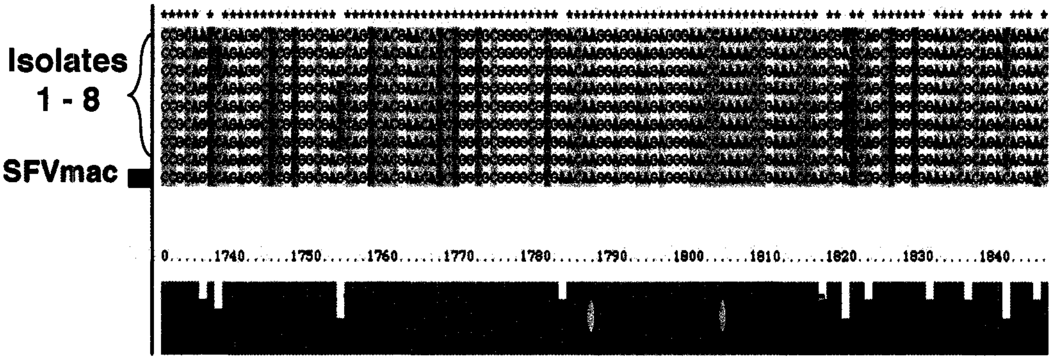

Figure 3.1. Sequence similarity between the *gag* gene from eight primary FV isolates and SFVmac.
A. Percent identity matrix. The isolate numbers are noted, and numbers in white boxes or gray boxes across the diagonal represent the percent nucleic acid similarity in the viral *gag* sequences. B. The qPCR design within the highly homologous GR box region at the 3' end of *gag*. Forward (F) and reverse (R) primers and probe designed for this research are indicated. The reverse primer was designed to be homologous to the eight isolates but to differ from SFVmac. The numbers along the ruler indicate the base of the FV *gag* gene. The depth of the white bars below the ruler represents variation at that base position.

Table 3.1. Status of SIV$^+$ animals at necropsy.

*DPI, days post infection. †SIV load is given as SIV gag RNA copies per ml of plasma. ‡ Absolute CD4+ T cell counts per ul in the blood. § Percentage of CD4+ cells of total CD3+ lymphocytes in bronchoalveolar lavage lymphocytes. ¶ Percentage of CD4+ cells of total CD3+ jejunal lamina propria lymphocytes. $ Not determined.

Animal	**SIV strain**	**DPI***	**SIV load†**	**CD4 (blood)‡**	**%CD4**		**Opportunistic infection(s)**
					BAL§	**LPL¶**	
SIV$^+$ 1	mac239	554	5 X10^6	415	0.25	0.28	CMV, cryptosporidium
SIV$^+$ 2	mac239	491	3 X10^6	399	0.4	ND$	CMV
SIV$^+$ 3	mac155T3	380	6 X10^5	407	0.42	1.23	CMV, pneumocystis
SIV$^+$ 4	mac155T3	730	6 X10^5	70	0.10	ND$	CMV, cryptosporidium

Table 3.2. Tissue distribution of FV RNA in tissues from normal and SIV$^+$ immunosuppressed animals.

This table summarizes the data for the four SIV$^-$ animals in shown in Figure 4 and the five SIV$^+$ animals shown in Figure 5 with the exception of PBMC, which was evaluated from 8 normal and 8 SIV-infected RM. RNA was analyzed by qRT-PCR as described in Materials and Methods. A + indicates that at least one animal in the group had significant levels of RNA in the tissue. The lower limit of the assay was 50 FV *gag* copies per 10^4 cell equivalents of RNA.

Tissue	**FV RNA**	
	SIV-	**SIV$^+$**
Buccal epithelium	+	+
Pharyngeal epithelium	+	+
Tongue	+	+
Tonsil	+	+
Lung	+	+
Small intestine	-	+
Mesenteric lymph node	-	+
Parotid salivary gland	-	-
Colon	-	-
PBMC	-	-

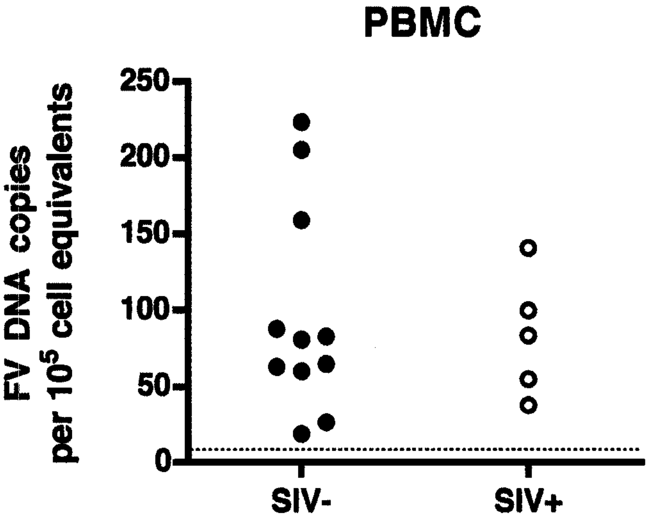

Figure 3.2. FV DNA loads in peripheral blood mononuclear cells of SIV-infected and SIV-uninfected rhesus macaques.

Each symbol indicates the mean normalized FV DNA level from two independent assays from an individual animal, run in duplicate. The lower limit of detection of the assay is indicated by the dotted line. All animals that were FV negative by ELISA had DNA levels below the level of detection, and are not shown.

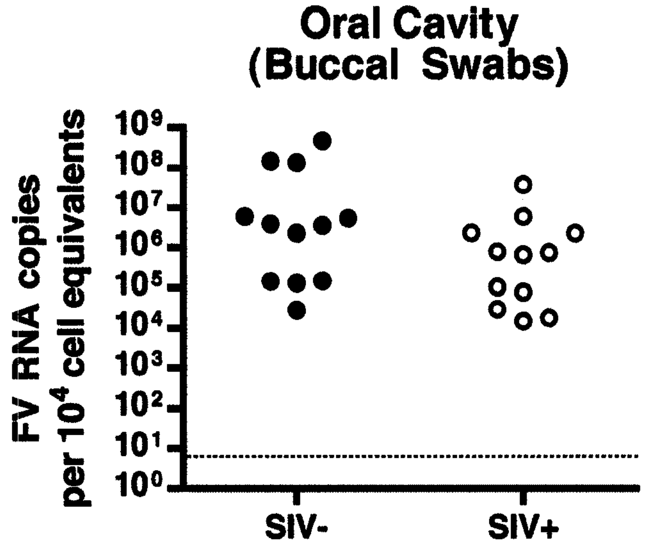

Figure 3.3. FV RNA loads in buccal swabs of SIV$^-$ and SIV$^+$ RM.

RNA levels from buccal swabs were normalized to cell equivalents using a qRT-PCR for 18S RNA. Each symbol indicates the FV RNA mean value from two independent assays, each run in duplicate, from an individual animal. The lower limit of detection of the assay is indicated by the dotted line. All animals that were negative by ELISA had RNA levels below the level of detection and are not shown.

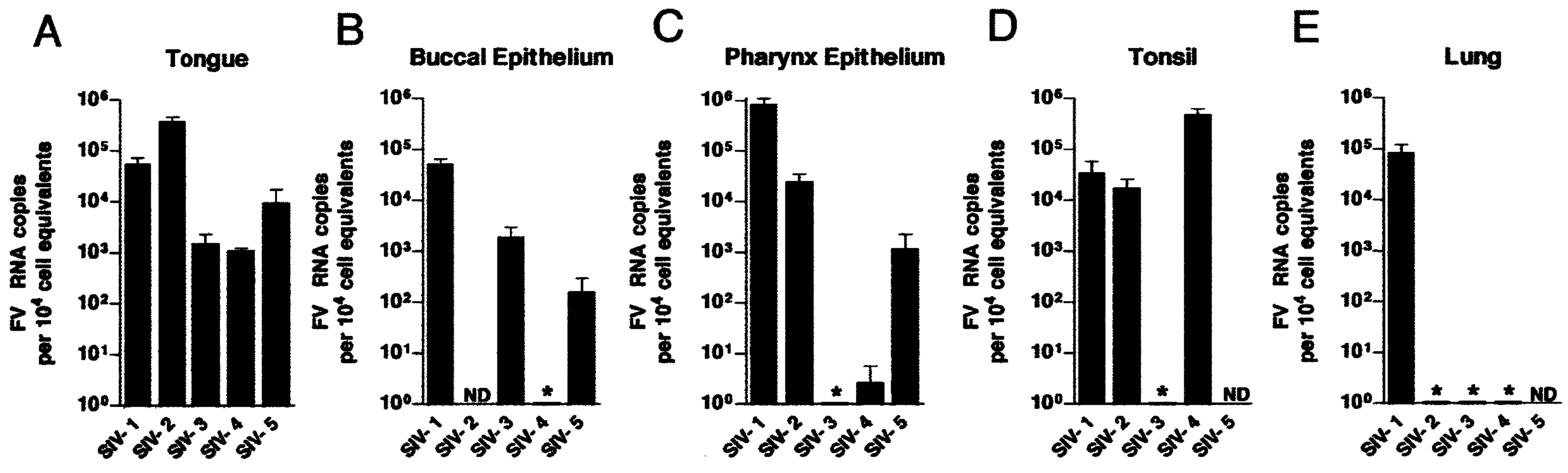

Figure 3.4. FV RNA levels in permissive tissues of normal, SIV- RM.
Results of qRT-PCR for FV RNA in tissues from five SIV$^-$ RM are shown. Each bar represents the mean viral load from three independent qRT-PCR for one animal. Viral loads were normalized to cell equivalents by qRT-PCR for 18S RNA. Error bars represent the standard deviation. All FV seronegative RM were PCR negative for FV RNA in all tissues examined. ND = not determined. * = undetectable.

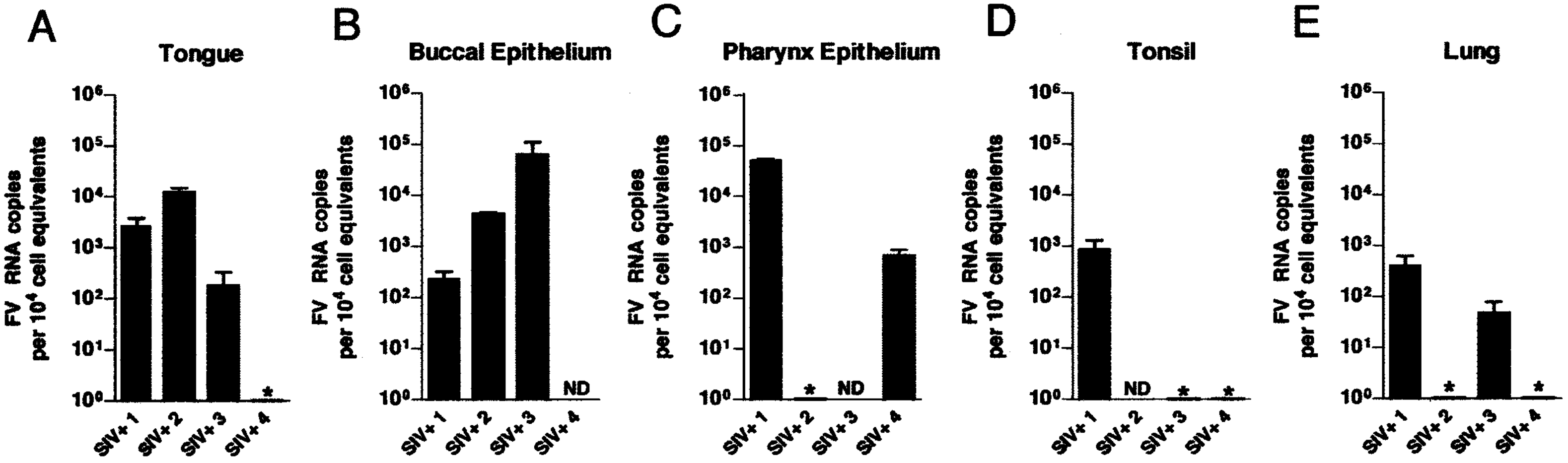

Figure 3.5. FV RNA levels in permissive oropharyngeal tissues of SIV$^+$ immunosuppressed RM.
Results of qRT-PCR for FV RNA in tissues from four SIV$^+$ immunosuppressed RM are shown. Analysis was done as in Fig. 3. Error bars represent the standard deviation. All FV seronegative RM were negative for FV RNA in all tissues examined by PCR. ND = not determined. * = undetectable.

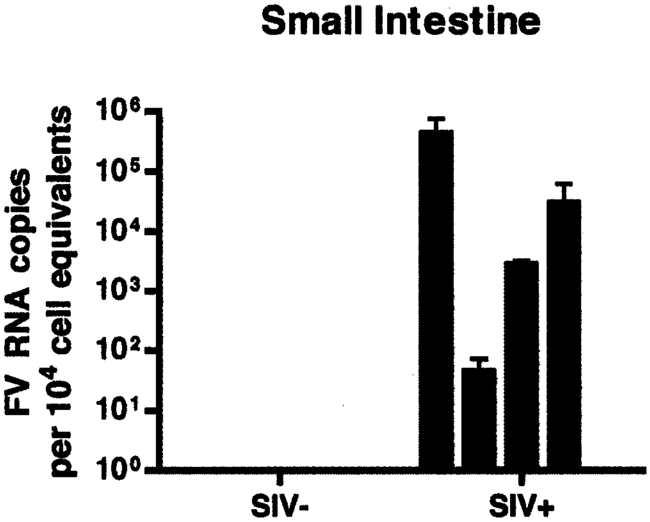

Figure 3.6. FV replication in the small intestine of SIV$^+$ immunosuppressed RM.

Results of qRT-PCR for FV RNA in four SIV$^+$ and five SIV$^-$ RM are shown. Each bar represents the mean viral load from three independent qRT-PCR assays for one animal. Viral loads were normalized to cell equivalents by a qRT-PCR for 18S RNA. Error bars represent one standard deviation.

Chapter IV: The Cell-Specificity of Foamy Virus Replication

Background

An outstanding yet fundamental issue is identification of the cell type that is permissive for FV replication in vivo. The oropharyngeal tissues permissive for FV replication in immunocompetent hosts are limited to buccal and pharyngeal epithelium, tongue, tonsil, and lung (at up to 10^3copies/cell), as determined by my research (Chapter III). These are all mucosal tissues, a tissue type which forms an interface between the environment and the animal, and is the site of encounters with environmental antigens. For example, the oral microbial community of humans includes over 300 bacterial species in addition to viruses and fungi (90). Thus, the oral mucosal tissues are the 'front-line' for microbial encounters and are characterized by more inflammatory cytokines and activated lymphocytes than the blood or secondary lymphoid tissues (118).

FV permissive tissues include those classified as keratinized mucosa (due to their outer cornified epithelial layer) such as the dorsal tongue, as well as nonkeratinized mucosa such as the buccal and pharyngeal epithelium (27, 31, 148). The oral mucosal epithelium is comprised of layers of squamous epithelial cells with an underlying basement membrane composed of basal cells, from which the more differentiated epithelial cells originate (104). Each stage of epithelial cell differentiation is marked by the expression of a unique collection of proteins, including well-characterized specific keratin pairs and transcription factors (27). The complete differentiation process from a basal cell to terminally differentiated, shed epithelial cell takes about seven days (28, 32, 104) (Beverly Dale-Crunk, personal communication).

The epithelium also contains tissue macrophages and dendritic cells, specifically Langerhans cells. Inflammation is associated with T cells and monocyte infiltrates scattered throughout the epithelium (32, 148). Below the epithelial basement membrane is the mesenchymal (connective) tissue, which contains mostly fibroblasts, leukocyte-derived cells and other connective tissue-associated cells such as endothelial cells, which line the venules and arterioles (31, 147). The mesenchymal tissue adjacent to the basement membrane includes the lamina propria, a loosely organized lymphoid structure (31, 104). Thus, within the oropharyngeal tissues, there are many possible cell types that might be permissive for FV replication.

Not only is the cellular specificity of FV replication unknown, but also the means of FV transmission between hosts and spread within a host are unknown. Further, it has not yet been proved that saliva is the mode of transmission. It may be that FV infection initiates in the oral cavity, and then disseminates within a host. Alternatively, it may be that peripheral sites initially become infected, and infected cells then traffic to multiple tissues, including the oral cavity, the unique qualities of which allow support of viral replication. Recent data support the latter model. Unpublished studies on early juvenile FV seroconvertors indicate that

PBMC are latently infected before FV RNA is detectable in the oral cavity (168). In two recent published studies, whole blood was transfused from FV$^+$ macaques into FV-uninfected macaques, resulting in acquisition of FV infection by the naïve animals (18, 73). One of these studies reported that PBMC were FV DNA$^+$ at 8 weeks p.i., well before FV RNA was detectable in the saliva at 29 weeks p.i. (18). However, this study utilized transfusion to transmit the virus, which may not represent the natural mode of transmission or intrahost spread. In animal models and zoonotic infection, acquisition of FV infection has been associated with biting or licking. Thus, during natural infection the virus may be transmitted by saliva, entering a new host at a heterologous (mucosal) site, such as the skin, or via the oral cavity, but not likely through the blood.

I previously proposed (Chapter III) that sloughed-off cells of the oral cavity are the cells permissive for FV replication, and may be the cellular source of transmitted virus. Evidence for this proposition includes the high levels of viral RNA in swab material, which includes saliva and sloughed-off cell fractions; the lack of viral replication in salivary glands, and the highly cell-associated character of FV in vitro. In vitro, fibroblasts and epithelial-derived cell lines are permissively infected while most leukocyte-derived cells are infected until activated, when they become permissive (99). In vivo, PBMC are latently infected at low levels (Chapter III), and the CD8$^+$, CD4$^+$, CD14$^+$, and CD19$^+$ subsets of PBMC have all been found to harbor FV DNA (163). However, in vivo, no viral RNA could be detected in the blood or in PBMC (Chapter III). Taken together, these data indicate that in vivo, migrating leukocyte-derived cells are likely to be involved in the dissemination of FV to widespread tissues, either as the source of the detectable FV DNA, or by producing a low and undetectable level of activation-induced virus production, which then results in a low level of viral infection within a tissue. However, it is not known what causes significant viral replication to occur exclusively in oropharyngeal sites, and not in other tissues where viral DNA is found. The SIV-induced transformation of the jejunum into a permissive site for FV replication, as discussed in Chapter III, may provide insight into what microenvironmental conditions support viral replication. But, until the cell type permissive for FV replication in the oropharyngeal tissues has been identified, it is difficult to choose between the many theoretically possible trajectories.

In situ hybridization (ISH) has become the gold standard to determine viral tropism and to localize viral replication within a tissue. In seminal papers by Haase and colleagues (13, 14, 45) ISH was used to identify sites of latency and replication of the equine retrovirus, visna virus. ISH has also been utilized to localize measles virus (46), HBV (10), EBV (40, 76), and Dengue virus (62) amongst others. More recently, in human and NHP models, ISH has been used in conjunction with immunohistochemistry (IHC) to characterize cell types infected with human or simian immunodeficiency viruses (176). Utilizing dual IHC and ISH, a number of studies have elaborated the mechanism of HIV- or SIV-induced CD4$^+$ T cell loss in mucosal sites such as the ileum and colon (17, 81).

In order to identify the tropism of FV replication, I developed an ISH assay for FV RNA to localize viral replication within tissues to the cellular level. I utilized IHC as a complementary technique to confirm what cell type was permissive for FV replication in vivo. This has allowed us to begin to develop a model of FV infection that explains both viral replication in the oropharyngeal tissues and the ubiquitous in vivo presence of provirus.

Methods

Animals and Tissues

Rhesus macaques (RM) of Indian origin between the ages of 2 and 21 years, of either sex, were bred and housed at the Oregon National Primate Research Center (ONPRC). All studies were conducted in accordance with the standards of the Center's Animal Use and Care Committee and the approved protocols in the *Guide for the Care and Use of Laboratory Animals* (115), as previously described (Chapters II and III). Animals were screened for FV by the presence of FV RNA in oral swabs (described in Chapter III). Tissues obtained at necropsy, including the buccal and pharyngeal epithelium, tongue, jejunum, and tonsil, were either placed in 10% neutral buffered formalin (NBF) for in situ hybridization and immunohistochemistry, or in RNAlater (Ambion) for RNA isolation and quantitative RT-PCR for FV *gag* RNA (described in Chapter III).

Cells

Telomerized fibroblasts (TF), an RM fibroblast cell line expressing human telomerase (74) were derived from an FV$^-$ neonatal RM and were grown in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (Cellgro). When TF were ~50% confluent, they were infected with FV isolate 5, a primary FV strain that was isolated from an ONPRC RM using the method described in Chapter III. When both uninfected and FV-infected TF were confluent, and when the FV infected TF cells exhibited syncytia but not significant cell death, the cells were harvested by scraping with a rubber scraper and spun at 1200 rpm for 10 min. The cell pellets were washed in PBS and treated with 10% NBF for 18 hrs. The fixed cell pellets were then embedded in paraffin.

Probe generation

Sense (s) and antisense (as) regions of FV*gag* and β-actin RNA were generated to use as RNA probes. First, a 679-base region of FV*gag* (bases 1253 to 1932) of FV isolate 5, Genbank accession number DQ120934, was PCR-amplified from cDNA prepared by oligo dT priming of RNA extracted from FV isolate 5-infected TF, using primers (FV*gag*1316_F1 CTGGACAAGCTGTAGTTACTGCTAT; FV*gag*R1, GTTCCCTTGATTTCCGCTTCCAGAG). A 791-base region (bases 10 to 801) of β-actin (similar to Genbank accession number DQ894128) was PCR-amplified from cDNA prepared by oligo dT priming of RNA isolated from uninfected TF, using primers (bactin-F GATATCGCCGCGCTCG-TCGTCGAC; bactin-R, CAGGAAGGAAGGCTGGAAGAGTGC). The PCR-amplified products were cloned by standard methods into the pCR4-TOPO vector (Invitrogen), downstream of the T7 RNA polymerase priming site. Clones with the FV*gag* and β-actin regions in both sense and antisense orientations were verified by both sequencing and restriction digestion. Each construct was linearized with Pme I (NEB), as confirmed by gel analysis, and used for in vitro transcription according to manufacturer's instructions for the Riboprobe System (Promega). Briefly, 1 μg of each template was added to a reaction containing transcription buffer (40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.05% Tween-20); 10 mM DTT; 0.5 mM each of rATP, rGTP, rCTP, and 0.005 mM rUTP; 125 uCi ^{35}S UTP; 40 units of RNasin ribonuclease inhibitor, and 20 units T7 RNA polymerase. The reaction was incubated at 37°C for 75 min. After incubation, one unit of RQ1 DNase was added and incubated further for 15 min at 37°C to degrade the template DNA. The reactions were purified by using the RNEAsy mini kit (Qiagen) and eluted in 100 μl of RNase-free water. The RNA was precipitated by adding 3 μl of 7.5M LiCl (Ambion), 1.5 μl of linear acrylamide (Ambion) and 250 μl of ethanol, and placed at –20°C overnight. The reactions were centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant removed. The pellets were washed with 70% ethanol and centrifuged for an additional 10 min, the supernatant was removed, and the pellets were allowed to air dry. The pellets were resuspended in RNase-free water with 10 mM DTT. Standard scintillation counting was used to determine radioactive incorporation into each ^{35}S-labeled probe.

In situ hybridization for FV RNA

Formalin-fixed, paraffin-embedded TF and tissues were cut into 4-μM sections, placed on positively charged slides (Fisher), and baked at 56°C for 30 min. The paraffin-embedded sections were deparaffinized by immersing them 3× in RNase-free xylene for 10 min, then 3× in 100% ethanol for 2 min, then 2× in 95% ethanol for 2 min, and finally 1× in 70% ethanol for 2 min. Sections were then washed 3× in 0.5X SSC for 30 sec. All slides were treated with 25 μg/ml Proteinase K (Sigma) at 37°C, the tissues for 30 min and the TF cells for 15 min. Subsequently, the slides were washed 3× in 0.5X SSC and dehydrated by subsequent immersion in graded ethanols: 1× in 75% ethanol, 2× in 95% ethanol, and 2× in 100% ethanol. A prehybridization solution of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris, 5 mM EDTA, 1X Denhardts, 10% dextran sulfate, 10 mM DTT, and 500 μg/ml yeast tRNA was placed on tissue sections at 100 μl/section, and the sections prehybridized for 2 hrs. at 50°C. The sense and antisense FV*gag* and β-actin probes were denatured by incubation at 95°C for 3 min, cooled on ice, then added to fresh prehybridization buffer to yield a hybridization solution with 750,000 cpm of ^{35}S-labeled probe per 50 μl. The hybridization solution was added to the sections at 50 μl per slide, and incubated for 18 hrs. at 50 °C. After hybridization, tissues were washed 3× in 2X SSC for 30 sec, then treated with RNase A (Sigma) at 20 μg/ml for 30 min at 37°C to degrade the residual single-stranded RNA probe. Tissues were washed again, 3× in 2X SSC for 30 sec, and then washed 3× in 0.1X SSC/0.5% Tween-20 for 45 min, at 50°C degrees. Tissues were washed 3× in 2X SSC for 30 sec and dehydrated in graded ethanols as follows: 2 min each of 50% ethanol with 0.3M ammonium acetate, 70% ethanol with 0.3M ammonium acetate, 90% ethanol with ammonium acetate, and 2× in 100% ethanol. Slides were allowed to air-dry, and dipped in Kodak NTB2 photographic emulsion that was diluted 1:1 with ddH_2O. Slides were allowed to air dry in a dark room, then placed in slide boxes which were sealed with electrical tape and wrapped in aluminum foil. Slides were left in the dark at 4°C. Each ISH run included duplicate slides for two exposure times. After 12–14 days, slides were developed to determine whether the exposure time was sufficient. The second exposure was empirically determined to be optimal after 18 days incubation at 4°C. Slides were developed by incubation in Kodak D-12 developer for 5 min at 12–15°C, rinsed in ddH_2O, and then incubated in Kodak rapid fix for 5 min at 12–15°C. Slides were then stained with hematoxylin and eosin, and dehydrated by treatment with the following series: 2× 95% ethanol; 2× 100% ethanol; 3× xylene substitutes. The tissue sections were then coverslipped with Histoclear mounting solution (Shandon). Slides were viewed either with an Olympus BH-2 microscope or with a Nikon eclipse E800 microscope. Images were taken with either a high-resolution digital camera (Olympus), or with a Cool Snap color video camera (Photometrics) using Metamorph software, version 5.0 (Universal Imaging Corporation).

Each tissue or cell sample was hybridized with the sense and antisense FV*gag* and β-actin probes. The ISH assay was first optimized for the TF controls, and all samples that demonstrated specific signal were confirmed in at least three independent ISH assays, with two time points per assay.

Immunohistochemistry for cell type-specific markers

Slides with serial sections of tissues were deparaffinized as described for the ISH slides, and rehydrated in Dako Wash Buffer. CD45, cytokeratin, and CD1a slides were steamed for 20 min in preheated Target Retrieval solution, pH6 (Dako), in a steamer (Black and Decker), and cooled for 20 min. S100 slides were not pretreated. Slides were rinsed 3× in wash buffer and all subsequent staining steps were performed at room temperature using a Dako Autostainer. Endogenous peroxide activity was blocked using 3% H_2O_2 for 8 min followed by protein blocking by incubation in 15% swine serum and 5% human serum in TBS containing 1% BSA for 10 min. All antibodies were incubated on the tissue for 30 min and then washed with wash buffer. CD45 LCA (Dako) was used at 4.7 μg/ml, S100 (Dako) was used at 0.1 μg/ml, CD1a (Dako) was used at 40 μg/ml, and Cytokeratin 8.13 (Sigma Aldrich) was used at a dilution of 1:20. S100 was detected by Envision Plus, HRP rabbit-specific polymer (Dako) for 30 min. The other antibodies were detected by Envision Plus, HRP mouse-specific polymer (Dako) for 30 min. The staining for all slides was visualized with 3,3′-diaminobenzidine (DAB) (Dako) for 7 min, and the sections were counter-stained with hematoxylin (Dako) for 2 min. Concentration-matched isotype control slides were run for each tissue sample (Jackson ImmunoResearch Laboratories). Images were taken with a Nikon E800 microscope equipped with a Cool Snap color video camera (Photometrics), using Metamorph software, version 5.0 (Universal Imaging Corporation).

Results

Validation of ISH for FV RNA

First, the ISH assay was tested on sections prepared from uninfected TF, and on sections from TF infected with FV in vitro. I observed foci of positive signal overlying the nuclei of infected cells after ISH with the FV as *gag* probe (Figure 4.1b). No significant hybridization was seen in the uninfected TF (Figure

4.1d). ISH with the FV s *gag* probe revealed only very low background signal (Figure 4.1a, 4.1c). ISH with the as β-actin probe showed a different pattern of expression than that seen with the FV as *gag* probe—more diffuse, yet homogenous (Figure 4.3b, 4.3d). Hybridization with the s β-actin probe showed only background signal in both the uninfected and infected TF (Figure 4.2a, 4.2c). These observations indicated that the assay and probes were specific, and that after an 18-day exposure the FV as *gag* hybridization signal was robust.

Screening of oropharyngeal tissues for FV RNA levels before ISH

Before performing ISH, I screened oropharyngeal tissues for FV RNA levels using qRT-PCR for FV *gag*, as described in Chapter 3. I identified tissues from three FV^+ macaques that had detectable levels of FV RNA, with levels ranging from 35 to 3200 copies per cell equivalent (Figure 4.3). For evaluation by ISH, I chose tissues from FV^+ RM that had >100 FV *gag* copies per cell equivalent, namely, the tongues from all three FV^+ animals, and the pharyngeal epithelium and tonsil from FV^+ 1. As a control, I chose the tongue from FV^- 1 (Figure 4.3).

FV RNA localizes to a distinct layer of the superficial epithelium in the pharyngeal epithelium

The FV RNA$^+$ regions were most striking in the tissues from FV$^+$ 1, the RM with the highest FV RNA levels as detected by qRT-PCR. In the pharyngeal epithelium, the FV RNA$^+$ cells were localized to the outer epithelium, specifically to the superficial stratified squamous epithelium (Figure 4.4a–c). The viral RNA consistently localized to this differentiated epithelial cell layer, and was not evident in basal cells along the basement membrane. The underlying mesenchyme and other sites within the tissues were carefully examined and no significant signal indicative of FV RNA was observed. The FV as *gag* signal was considered specific only if a serial section of the tissue hybridized to the FV s *gag* probe showed no signal where the FV as *gag* signal was observed. A serial section probed with the FV s *gag* probe is shown (Figure 4.4d–f).

FV replication is focally distributed and appears in cells sloughing off from tissues

It appears that FV RNA is usually concentrated in discrete foci rather than diffuse, with a low level of viral RNA spread throughout many cells. In the pharyngeal epithelium of RM FV$^+$ 1 (Figure 4.4), for example, 6 foci were identified in one tissue section. Two other foci of FV replication within the same pharyngeal epithelium section are shown (Figure 4.5). These foci also localized to the outer layer of the epithelium, where the FV-permissive cells often appeared to be in the process of sloughing off the tissue (Figure 4.5). The tonsil from RM FV$^+$ 1 also demonstrated this pattern of cellular specificity and focal distribution (Figure 4.6).

FV RNA localizes to the superficial epithelium in the keratinized tongue

In the keratinized epithelium of the tongue, FV RNA also localizes to the superficial epithelium. The foci of FV RNA were excluded from the keratinized region (demarcated by dark pink staining), but consistently localized to the superficial differentiated epithelium of the tongue from FV$^+$ 1 RM (Figure 4.7). This pattern of FV replication was also seen within the tongues from FV$^+$ 2 and FV$^+$ 3 RM, although the pattern was less striking and somewhat more diffuse than that of FV$^+$ 1 RM (Figure 4.8). Overall, fewer foci of FV replication were seen in the tongue from FV$^+$ 2 and FV$^+$ 3 (2–3 foci/tissue section) compared to those from FV$^+$ 1, as would be expected based on the RNA levels in these tissues, as determined by qRT-PCR. I observed no FV-specific signal in tissues from FV$^-$ RM. One ISH of the tongue from FV$^-$ 1 RM is shown (Figure 4.9).

Epithelial but not leukocyte-derived cells localize to sites of FV replication

Next, antibodies specific to epithelial (cytokeratin), leukocyte-derived (CD45) and dendritic cells (CD1a and S100) were utilized to further characterize the cell type expressing FV RNA. Immunohistochemistry with these antibodies was performed with tissue sections adjacent to those used for ISH. The results from cytokeratin staining of the pharynx epithelium are shown (Figure 4.10). Cytokeratin expression was pronounced and colocalized with the FV RNA in the pharynx epithelium (Figure 4.10a, 4.10b). Tissue sections were also stained with an antibody to CD45 (Figure 4.10c). The CD45-expressing cells are present in the epithelium, but at sites deeper within the epithelium, along the basement membrane, and did not significantly colocalize to the FV RNA$^+$ regions (Figure 4.10a). In the tongue a similar distribution of staining was observed (Figure 4.11). Sections stained with a matched isotype control showed only low levels of background staining, and confirmed the specificity of the antibodies used (data not shown).

To examine dendritic cells (DC), specifically Langerhans cells, which are associated with differentiated epithelium, CD1a, a Langerhans cell marker, was used to identify these cells within human skin. However, when this antibody was used for the oral epithelium of RM, we found little to no detectable staining above background (data not shown). This antibody does not appear to significantly crossreact with the RM antigen to yield specific staining of Langerhans cells. Therefore, another DC marker, S100, was used. In human skin tissue employed as a positive control, staining could be detected within the epithelium. However, when the pharynx epithelium, tonsil and tongue tissues from RM were stained with the S100 antibody, only a faint (but specific) signal in the epithelium and darker staining along the mesenchyme was observed (data not shown). These results were inconclusive, and further testing would be necessary to determine whether DCs colocalize with FV RNA$^+$ sites.

Superficial epithelial cells are the dominant cell permissive for FV replication in multiple oropharyngeal tissues

Distinct FV RNA$^+$ regions were found within all tissues examined from the three FV$^+$ RM. The FV RNA$^+$ regions were limited to discrete foci within each tissue, which may suggest that cells only at a specific differentiation stage are permissive for FV replication. Taken together, these data show that FV replicates in the suprabasal superficial layer within the oral mucosal epithelium. The most abundant cell type in this region is the differentiated squamous epithelial cell (27, 31, 104, 148). The majority of FV-permissive cells observed by ISH are cytokeratin- but not CD45-expressing cells. Therefore, we can conclude that epithelial cells are the dominant permissive cell type for FV replication. However, other cell types, such as Langerhans cells, may be present in this layer. For this reason, we cannot rule out the existence of other permissive cell types in the superficial epithelium. Further, this is not to say that CD45-expressing cells are not involved, for example, in spreading the virus to a cell type that is or becomes permissive. If these cells had a low level of viral production, it would likely be undetectable by ISH.

Discussion

This is the first description of cells that are permissive for FV replication in vivo. I have shown that replication is highly focused, in differentiated superficial epithelial cells of the oral mucosa. FV RNA was restricted to a discrete superficial epithelial cell layer and was not observed in the less differentiated basal epithelial cells. Many of these permissive superficial cells appear to be in the process of being shed from the tissue. The robust replication of the virus in this sloughed-off cell type also explains efficient salivary transmission. This highly focused replication in a potentially dispensable cell type may account for the innocuous character of FV infection in vivo.

These observations raise several questions. How is FV replication localized to a specific, distinct layer of the superficial epithelium? What determinants are involved in its cell-specificity? How is FV replication restricted to discrete foci? Why does FV replication not spread throughout a tissue? I discuss below several possible hypotheses and models that address these questions (Figure 4.12).

One model of the localization of FV-infected cells to the oropharyngeal tissues is a 'leukocyte-spread' model. Leukocytes are likely to be a latently infected reservoir for FV (Chapter III). Leukocytes normally migrate throughout tissues, including the oropharyngeal tissues. This model is consistent with latent infection of PBMC before the oral cavity becomes permissive for FV replication (18, 168). This mode of spread, if involving $CD4^+$ T cells, would also be consistent with the trend towards lowered FV RNA loads in the oral cavity with SIV-immunosuppression (Chapter III). An alternate model is that oral mucosal cells, in close contact with the oral environment and saliva, are initially infected by incoming virus rather than by a cell within the tissue. The leukocyte-spread model is most congruent with all that is known about FV infection.

I propose two competing models to account for how FV infection is localized in the differentiated epithelial cells. In the 'precursor-cell latency' model, the latently FV-infected leukocyte spreads FV to a precursor of the superficial epithelial cell, for example, a generative basal epithelial cell. In vitro, leukocyte-derived cells infected with FV produce only low levels of virus, but co-culture between infected leukocytes and uninfected, epithelial-derived cells results in a permissive, highly cytopathic infection of the epithelial cells (99). Since leukocytes are normally localized to the mesenchymal-epithelial border, i.e., in close contact with basal cells, they could presumably transmit the virus to basal cells. Then, a stimulus would activate replication specifically in a superficial epithelial daughter cell, a cell type permissive for FV. This stimulus could be endogenous, such as cell differentiation, or exogenous, such as tissue damage that activates cell division, as discussed later.

Alternatively, the 'direct infection' model proposes that virus is transmitted from a persistently infected (and perhaps activated) leukocyte, directly to the differentiated superficial epithelial cell, which is permissive for FV. In this model, no latent infection develops in basal epithelial cells. However, our IHC analyses indicated no significant leukocyte infiltrate in FV RNA-positive regions, a presence we should have detected if leukocytes were directly involved. Langerhans cells could spread virus to superficial epithelial cells, but this will remain an outstanding issue until a more quantitative colocalization analysis of Langerhans cells and FV RNA is performed. This direct infection model does not fully explain why a specific epithelial layer and not underlying layers are consistently permissive, and would predict a more heterogeneous pattern of viral replication than we observe. Therefore, the precursor-cell latency model is favored.

The precursor-cell latency model requires an additional explanation of how FV replication is activated in the superficial epithelial cells which become permissive for FV. I propose two alternative hypotheses. The 'differentiation-activation' hypothesis proposes that a specific phase of epithelial cell differentiation activates FV replication. This pattern of activation has been observed for other viruses. For example, Epstein-Barr virus (169) and Kaposi sarcoma herpesvirus (KSHV) (63) as well as human papilloma virus (HPV) (111) [reviewed in (89)] establish latent infections in basal epithelial cells of the mucosa. After epithelial cell differentiation, viral replication is activated, resulting in a lytic infection and virion production. Therefore, it seems plausible that foamy virus initially infects basal epithelial cells, and then is activated by epithelial cell differentiation. The herpesviruses EBV and KSHV are also associated with salivary transmission. Thus, this may be a mechanism common to many viruses, which has evolved for efficient transmission.

In contrast, the 'division-activation' hypothesis suggests that the stimulus that triggers FV activation is exogenous in nature, e.g., tissue damage. In the epithelium, basal cells are the only cells that normally divide. Differentiated cells usually do not divide, except under circumstances such as wound healing (104, 148) (personal communication, Beverly Dale-Crunk, 2007). In vitro, FV is able to infect stationary cells but requires cell division for productive infection (8, 157). Therefore, cell division induced by tissue damage could activate FV replication in differentiated cells. This model explains the patchy distribution of foci as well as limited FV replication; however, two observations are not consistent with it. One is the lack of an obvious wound and apparent leukocyte infiltrate found at these permissive sites in my ISH and IHC studies. Second, this model predicts that latently infected basal cells, which are dividing, would be permissive, which is not the case. Further, the division-activation hypothesis also implies that efficient viral transmission is left to a rare, chance event, which would be unlikely to result in efficient viral transmission, and which stands in contradiction to the high levels of FV replication which we consistently observe in the oropharyngeal tissues of RM.

The differentiation-activation hypothesis is thus preferred, although it is in contradiction with the in vitro studies that indicate a restriction of FV replication in nondividing cells (8, 157) This finding may have been due to the fact that the in vitro studies were performed in cell lines that don't represent the differentiation state, specific keratin networks, extracellular matrices, or cell turnover rates that exist within mucosal epithelial tissues.

A self-limited, focal distribution of FV replication was observed in vivo, which stands in contrast to the phenotype of FV in vitro, where viral replication results in death of entire cultures. Perhaps the evolved kinetics of viral infection in vivo simply allows no time for adjacent differentiated epithelial cells, which are not latently infected, to become permissively infected. Transformed epithelial cells divide about once every 24 hours in vitro, but it often takes up to a week after FV infection (at a multiplicity of infection of one) for virally-induced CPE to spread throughout a culture. In contrast, in vivo superficial cell layers turn over in a much shorter period, with estimates of 3 hours for the most terminally differentiated cells (28). The short turnover time of superficial epithelial cells is not long enough for a new round of the viral infection in adjacent, uninfected superficial cells, and for another round of the viral life cycle, before they are sloughed off into the saliva. Thus, viral replication is regulated so that cell death and shedding occur before the virus ravages the entire epithelium.

In this 'kinetic' model, superficial epithelial cells would become permissive only at a late stage, with very little replication actually occurring in the tissue, a scenario which would tend to minimize tissue damage. If this were the case, then we would expect cells in saliva to harbor more FV RNA than does mucosal epithelial tissue. This is indeed what we observed (Chapter III). We found the highest levels of FV replication in oral swabs; up to 5×10^8 FV RNA copies per 10^4 cell equivalents, much higher levels than in the originating tissues. Interestingly, in vitro time-course studies indicate that FV is stable and remains infectious for over 18 hours after CPE is observed and after dying cells are released into culture supernatant (Chris Meiering, personal communication). The DNA genome and other unique features of FV may contribute to this stability and efficient preservation.

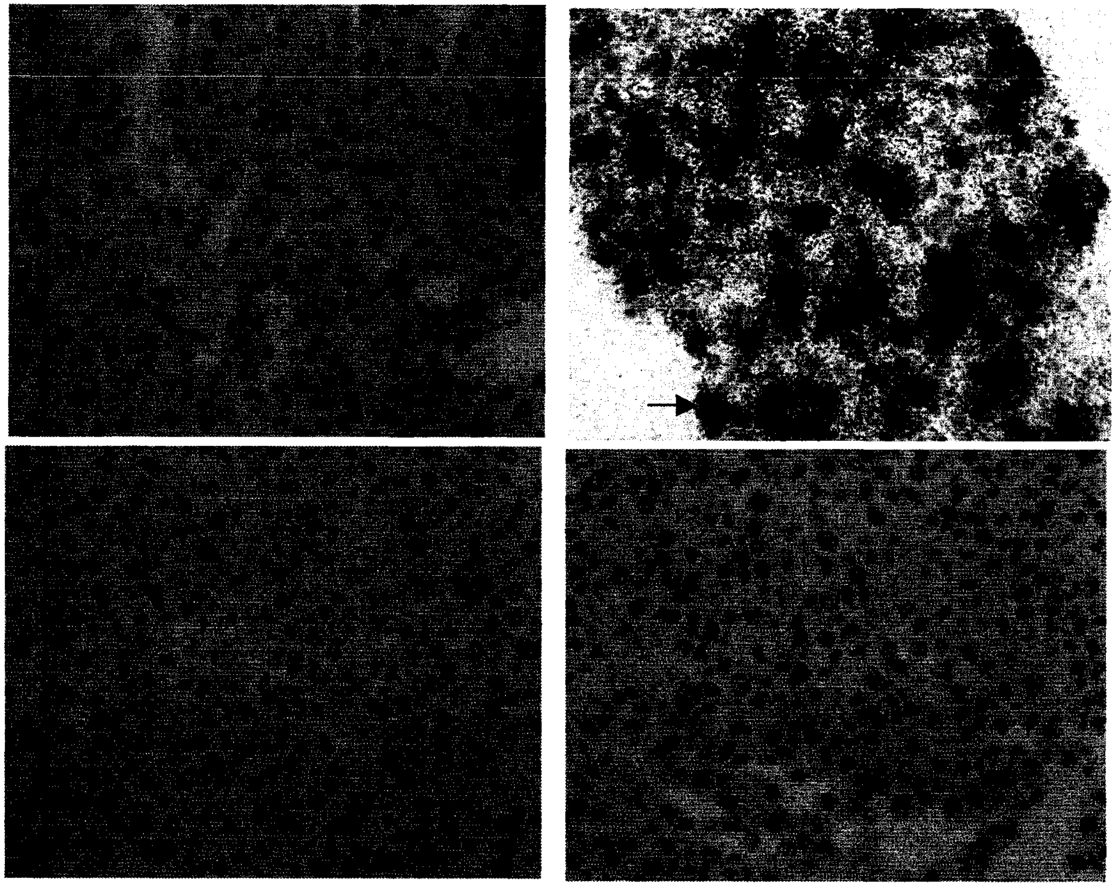

Figure 4.1. FV replication in FV-infected macaque fibroblasts.
Detection of FV RNA by in situ hybridization of infected (a, b) or uninfected (c, d) telomerized fibroblasts probed with a sense (a, c) or antisense (b, d) FV *gag* probe. Positive regions are indicated by silver grains that appear as dark deposits in a plane above the cells. Arrows indicate FV RNA+ signal. The cells were counterstained with hemotoxylin and eosin. Bright-field microscopy

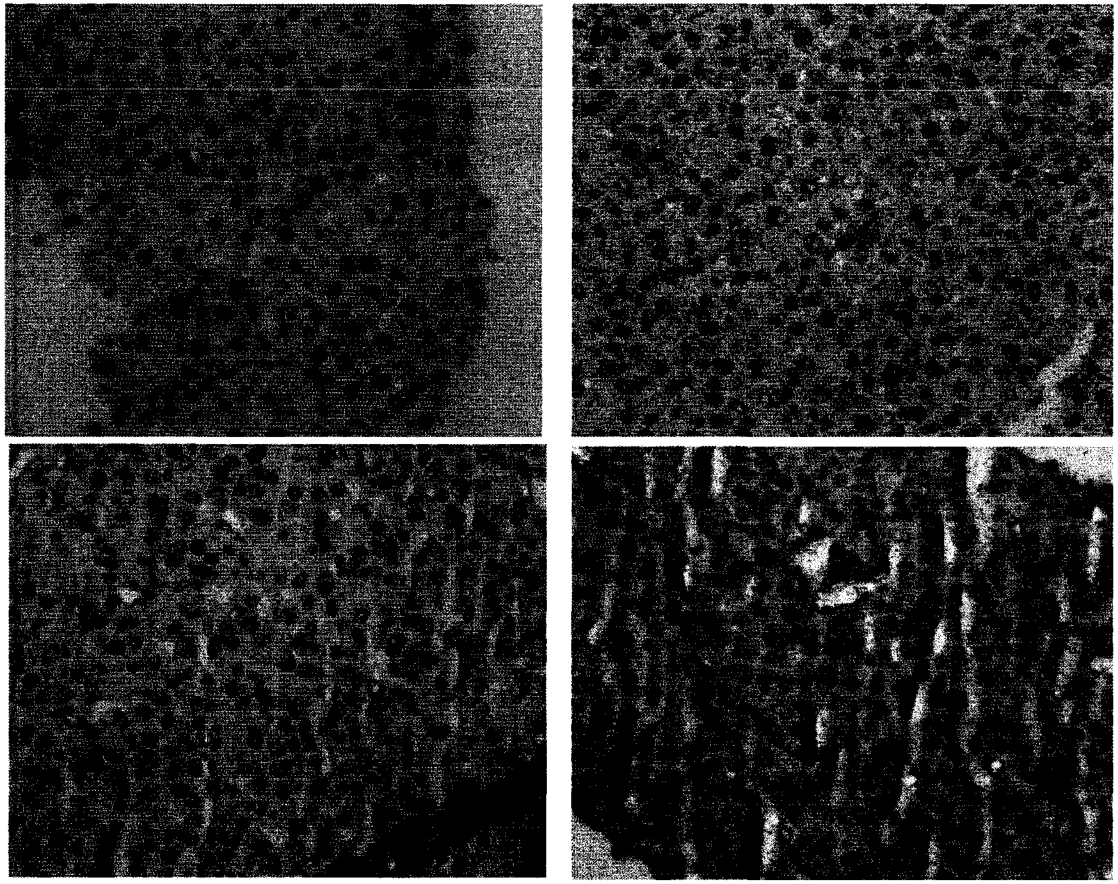

Figure 4.2. β-actin RNA in FV-infected and -uninfected macaque fibroblasts.
Detection of β-actin RNA by in situ hybridization of infected (a, b) or uninfected (c, d) telomerized fibroblasts probed with a sense (a,c) or antisense (b, d) β-actin probe. Positive regions are indicated by silver grains that appear as dark deposits in a plane above the cells. The cells were counterstained with hemotoxylin and eosin. Bright-field microscopy images were taken at 400X magnification.

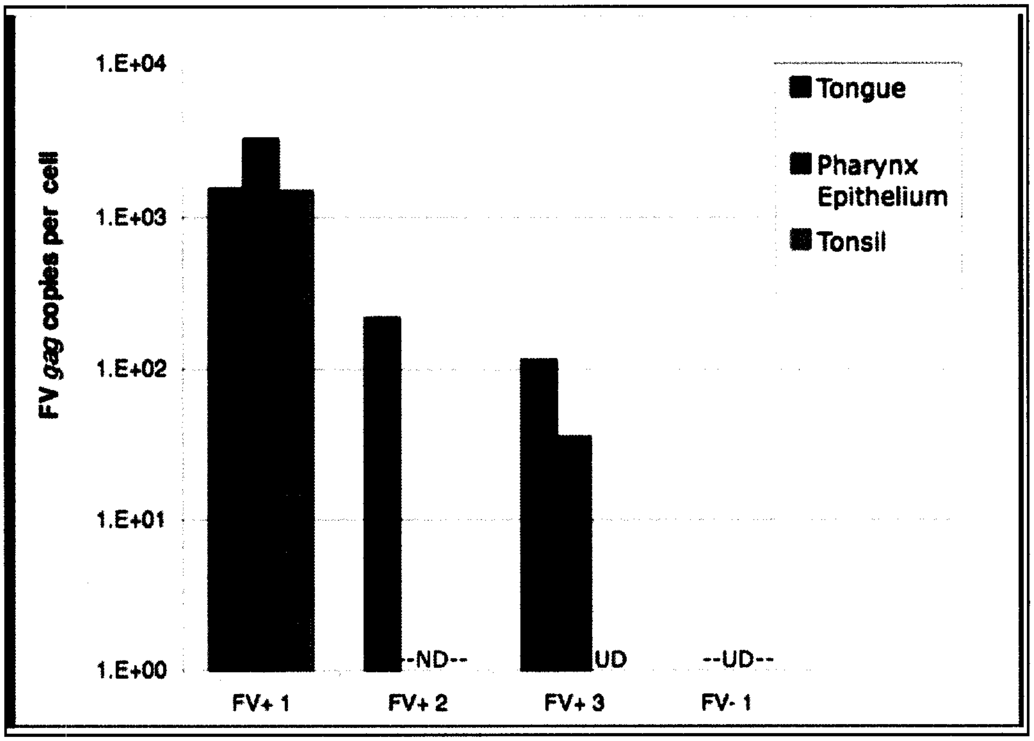

Figure 4.3. FV RNA levels in oropharyngeal tissues.
Tissues from three FV+ macaques (FV+ 1, FV+ 2, and FV+ 3) and one FV- macaque (FV$^-$ 1) were screened for FV RNA levels by qRT-PCR, before use in ISH. ND, not done; UD, undetectable.

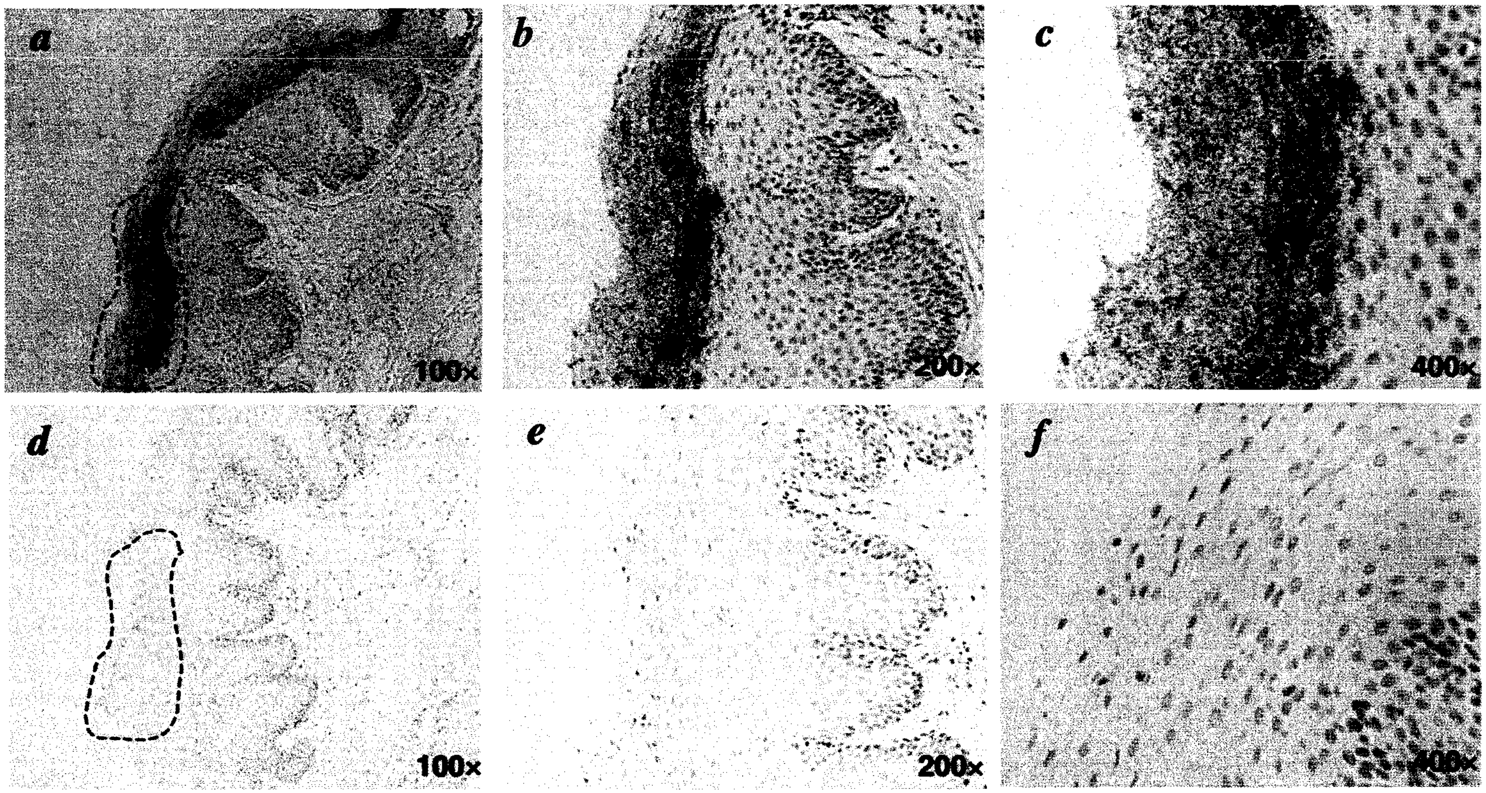

Figure 4.4. FV replication localizes to the superficial epithelium in the pharynx epithelium.
FV RNA was detected by in situ hybridization of the pharynx epithelium with an antisense (a-c) and sense (d-f) FV *gag* probe. One FV RNA+ region from FV+1 RM is shown at 100X (a, d), 200X (b, e), and 400X (c, f) magnifications. Arrows indicate FV RNA+ sites. Dotted lines outline the region that showed FV RNA+ signal using the FV as *gag* probe. No background signal is observed in the same region of the tissue with the sense FV *gag* probe (d-f)The cells were counterstained with hemotoxylin and eosin.

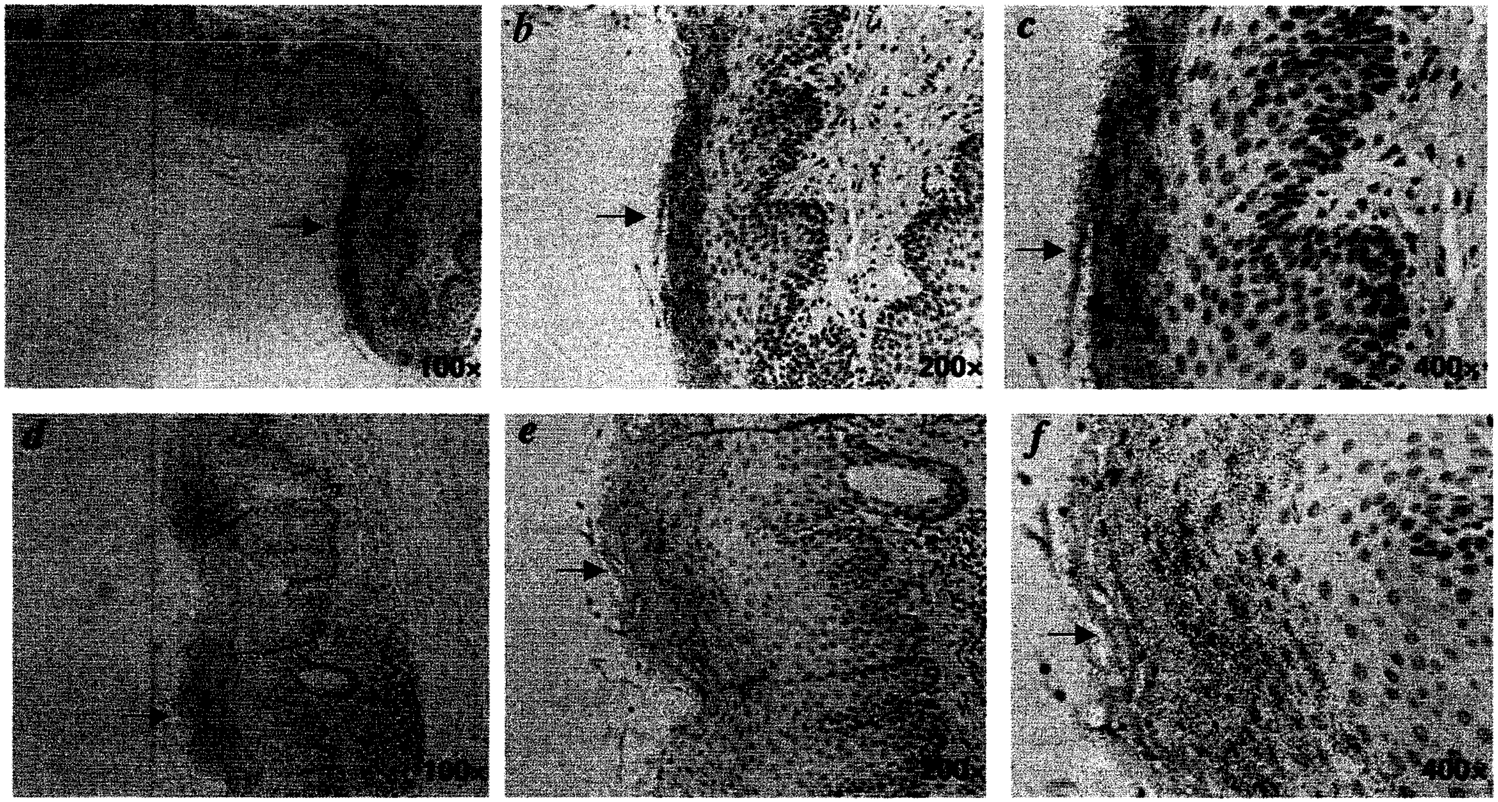

Figure 4.5. FV replication in the pharyngeal epithelium of FV+1 RM.
Some FV RNA-positive cells appear to be sloughing off of the tissue. FV RNA was detected by in situ hybridization of the pharynx epithelium with an antisense FV *gag* probe. Two FV RNA-positive regions of the pharynx epithelium are shown at 100X (a, d), 200X (b, e), and 400X (c, f) magnifications. Serial sections probed with the FV *gag* sense probe showed no signal (data not shown). Arrows indicate FV RNA+ sites. The cells were counterstained with hemotoxylin and eosin.

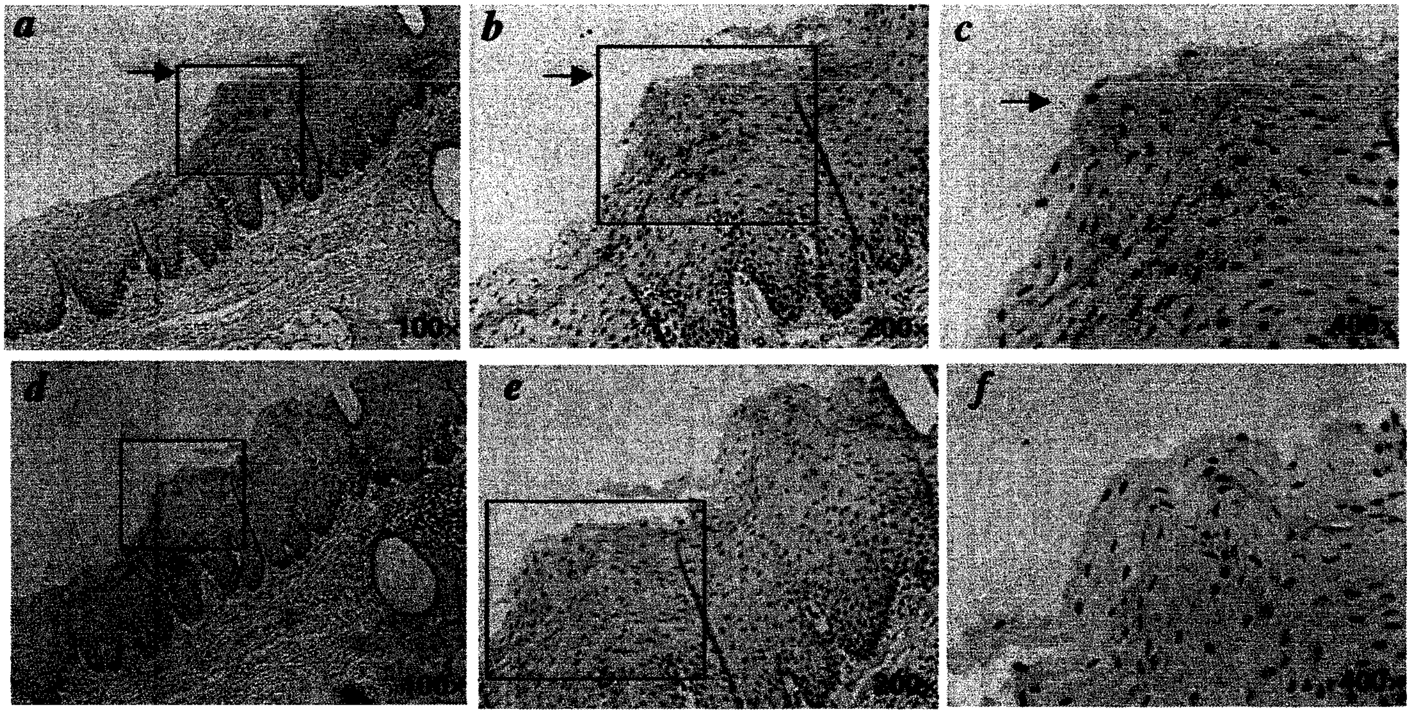

Figure 4.6. FV replication in the superficial epithelium of the tonsil of FV+1 RM.
FV RNA was detected by in situ hybridization of the tonsil with FV antisense (a-c) and sense (d-f) *gag* probes. One FV RNA+ region from FV+1 RM is shown at 100X (a, d), 200X (b, e), and 400X (c, f) magnifications. In this region, cells appear to be sloughing-off, and a vessel is in close proximity. No signal is seen in the serial section in the same region probed with the antisense probe (d-e). Arrows indicate regions of FV RNA+ signal. Boxes outline the region that showed FV RNA+ signal using the FV as *gag* probe. The cells were counterstained with hemotoxylin and eosin.

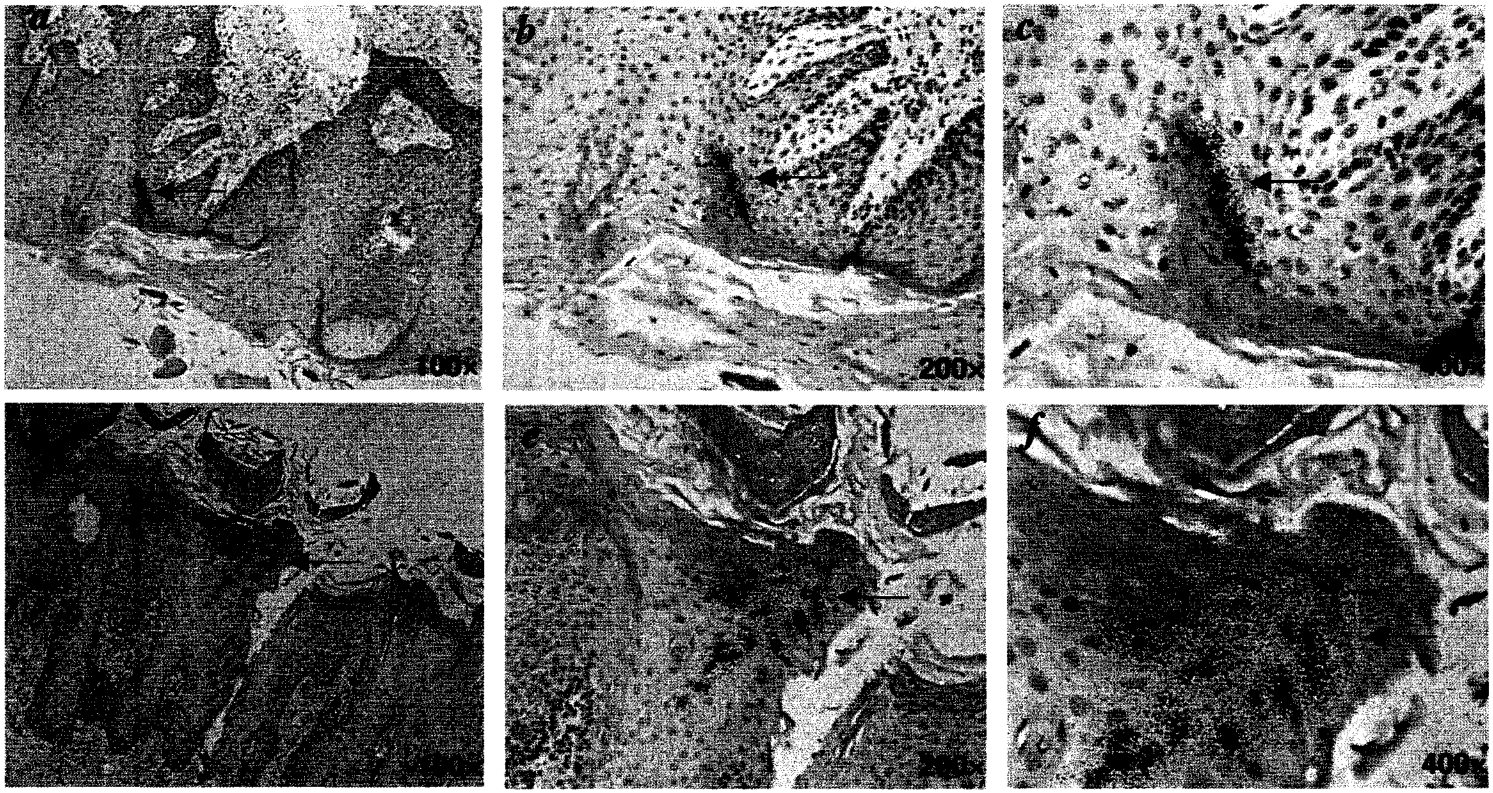

Figure 4.7. FV replication localizes to the superficial epithelium in the tongue of FV+1 RM.
Permissive cells appear adjacent to, but mostly excluded from the keratinized regions, which are demarcated by the dense pink staining. FV RNA was detected by in situ hybridization of the tongue probed with FV antisense *gag* probe. Two FV RNA+ regions from FV+1 RM are shown at 100X (a, d), 200X (b, e), and 400X (c, f) magnifications. Serial sections probed with the FV sense *gag* probe showed no signal in the same region (data not shown). Arrows indicate FV RNA+ sites. The tissues were counterstained with hemotoxylin and eosin.

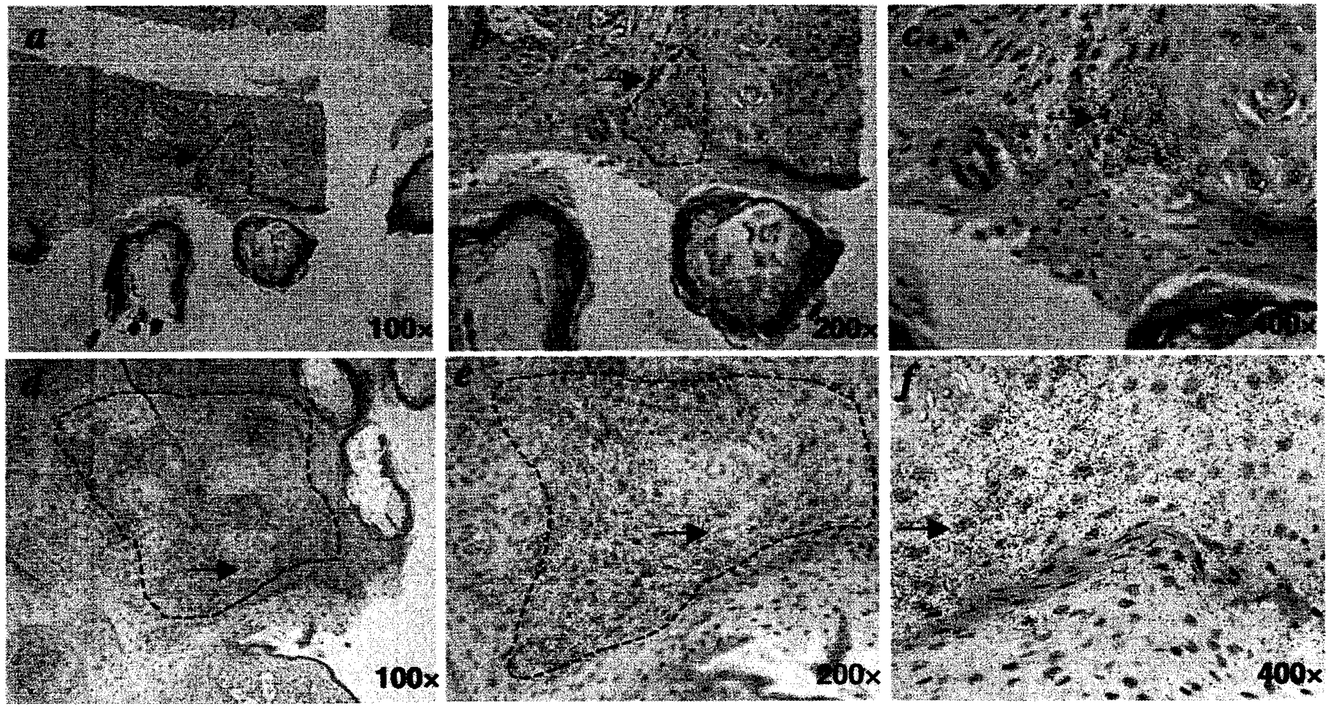

Figure 4.8. FV replication localizes to the superficial epithelium in the tongue of FV+2 and FV+3 RM.
FV RNA is mostly excluded from the adjacent keratinized layer. FV RNA was detected by in situ hybridization of the tongue tissue with the FV antisense *gag* probe. FV RNA+ regions from FV+2 (a-c) and FV+3 (d-f) RM are shown at 100X (a, d), 200X (b, e), and 400X (c, f) magnifications. Serial sections probed with the FV sense *gag* probe showed no signal (data not shown). Arrows indicate FV RNA+ sites. Dotted lines outline the FV RNA+ regions. The tissues were counterstained with hemotoxylin and eosin.

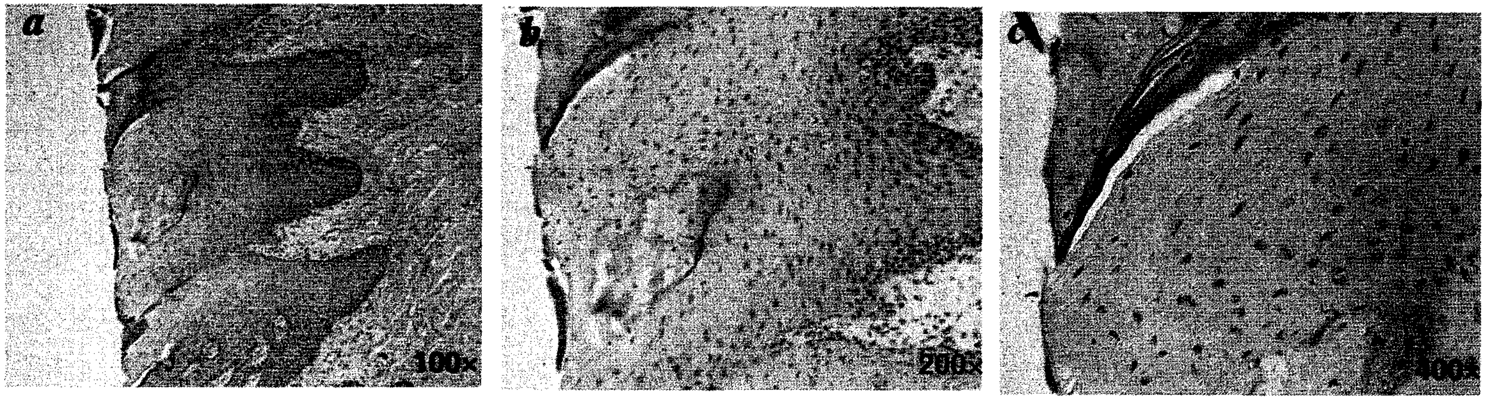

Figure 4.9. The absence of FV RNA-specific signal in the tongue of an FV- macaque.
In situ hybridization of a tongue section from an FV- macaque probed with a FV antisense gag probe. No FV RNA+ regions were seen in the tissue. A representative region is shown at 100X (a, d), 200X (b, e), and 400X (c, f) magnifications. The tissues were counterstained with hemotoxylin and eosin.

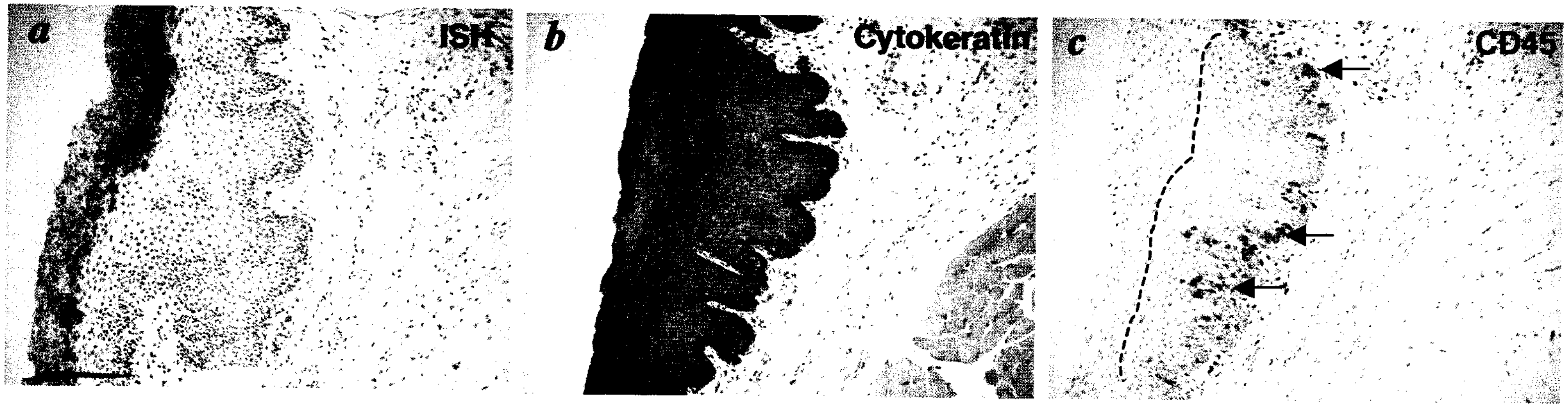

Figure 4.10. Cytokeratin but not CD45 expression significantly localizes within FV RNA+ regions of the pharyngeal epithelium.

Serial sections to those used for in situ hybridization (a) were used for immunohistochemistry with antibodies to cytokeratin (b) or CD45 (c). Results are shown at 100X magnifications. The brown deposits represent specific staining. Dotted lines show the FV RNA+ regions of the tissue. Arrows identify CD45-specific staining. The matched isotype controls showed little background staining, with the exception of the outer cornified cell layer (data not shown). The ISH tissues were counterstained with hemotoxylin and eosin, and those for IHC were counterstained with hemotoxylin.

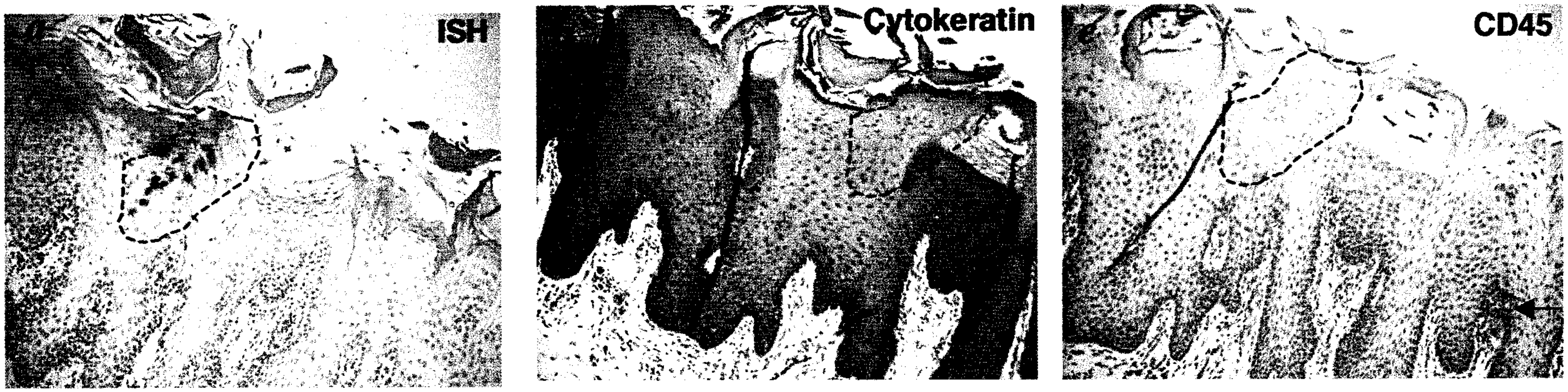

Figure 4.11. Cytokeratin but not CD45 expression significantly localizes within FV RNA+ regions of the tongue. Serial sections to those used for in situ hybridization (a) were used for immunohistochemistry with antibodies to cytokeratin (b) or CD45 (c). Results are shown at 100X magnifications. The brown deposits represent specific staining. Dotted lines show the FV RNA+ region of the tissue. Arrows identify CD45-specific staining. The matched isotype controls showed little background staining, with the exception of the outer cornified cell layer (data not shown). The ISH tissues were counterstained with hemotoxylin and eosin, and those for IHC were counterstained with hemotoxylin.

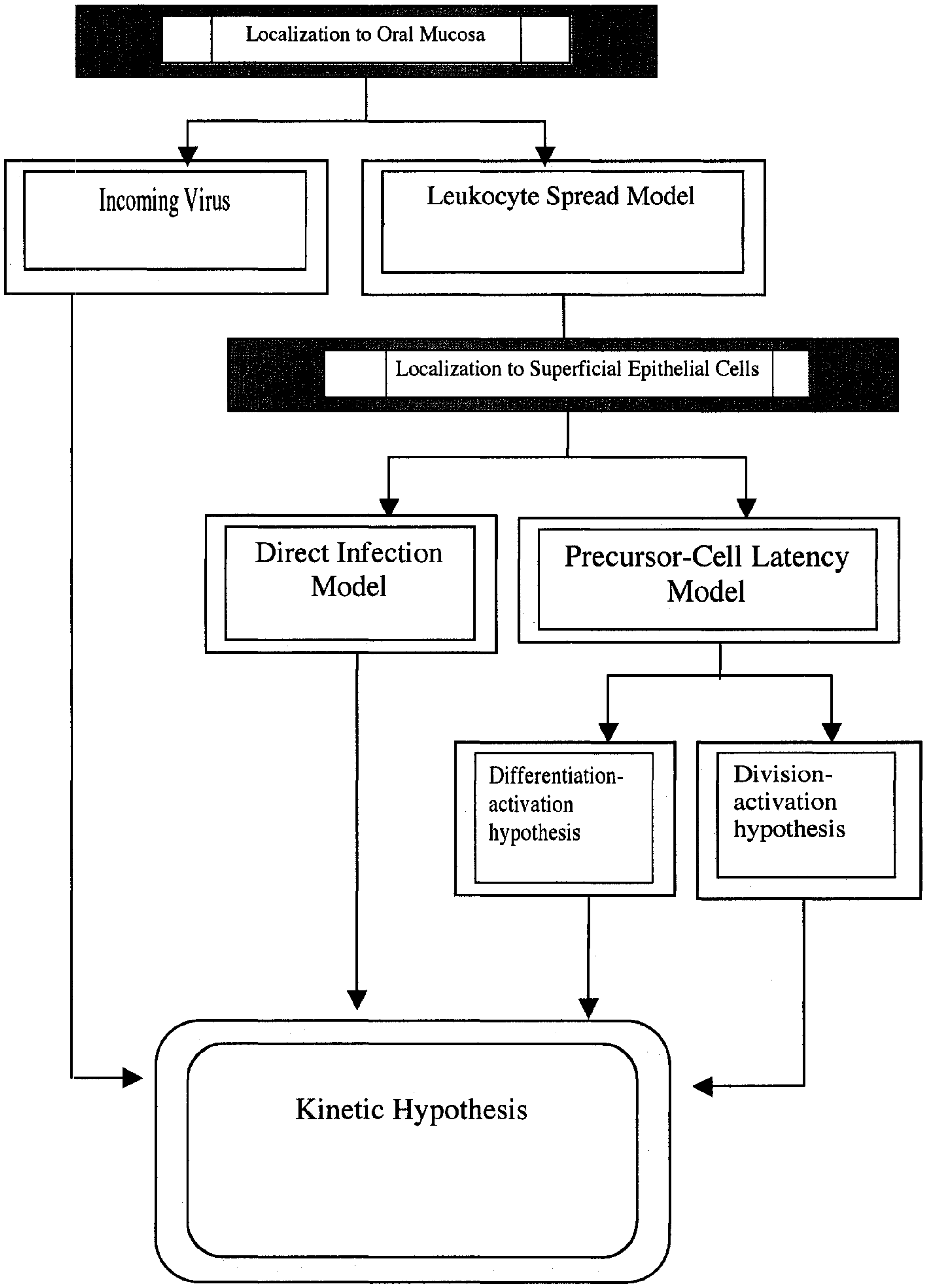

Figure 4.12. Possible explanatory models of the focal and limited replication of foamy virus within the superficial epithelium of the oral mucosa. Shown is a decision-tree indicating the relationships between the models and hypotheses presented in Chapter 4. Observations are in blue boxes; models and hypotheses are in yellow boxes.

Chapter V: Conclusions and Future Directions

Foamy viruses have cospeciated with primate hosts and thus provide a prime model for long-term virus–host evolution. The studies outlined in the previous chapters have informed knowledge in three major areas of foamy virus–host interaction that were not previously understood: host immune responses to FV infection, tissue sites of FV replication in normal and immunosuppressed hosts, and the cellular sites of FV replication (Table 5.1). I now summarize my findings, and then suggest an overall model of FV infection.

Mucosal and Systemic Immunity to FV Infections

What I and others have found with FV infection is a scenario where there is no IgA—a hallmark of mucosal immune response—in the saliva, yet significant levels of systemic antibodies, including antiviral sera IgA and envelope SU-specific IgG in some hosts. I suggest that the presence of IgA and SU-specific IgG may simply mark the onset of new viral infection. Transient, brisk IgA production is also associated with initial infection by Epstein-Barr Virus (EBV) (78, 125).

I found relatively low frequencies of FV-specific T cells in the blood. My preliminary data suggest the presence of more FV-specific CD4$^+$ T cells in mucosal sites than in the blood. Yet if virus-specific T cells are present in the mucosa, then how is it that another type of mucosal immunity is not present, namely that of salivary IgA? There are some examples of infections that suppress mucosal IgA production. For example, HIV and SIV infections are associated with very low levels of mucosal IgA (102, 134).

The innate immune system is also a major determinant in the potency of adaptive immune responses. Therefore, it may be that the suppression of innate and intrinsic immunity by FV in vitro (79, 88, 123, 128, 130) also occurs in vivo, and results in suppression of some host adaptive immune features such as mucosal IgA production.

I found high-titer FV Gag-specific IgG in the blood, so a generalized 'immunologic ignorance' is certainly not operative. The route of antigen presentation to the host immune system significantly affects the adaptive immune responses that follow. Since viral replication is not detectable, and thus, is self-limiting, in the blood (Chapter III), viral antigen must be presented at sites other than the blood in order to induce these systemic antibodies. Therefore, viral antigen within the oral cavity or at the site of initial infection may stimulate systemic immunity.

My data suggest that T cell and antibody responses are independently regulated. This independence may reflect the split between systemic immune responses to antigen generated at the infection site, and immune response in the oropharyngeal tissues, the site of ongoing viral replication. Interestingly, a transient T cell-immunosuppression with primary FV infection has been observed in small animal models (56, 133). If this occurred during natural infection of RM, this could result in suppression of host systemic T responses and maximize viral dissemination by suppressing leukocyte activation. Also, sera IgA production has been associated with immunosuppressive states in some persistent viral infections (such as EBV) (3, 78).

My findings are compatible with a model in which the site of initial infection is different from the site of ongoing viral replication. Systemic immune responses may be stimulated at the initial infection site. Antiviral sera IgA production would be induced at the site of initial infection but not in the oral cavity, which eventually becomes the site of viral replication. The initial site of infection may also be where leukocytes are latently infected with the virus, later migrating to other mucosal sites, including the oral cavity. This idea would explain why some recent studies, albeit preliminary, have found FV DNA in PBMC before FV RNA is detected in the oral cavity of newly FV-infected macaques (18, 168).

The Role of Host Immunity in Controlling Systemic FV Replication

My examination of tissues in normal, immunocompetent hosts revealed viral replication limited to the oropharyngeal tissues, more specifically to oral mucosal tissues. The salivary glands were nonpermissive for FV replication, while the buccal and pharyngeal epithelium, tongue, tonsil and lung were permissive. All other sites, including the blood, were nonpermissive with no detectable viral RNA, despite widespread viral DNA. In SIV-infected hosts, I found an extension of FV replication to the jejunum, but not to any other sites including the blood. And, in fact, there is a statistically significant trend towards lowered viral loads in the oral cavity and in oropharyngeal tissues of the SIV-infected RM (Chapter III).

The lack of viral replication in the blood of SIV-immunosuppressed hosts, and the relatively low level of systemic virus-specific T cells, argue that the host immune system is not the major factor in systemic immune control of viral replication. In the blood, it appears that the limit to FV replication is primarily under viral control. Further, my data suggest that the immune system may even support replication, by spreading FV to the oral cavity. The significant trend towards slightly lowered viral RNA loads in the oral cavity of SIV-immunosuppressed animals is suggestive of a CD4$^+$ T cell-mediated viral spread.

The extension of FV replication to the jejunum, but not to other significantly CD4$^+$ T cell-depleted sites in SIV-infected animals, argues against a systemic immune control model. However, these results indicate that there are important differences between the jejunum, and other organs such as the colon. One key may be the differences in tissue structure. The jejunum is made up of villae comprised of epithelial tissue and underlying lamina propria, in contrast to the lack of villae in the colon (31). With SIV infection, fusion of villae, an increase in epithelial cell turnover, and crypt cell hyperproliferation are observed in the jejunum (42, 72, 132, 151). Perhaps these changes make the jejunum similar to an oral cavity tissue in significant ways that cause it to become permissive for FV replication.

Cell-Specificity of FV Replication

My observations demonstrate that the permissive niche for FV replication is the superficial epithelial cell (Chapter IV), resulting in virus being sloughed off into the oral cavity along with cells. Viral replication is highly focal, limited to discrete pockets, and localized to a specific uniform layer of differentiated superficial epithelial cells. This pattern of replication appears to minimize damage to the host tissue while maximizing transmission efficiency. I have proposed several models to explain these observations (Chapter IV), and summarize them here.

FV-infected leukocytes may occasionally infect precursors of superficial epithelial cells in the oral mucosa, at a low level (the 'leukocyte-spread' model). Viral latency may be established in this precursor cell, perhaps a basal cell, followed by activation of viral permissivity in suprabasal epithelial cells (the 'precursor-cell latency' model). Then, FV is activated by signals specific to a certain stage of cell differentiation (the 'differentiation-activation' model), a mechanism utilized by other viruses such as HPV (89, 111), KSHV (63), and EBV (169). This best explains the heterogeneous pattern of FV replication within a distinct layer of superficial epithelium. This pattern of limited viral replication, in contrast to the phenotype of FV in vitro, results from the fact that the short turnover time of superficial cells is not long enough for a new round of the viral infection in adjacent, uninfected superficial cells, and for another round of the viral life cycle before they are sloughed off into the saliva (the 'kinetic' hypothesis).

Integrated Model of FV Transmission and Within-Host Spread

I am now able to present an overall model of FV infection, albeit a preliminary and partially speculative one, that integrates my findings with others' observations. I suggest that the sites of initial infection and of ongoing viral replication are different. I propose that the virus enters hosts through the skin as via a bite or wound. The virus is then taken up by antigen-presenting monocytes, which encounter and infect lymphocytes, including CD4$^+$ T cells. This initial infection stimulates systemic immune responses, including envelope-specific IgG and antiviral IgA production, and perhaps a low level of FV-specific CD4$^+$ T cells.

Latently FV-infected CD4$^+$ T cells then circulate throughout tissues, resulting in dissemination of low levels of FV DNA to all tissues, as observed (Chapter III) (37). Within the oropharyngeal epithelium, activation of these latently infected T cells result in the infection of a precursor cell of the superficial epithelial cell. This infection is latent until a specific phase of epithelial cell differentiation activates viral replication. Thus, FV replication is effectively localized to a distinct outer layer of the superficial epithelium. I propose that it is the timetable of differentiation and turnover of epithelial cells in the oral mucosa that render these tissues uniquely permissive for FV replication. FV replication is focused in soon-to-be-shed superficial epithelial cells. This strategy of viral replication promotes viral transmission, while utilizing a dispensable cell type. FV infection then spreads to a new host via these shed cells.

The question "why is foamy virus not pathogenic in its host?" to which I have begun to provide an answer, can be reframed as "why is FV so cytopathic in vitro?" In vitro studies with FV infection have been performed in cell lines that do not represent the differentiated cell types or the cell turnover rates that exist within epithelial tissues—the in vivo reality with which FV has so intimately coevolved. What FV does to epithelial cells in vitro is the same as what it does in vivo—but the realities in vivo are cell differentiation and epithelial cell turn over, so timing is everything. In cells that don't turn over, shed and die as they do within a tissue, we observe an artifact of immortalized cells. Because this artifact of in vitro systems may be recapitulated by carcinogenesis in vivo, FV may have useful oncolytic properties. In the following section of this chapter, I discuss ways to further examine the hypotheses I have outlined.

Future Research

Elucidation of the cellular specificity of FV latency and replication

Laser-capture microdissection (lcmd) has been useful in separating various subtissue structures, including cells, from tissues, in order to analyze gene expression in separated cell populations (36, 92). This technique could be applied to sections contiguous to those used for FV RNA in situ hybridization (ISH), so that FV RNA$^+$ regions can be aligned and easily identified. Superficial FV-permissive cells could be separated from underlying epithelial cells and leukocytes to determine whether provirus is present in these nonpermissive cell populations. DNA and RNA from each cell population would be isolated, and the quantitative PCR and RT-PCR that I developed would then be used to test each of these separate cell populations for presence and levels of FV DNA and RNA. This separation would also allow testing of whether leukocytes adjacent to basal cells and underlying regions of FV RNA$^+$ differentiated epithelial cells are enriched for latent provirus. Permissive cells could also be isolated for microarray-based analyses to characterize the differentiation and cell cycle markers they express. Lcmd could also used to isolate basal cells and test whether differentiation of these cells results in FV replication, using an in vitro system, described below. One caveat is that if the originating, latently infected cell was not a basal cell or leukocyte, then there would be no precursor cell remaining within the tissue. A limitation of such studies of provirus localization within a tissue is that only a single time point is examined.

A more controllable system, where cell differentiation can be followed and various stimuli can be introduced, would be useful in studying the mechanics of viral activation. An organotypic raft culture of epithelium is an in vitro system that closely mimics the native three-dimensional epithelial structure and recapitulates in vivo epithelial differentiation. This system has been shown to create distinct layers of differentiated stratified epithelia characterized by specific keratin pairs and other differentiation markers observed in vivo (107, 122). This organotypic raft culture has been utilized for many oral epithelial tissues, including the tonsil and buccal epithelium (63). Such a system has been used to demonstrate that HPV as well as KSHV infect basal epithelial cells and are activated with epithelial cell differentiation (63, 105), and to study adenovirus differentiation-dependent replication in the contexts of both normal and HPV-transformed epithelia (113).

An organotypic raft culture of oral epithelium could be utilized to study FV infection and the induction of viral replication. This highly controlled model would allow for in vitro viral infection into different cell types and into epithelial cells at different differentiation stages. Exogenous stimuli such as tissue damage or microbial antigens could be introduced to determine whether FV replication is activated by these stimuli. One promising avenue might be to add leukocytes at the base of a culture in which fibroblasts are embedded in a collagen matrix. It may be possible to develop this system, mostly described for human cells, for cultures of RM cells. Of course, extensive development would be required to determine the details of research using such a system.

Other methods, such as flow cytometry-based cell sorting, could be utilized to separate specific leukocyte subpopulations to determine the level of provirus in each. Only one study has included this type of analysis, but this approach warrants further examination. For example, a fluorescence-activated cell sorter (FACS) could be used to determine whether CD4$^+$ T cells or other cell types harbor provirus, and whether a specific subpopulation of CD4$^+$ T cells such as those with memory or tissue-specific homing markers, or FV-specific T cells, are enriched for provirus. Utilizing the qPCR and qRT-PCR that I developed, this issue can be evaluated in a highly sensitive and quantitative manner. This would aid in elucidating the model of viral spread within a host.

ISH studies should be extended to further evaluate FV RNA in the jejunum of SIV-immunosuppressed RM, since this would be useful to understand the mechanism of expanded FV replication there. Fluorescence in-situ hybridization and in-situ PCR have been developed to sensitively measure low copy proviral DNA of a number of retroviruses (6, 29, 156). In order to localize provirus, this method could be used with serial sections similar to those used for ISH for FV RNA. These techniques could be developed to determine whether latently FV-infected basal epithelial cells underlie sites of FV replication. Ideally, a dual DNA–RNA ISH could be developed to simultaneously localize provirus and viral RNA. However, a caveat to this approach is that the lower limit of detection of the technique is close to that of the DNA levels in oral tissues: ~1 copy per 1000 cells. Immunohistochemistry (IHC) in conjunction with ISH should be utilized to identify what well-characterized differentiation and cell cycle markers are co-expressed in FV RNA-positive cells, in order to delineate at what stage of cell differentiation FV becomes permissive.

Experimental infection

More in vivo experimentation should be undertaken to test the hypotheses outlined here. The development of an experimental infection model will provide a tractable system in which to follow viral spread and evaluate the dynamics of immune responses. Now that we know the permissive cell type, we could test whether the transmission of sloughed-off cells to a new host via the skin (subcutaneously) or to the oral cavity result in the same outcome as natural infection. Using the assays I've developed, including quantitative ELISA for FV Gag IgG and IgA and SU-specific IgG, and extending the FV-specific CD4$^+$ T cell assays to include FV-specific CD8$^+$ T cells, immunologic parameters can be evaluated in RM. Also, the quantitative PCR, RT-PCR and ISH assays that I have developed will be useful in RM experimental infection models. Since we would want to mimic the natural route of infection, we would need to test multiple sites of inoculation, since the natural route remains undetermined. A small animal model, such as mice, should not be ruled out, and should include a close evaluation of the oral cavity to determine whether this model mimics viral replication in natural infection.

Oncolytic properties of FV infection?

Other retroviruses such as HTLV and MMTV are associated with oncogenesis, while no association with FV has been demonstrated. Interestingly, cancer gene therapy studies with FV vectors in mice with solid tumors (48) have revealed an oncolytic effect of FV itself, even in the absence of an inserted cancer suicide gene. Could this observed oncolytic effect simply be a result of FV induction of CPE in cancer cells, which do not go through a natural cell death and are not shed from their tissue? These observations warrant more investigation of the targets of FV-induced CPE to determine whether FV could be used and developed to specifically target and induce CPE in cancer cells. The organotypic raft model may be a useful system to initiate these studies. For example, differentiated superficial epithelial cells could be transformed, either by transfer of a specific oncogene or utilizing a virus that induces tumorigenesis in differentiated epithelial cells, such as HPV or KSHV. We could then determine whether FV induces CPE specifically in transformed cells, and has oncolytic properties in this system.

Do rhesus monkeys and other nonhuman primates get oropharyngeal cancers? These cancers are documented in humans, but reports on RM are virtually nonexistent, and their occurrence is rare in primates in captivity (Anne Lewis, personal communication). Could FV act as a selective pressure against tumorigenesis in the oral cavity in nonhuman primates? There is indication that

EBV (75, 145) and HPV (44, 110) are associated with oropharyngeal carcinomas in humans, but few papers exist for nonhuman primate models, although the simian homologue of EBV is endemic to RM. Epidemiologic studies of oropharyngeal cancers in NHP infected with FV and EBV should be undertaken.

Interestingly, FV transgenic (Tg) mice, engineered to express the FV genome under its own LTR promoter exhibited pathology restricted to the neurologic system (12) The observation that FV has oncolytic properties may provide some insight into why the FV-Tg mouse model appears to be an anomalous model of in vivo FV pathology. Neurons are the longest-lived differentiated cell in the body; they are in a state of terminal differentiation, but rarely experience cell death and turnover. It may be this combination of differentiation state combined with a long-lived phenotype that renders these neurons a target of FV pathology. This infection of neurons may be an example of FV as a 'misplaced microbe'—misplaced into a new environment with important differences from its natural niche. It may be that long-lived cells at a particular differentiation state are most susceptible to FV-induced cytopathicity.

Opportunist, commensal, or mutualist?

When FV finds itself in particular non-natural settings such as in tissue culture, or neurons, it has the potential to induce pathology, making FV an opportunist in at least some situations. Whether FV is an opportunist in the jejunum of SIV-immunosuppressed hosts is still an outstanding question. The significant levels of FV replication there could presumably induce a pathology, and warrants closer analysis of FV involvement in the previously described 'generalized giant cell disease' (82) and cytopathic vacuoles in crypt epithelial cells in the jejunum of SIV-infected macaques (151). However, in vivo, in natural infection, FV is a commensal with a tissue and cellular niche that limits viral replication to a superficial site, while maintaining high levels of viral transmission.

Due to the long-term coevolution of FV and its hosts, it is reasonable to ask whether FV infection provides any advantages to its host. Specifically, could FV infection be mutualistic, where both the host and virus benefit? The answer to this, of course, will require more extensive study. The possibility that FV infection takes on an oncolytic property speaks to this issue—perhaps FV inhibits oropharyngeal squamous epithelial cell cancers by inducing cytopathicity. Further, FV might provide some other advantages to its host, such as promoting the release or elimination of shed cells.

Two major questions that are still outstanding are "why is there no human-specific FV?" and "why don't humans horizontally transmit FV?" Perhaps the answers are inextricably linked to behavior that, such as biting or grooming, was lost in the evolution of humans, leading to loss of FV as well. Or perhaps some biological property, such as lack of permissivity in the oral cavity, is responsible. Alternatively, the loss of FV in humans could be due to competition between FV and other microbes.

While many questions remain outstanding, our studies indicate that FV has evolved an innocuous niche within its host, and that many of its features are virally controlled, but likely require signals—either differentiation or activation cues—from its host. Host T cell immunity does not limit viral replication and, in fact, viral spread within a host is likely to rely on the immune system itself, specifically on lymphocyte trafficking. My studies suggest that FV may have established a niche that relies upon a fundamental aspect of host biology, namely, epithelial cell differentiation and turnover. Many features of foamy virus that differ from those of the other retroviruses, such as the timing of reverse transcription, the DNA genome, the unique internal promoter and the Bet protein, are all likely to be related to the strategies of viral within-host spread and timing of viral replication that result in the focal and limited viral replication that have been observed in vivo.

Table 5.1. Summary of the features of foamy virus–host interactions revealed by these studies.
UD = undetectable.

Feature	Finding
Sites of replication	Oropharyngeal-associated tissues including buccal and pharyngeal epithelium, tongue, tonsil and lung, but not salivary glands Highest levels in oral swabs: 1.4–4.7 $\times 10^4$ FV RNA copies/cell
Cellular tropism	Superficial differentiated epithelial cells
Virus-specific $CD4^+$ T cell frequencies	In PBMC: UD–0.23% In BAL (lung): UD–4.6%
Virus-specific antibodies	Plasma: Gag IgG titer 356–8530 In some hosts, Plasma Gag IgA and SU-Env IgG Saliva: Gag IgG but no Gag IgA
Replication limited by $CD4^+$ T cell immunity?	No

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Vita

Shannon Murray grew up in Baltimore, Maryland, and was exposed to people from many cultures and socio-economic backgrounds during her upbringing. This influenced her in exploring many areas of study including international studies, third world development, computer science, and biology. At the University of Maryland in Baltimore County she studied International Studies and Biology, and in 1995 earned a Bachelor of Science. She became deeply fascinated with immunology after taking a graduate immunology course taught by Dr. Suzanne Ostrand-Rosenberg. She chose to continue her biology and immunology studies, and completed a Master of Science in Applied Molecular Biology from the University of Maryland in 1998. Shannon then became a research assistant at the Laboratory of Immunology at the Gerontology Research Center, National Institute of Aging, National Institutes of Health, where she researched telomere length and telomerase expression in lymphocytes during aging. In 2000, she moved to Seattle to join the Immunology Department at the University of Washington as a doctoral student. In 2002, she joined Maxine Linial's laboratory, and became part of the Molecular and Cellular Biology Program. Her research has been published in *Journal of Virology* and *Journal of Medical Primatology.* In 2007, she completed a Doctor of Philosophy at the University of Washington in Molecular and Cellular Biology.