Mucosal Simian Immunodeficiency Virus Transmission in African Green Monkeys: Susceptibility to Infection Is Proportional to Target Cell Availability at Mucosal Sites

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African green monkeys (AGMs) are naturally infected with a simian immunodeficiency virus (SIV_{agm}) that is nonpathogenic in its host. Although SIV_{agm} is common and widespread, little is known about the mechanisms that govern its transmission. Since the earliest virus-host interactions may provide key insights into the nonpathogenic phenotype of SIV_{agm}, we developed a mucosal transmission model for this virus. Using plasma from an acutely infected AGM as the virus inoculum, we exposed adult and juvenile AGMs, as well as pigtailed macaques (PTMs) as a nonnatural host control, by mucosal routes to increasing titers of virus and compared the doses needed to establish a productive infection. Four juvenile and four adult AGMs as well as two PTMs were intrarectally (IR) exposed, while two additional adult female AGMs were intravaginally (IVAG) exposed. No animal became infected following exposure to 10⁵ RNA copies. Both PTMs but none of the AGMs became infected following exposure to 10⁶ RNA copies. Finally, all adult AGMs and two of the four juvenile AGMs became infected following exposure to 10⁷ RNA copies, acquiring either one (2 IR infected juveniles, 1 IR infected adult, 2 IVAG infected adults) or two (3 IR infected adults) transmitted founder viruses. These results were consistent with immunophenotypic data, which revealed a significant correlation between the percentage of CD4⁺ T cells expressing CCR5 in the mucosa and the susceptibility to infection, in terms of both the viral dose and the numbers of transmitted founder viruses. Moreover, studies of uninfected AGMs showed that the fraction of CCR5-expressing CD4⁺ T cells increased significantly with age. These results indicate that (i) AGMs are readily infected with SIV_{agm} by both intrarectal and intravaginal routes, (ii) susceptibility to infection is proportional to the number of available CCR5⁺ CD4⁺ target cells in the mucosa, and (iii) the paucity of CCR5⁺ CD4⁺ target cells in infant and juvenile AGMs may explain the near absence of vertical transmission.

Simian immunodeficiency viruses (SIVs) naturally infect over 40 nonhuman primate (NHP) species in sub-Saharan Africa (4, 71). Many of these infections represent virus-host relationships that are evolutionarily older than human immunodeficiency virus (HIV) infection of humans (79), which may explain their lack of pathogenicity. Cross-species transmissions of SIVs from chimpanzees/gorillas (SIV_{cpz}/SIV_{gor}) and sooty mangabeys (SIV_{mm}) to humans have generated HIV type 1 (HIV-1) and HIV-2, respectively (10, 16, 34, 61, 64, 68). A key question that remains to be answered is why infections with the last two viruses are pathogenic, while natural host species exhibit a much more benign virus-host relationship.

To date, studies of natural NHP hosts have involved African green monkeys (AGMs), sooty mangabeys, and mandrills (69). Molecular epidemiological studies established that in these species the prevalence of SIV infection is high and increases with age (15, 20, 31, 58), suggesting sexual transmission as the main route of virus spread. Aggression and fights for dominance involving biting and exposure to blood are also known to occur in some species and may thus contribute to transmission (43). However, the mechanisms underlying natural SIV transmission are largely unknown.

Comparative studies with pathogenic HIV and SIV infections have revealed a number of similarities, including (i) comparable

levels of viral replication during acute and chronic infection (23, 44, 45, 48, 51, 56, 65), (ii) depletion of mucosal CD4⁺ T cells during acute infection (23, 51), (iii) increased levels of innate and adaptive immune responses during acute infection (5, 13, 28, 30, 35), (iii) preferential replication in short-lived activated CD4⁺ T cells (22, 55), and (iv) an inability of SIV-specific cellular and humoral immune responses to suppress virus replication (2, 12, 18, 19, 74, 80, 81). However, there are also key differences between natural SIV infections and pathogenic HIV/SIV infection, the most important of which is the fact that natural hosts are able to maintain normal levels of CD4⁺ T cells (23, 51) and generally avoid disease progression (57, 66). In addition, nonpathogenic SIV infections are characterized by (i) a near absence of vertical transmission (8, 54), (ii) an absence of chronic immune activation (23, 51, 53, 67), (iii) the preservation of mucosal CD4⁺ T cell

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homeostasis with normal levels of mucosal T helper type 17 (TH17) cells (6, 14), and (iv) an absence of microbial translocation (7, 50, 51). Thus, comparing and contrasting the immunologic, virologic, and clinical parameters of SIV infection in natural hosts with those of pathogenic HIV-1 infection of humans and SIVmac infection of macaques has the potential to elucidate the mechanisms that underlie disease progression in pathogenic HIV/SIV infections.

One approach to elucidate the mechanisms that govern the nonpathogenic phenotype of SIVagm is to focus on the earliest stages of infection. It has been shown that AGMs are able to induce a nonpathogenic outcome through the establishment of an anti-inflammatory milieu following the resolution of an initial stage of immune activation (30). Thus, studies of the earliest virus-host interactions may yield new insights into the mechanisms that are responsible for this. Moreover, most sexually acquired HIV-1 infections are caused by a limited number of transmitted founder viruses (32, 33). In contrast, infection by intravenous (i.v.) routes is usually associated with a less stringent bottleneck (1, 77). Because there may be a relationship between the number of transmitted variants and disease progression (62), we sought to establish a mucosal infection model for natural hosts using low-dose exposures to a virus inoculum directly derived from an acutely infected AGM and not passaged *in vitro* (48).

We have previously reported that, in contrast to Asian macaques and humans, natural host species such as AGMs, sooty mangabeys, mandrills, sun-tailed monkeys, and patas monkeys harbor low numbers of CD4⁺ CCR5⁺ target cells, especially at mucosal surfaces (49). It has been hypothesized that the reduced expression of CCR5 on CD4⁺ T cells, together with the low immune activation upon SIV infection, represents an evolutionary adaptation to limit CD4⁺ T cell destruction (69). In addition, a paucity of CCR5⁺ CD4⁺ T cells at mucosal sites may also explain the near absence of vertical transmission in natural hosts (8, 46, 54). In contrast, nonnatural hosts have significantly higher levels of CCR5⁺ CD4⁺ T cells in their mucosae, which has allowed us to test the hypothesis that susceptibility to SIV/HIV infection is a function of target cell availability. Toward this goal, we have performed mucosal transmission studies in AGMs and pigtailed macaques (PTMs). Since the fraction of CCR5-expressing CD4⁺ T cells is similar in the vagina and the rectum (72) and most data concerning mucosal transmission have been derived from intrarectally (IR) inoculated macaques, we have used the same route of exposure to infect AGMs. However, since it is unlikely that this mode of transmission contributes to the spread of SIVagm in the wild, we have also performed infection by the intravaginal (IVAG) route in two adult female AGMs. Here, we report a detailed analysis of the biological and molecular characteristics of these infections.

MATERIALS AND METHODS

Animals. The IR inoculation study included eight Caribbean AGMs (*Chlorocebus sabaeus*) and two PTMs (*Macaca nemestrina*) that were housed at the Tulane National Primate Research Center (TNPRC), which is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International facility. Of the eight animals, four were juveniles (aged between 3.4 and 3.6 years) and four were adults (aged between 6.4 and 8.6 years). The PTMs were adults (aged 5.9 and 6.2 years, respectively). All IR exposed NHPs were males.

The IVAG inoculation experiment included 2 AGM females (aged 8.4

and 8.6 years). These animals were housed at the RIDC animal facility of the University of Pittsburgh, which is an AAALAC International facility.

All animals were housed according to regulations set forth by the *Guide for the Care and Use of Laboratory Animals* (42) and the Animal Welfare Act. Animal experiments were approved by the Tulane University and University of Pittsburgh Institutional Animal Care and Use Committees (IACUCs).

In addition to the monkeys included in these mucosal transmission experiments, 65 blood samples, 36 jejunal biopsy specimens, and 15 esophageal biopsy specimens from a cohort of 65 SIV-uninfected *C. sabaeus* monkeys aged between 3 months and 24 years housed at Wake Forest University were available for quantitative analysis of age-related variations in the levels of CCR5⁺ CD4⁺ T cells in blood and at mucosal sites. The age distribution of the AGMs from which jejunal tissue was available is as follows: juvenile AGMs less than 4 years of age ($n = 23$; age <1 year, $n = 7$; age 1 to 2 years, $n = 4$; age 2 to 3 years, $n = 4$; age 3 to 4 years, $n = 8$) and adult AGMs ($n = 13$). Esophageal biopsy specimens were available only from infant AGMs that were less than 1 year of age and juvenile AGMs that were 1 to 4 years old. The protocols and procedures used in this study were approved by the Wake Forest University IACUC.

Dose-escalation infection studies. Eight AGMs and two PTMs were exposed three times at 2-week intervals to SIVagmSab92018 (48) by the IR route using a dose-escalation protocol, as follows: SIVagmSab92018 plasma stock containing 10^8 copies of SIVagm RNA per ml was diluted to 10^5 copies/ml and 1 ml was administered IR to each animal. Infection was monitored by real-time PCR (RT-PCR) performed on day 5 postinoculation (p.i.) and then at 3-day intervals. Animals which remained uninfected after the first exposure were inoculated a second time 2 weeks later with 1 ml of plasma containing 10^6 copies, followed by RT-PCR analysis to monitor infection. Animals which still remained uninfected after the second exposure were inoculated a third time with 1 ml of plasma stock containing 10^7 copies of SIVagmSab92018.

The two adult AGM females included in the IVAG inoculation experiments were also scheduled to receive escalating doses (10^7 , 10^8 , and 5×10^8 SIVagmSab92018 RNA copies) of SIVagm. However, since both females became infected after the first inoculation with 10^7 copies of SIVagmSab92018, subsequent exposures were canceled.

To minimize any experimentally induced alteration of the mucosa that might have impacted the efficacy of mucosal transmission, each dose of virus was administered only once (36). Also, IVAG exposure of the female AGMs was not performed at a specific stage of the menstrual cycle and did not involve hormonal pretreatment that may have altered the thickness of the vaginal mucosa.

The IR infected juvenile AGMs, the IVAG infected female AGMs, and the IR infected PTMs were euthanized at the peak of viral replication (days 12 to 15 postinfection [p.i.]), while the IR inoculated adult AGMs that became infected were followed for up to 100 days to compare the natural history of mucosally versus i.v. acquired SIVagm infections. The i.v. infected animals comprised historical control animals that have been described previously (48, 51, 55). The viral inoculum administered to the i.v. infected AGMs was identical to the ones used for mucosal inoculation.

Sampling of blood, peripheral LNs, and intestine. Blood was collected from all the animals at 3 time points prior to the initial virus exposure (days -35, -14, and -7 p.i.), at the time of SIVagm administration, and at days 6, 8, 10, and 12 p.i. For the adult animals that were followed for 100 days, blood collection after the peak of viral replication was as follows: weekly for 4 weeks and then every 2 weeks for the next 3 months. Lymph node (LN) biopsy specimens were collected only from adult AGMs on days -14, 8, 21, and 42 p.i. Intestinal biopsy specimens (proximal jejunum) consisting of approximately 10 to 15, 1- to 2-mm² pieces were obtained only from adult AGMs by endoscopically guided biopsy on days -18, 0, 8, 21, 29, 42, 72, and 100 p.i. To assess the availability of target cells at the inoculation site without facilitating transmission, rectal biopsies were performed on all animals 2 months prior to infection. Five i.v. in-

ected AGMs were used as controls, and the sampling schedule was similar.

Isolation of lymphocytes from blood, LNs, and intestinal and esophageal mucosae. Within 1 h of blood collection, whole blood was stained for flow cytometry. Plasma was aliquoted and stored at -80°C until viral load (VL) testing was performed. Peripheral blood mononuclear cells (PBMCs) were purified from whole blood by density gradient centrifugation using lymphocyte separation medium (LSM; Organon-Technica, Durham, NC). PBMCs were frozen at -80°C using freezing medium containing RPMI, heat-inactivated newborn calf serum (60%), and 10% dimethyl sulfoxide (DMSO).

Lymphocytes were separated from LNs by pressing tissue through a nylon mesh screen. Cells were filtered through nylon bags and washed with RPMI medium (Cellgro, Manassas, VA) containing 5% heat-inactivated newborn calf serum, 0.01% penicillin-streptomycin, 0.01% L-glutamine, and 0.01% HEPES buffer, as previously described (48, 51). Lymphocyte separation from pinch biopsy specimens was done as previously described (17, 50, 51, 55). Briefly, intestinal biopsy specimens were digested using EDTA followed by collagenase, and cells were then isolated using Percoll density gradient centrifugation (50, 51, 55). Within 30 min after separation, cells were stained for flow cytometry. Those cells not used for staining were frozen at -80°C in freezing medium.

Flow cytometry analysis of lymphocyte populations. Immunophenotyping of lymphocytes isolated from the blood, LNs, and intestine was performed using fluorescently conjugated monoclonal antibodies (MAbs) in multiparameter panels. Data were acquired on an LSR-II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star, Inc.). The following MAbs were used for flow cytometry: CD3-Pacific blue (clone no. SP34), CD4-allophycocyanin (APC; clone no. L200), CCR5-phycoerythrin (clone no. 3A9), HLA-DR-APC-Cy7 (clone no. L243), Ki-67-fluorescein isothiocyanate (FITC; clone no. B56) (all from BD Bioscience), and CD8 $\alpha\beta$ -Texas Red (clone no. 2ST8.5H7; Beckman Coulter). We and others have previously shown that all of these MAbs are cross-reactive with AGM lymphocytes (3, 18, 51, 81). Whole blood was lysed using fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences) and stained using a procedure previously described (48). Mononuclear cells from blood, LNs, and intestinal biopsy specimens were stained by incubation at 4°C for 30 min using an excess of MAb. Cells were then washed with phosphate-buffered saline (PBS) and fixed with BD stabilizing fixative (BD Bioscience). For intracellular stains, lymphocytes were fixed with 4% paraformaldehyde for 1 h. Cells were then washed with PBS, washed with a 0.1% saponin solution, incubated with Ki-67-FITC, washed with a 0.1% saponin solution, and then fixed with a BD stabilizing fixative. The absolute number of peripheral lymphocytes was determined by performing cell blood counts on each blood sample.

Viral load determination. Plasma VLs were quantified by RT-PCR, as previously described (48, 52, 55). Assay sensitivity was 100 RNA copies per 1 ml of plasma.

SGA of SIVagm env genes. To determine the number of transmitted founder viruses in newly infected animals, single-genome amplification (SGA) of viral sequences was performed as described previously (21). Briefly, viral RNA was extracted from plasma samples with an EZ1 virus minikit (version 2.0; Qiagen, Valencia, CA) and reverse transcribed using primer SIVagmENVoutR (5'-GTACCTGGCCCATCAGTGTAATTCTG C-3') and SuperScript III reverse transcriptase. The first-strand-synthesis reaction mixture contained $1\times$ reverse transcription buffer, 0.5 mM each deoxynucleoside triphosphate, 5 mM dithiothreitol, 2 units/ μl of RNase-OUT reagent, 10 units/ μl of SuperScript III reverse transcriptase, and 0.25 μM antisense primer. Dilutions of this cDNA were distributed in replicates of 16 PCRs to determine the dilution at which no more than 30% of reactions yielded amplicons, to ensure that most positive reaction mixtures contained a single template molecule. Full-length *env* genes were amplified by nested PCR using 1st-round sense primer SIVagmENVoutF (5'-CAGGTGCTGTAAGCCCAAGACACATC-3'), 1st-round antisense primer SIVagmENVoutR (5'-GTACCTGGCCCATCAGTGTAATTCTG

C-3'), 2nd-round sense primer SIVagmENVinF (5'-GCTATCATTTGTC CGCTTTGCTTCACTC-3'), and 2nd-round antisense primer SIVagmENVinR (5'-CTCACTGGGAAGCCAACCTCTTCTTC-3'). PCR was performed using Platinum *Taq* High Fidelity polymerase (Invitrogen, Carlsbad, CA) in the presence of $1\times$ PCR buffer, 2 mM MgSO_4 , 0.2 mM each deoxynucleoside triphosphate, 0.2 μM each primer, and 0.025 units/ μl of polymerase in a 20- μl reaction mixture. PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 56°C for 30 s, and 68°C for 5 min (first round) or 45 cycles with a 59°C annealing temperature (second round), followed by a final extension of 10 min at 68°C . Amplicons were inspected using 96-well E-gels (Invitrogen) and directly sequenced.

Statistical analysis. To compare measured variables at a given time point between IR and i.v. infected animals, we used the nonparametric Mann-Whitney test in Prism 5 (GraphPad) software. To analyze changes over time, we used linear mixed-effects models (60). Significance was assessed at the α equals 0.05 level. Prism 5 was also used to calculate the Mann-Whitney test and Spearman's rank-order correlation associated with the number of transmitted variants.

RESULTS

SIVagm is efficiently transmitted by mucosal routes. Most SIVs studied to date utilize CD4 and CCR5 as their receptor and coreceptor for gaining entry into target cells (71). However, the levels of CCR5 $^{+}$ CD4 $^{+}$ T cells in the respective host species differ, with naturally SIV-infected primate species generally exhibiting lower levels, especially in mucosal tissues (49). To directly compare the susceptibility to mucosal SIV infection in a natural and nonnatural host, we exposed eight AGMs and two PTMs to increasing doses of SIVagm by IR inoculation. We used uncultured plasma from an acutely infected AGM for our inoculum, since this infection stock has never been cultured. Virus inoculum was quantified at 1.83×10^8 SIVagmSab RNA copies/ml of plasma, and the titer was determined to be 1,580 50% tissue culture infective doses, as previously reported (48). The same virus stock was also used to infect PTMs, which have previously been shown to be susceptible to SIVagm infection (29). Finally, plasma from an animal with acute infection is known to be more infectious on a per-particle basis than plasma from one with chronic infection (40). To determine the lowest dose capable of establishing a productive infection, we exposed the animals to increasing copy numbers of SIVagm RNA. Each virus inoculum was independently examined by RT-PCR and determined to contain 1.18×10^5 , 1.07×10^6 , and 1.11×10^7 SIVagm copies/ml. Each dose was administered only once to prevent immune activation and inflammation. Moreover, rectal biopsies were performed 2 months prior to the first IR inoculation, so as to not increase the risk of SIVagm transmission through mucosal lesions or areas of inflammation. Vaginal biopsies, which are more traumatic, were not performed. As shown in Fig. 1, AGMs and PTMs varied considerably in their susceptibility to mucosally administered SIVagm. Neither PTMs nor AGMs became infected when exposed to an inoculum containing 10^5 copies of SIVagm. However, a 10-fold higher dose of 10^6 RNA copies was sufficient to infect both PTMs, while all eight AGMs remained uninfected (Fig. 1). An additional log unit increase to 10^7 RNA copies resulted in the infection of all adult and 2 of the 4 juvenile AGMs. These results show that (i) AGMs are susceptible to SIVagm infection transmitted by IR routes, (ii) adult AGMs require a 10-fold higher viral dose than adult PTMs to establish a productive infection, and (iii) juvenile AGMs seem to be less susceptible to IR infection than adult AGMs.

Because IR routes are unlikely to be relevant to the spread of

FIG 1 Development of a mucosal transmission model for SIVagm. PTMs (black solid lines), adult AGMs (red solid lines), and juvenile AGMs (orange solid lines) were exposed to sequential IR administration of 10^5 , 10^6 , and 10^7 SIVagm RNA copies. PTMs became infected after IR exposure to 10^6 SIVagm copies. All adult AGMs and half of juveniles became infected after a dose escalation to 10^7 SIVagm copies. Two female AGMs were exposed to an IVAG dose of 10^7 SIVagm copies. x axes represent the number of days that elapsed after each exposure; y axes represent SIVagm RNA loads/ml.

SIVagm in the wild, we also tested the IVAG transmission route in two adult female AGMs. Of note, our inoculation strategy did not involve two inoculations at a 4-hour interval, as recommended by the majority of vaginal exposure protocols (70). We also opted not to thin the vaginal mucosa through pretreatment with hormones (38, 41). Since IVAG transmission in rhesus macaques (RMs) requires viral doses 2 to 3 orders of magnitude higher than those for IR transmission (11), we reasoned that the same might be true for AGMs. We thus selected a starting dose of 10^7 RNA copies, which, based on data from rhesus macaques, seemed unlikely to be sufficient to establish a productive infection. However, as shown in Fig. 1, both female AGMs became infected after a single administration of 1.12×10^7 RNA copies into the vaginal vault. Although limited to just two animals, these results suggest that SIVagm is readily transmitted by IVAG routes using doses that are similar to those required for IR infection. In addition, it is clear that employing plasma containing large amounts of cell-free virus is an effective means of transmitting SIVagm through mucosal routes.

Mucosal SIVagm transmission is characterized by a genetic bottleneck. A substantial bottleneck occurs when HIV is transmitted through heterosexual contact, with only one virus being transmitted in about 80% of cases (32). The frequency of multiple-variant transmission is higher in men who have sex with men (~40%) and intravenous drug users (~60%), corresponding to the higher epidemiological risk observed in these groups (1, 37). A similar bottleneck has also been documented in experimentally infected rhesus macaques, where higher challenge doses resulted in a greater number of transmitted variants (39). In light of these data, we sought to quantify the number of transmitted founder viruses in both IR and IVAG infected AGMs (Fig. 2).

The number of transmitted variants was inferred from phylogenetic analyses as described previously (32, 33). Of the four adult IR infected AGMs, one acquired a single variant (HK14), while the other three each acquired two variants (Fig. 2). In animal FV83, a potential third variant was identified; however, the fact that this variant, which was represented by a single sequence, was a recombinant of the other two lineages present in the same animal strongly suggested that it was generated after infection. In contrast, both juvenile AGMs, which were infected with the same dose

of SIVagm, harbored only a single transmitted founder virus (Fig. 2) (adult, 1, 2, 2, 2; juvenile, 0, 0, 1, 1 [Mann-Whitney test, two-tailed, $P = 0.057$]). Thus, both the percentage of animals infected and the numbers of their transmitted founder viruses suggested that adult AGMs are relatively more susceptible to SIVagm than juveniles. Finally, each of the two IVAG infected females acquired a single transmitted founder virus, demonstrating that although these animals were infected following a single exposure, the dose was not overwhelming mucosal barriers.

Phylogenetic analysis of SGA-derived viral sequences also allowed us to assess the distribution of transmitted viruses within the challenge stock (Fig. 3). IR transmitted founder viruses were interspersed throughout the tree, excluding the possibility that only certain viruses within the challenge stock were fit for rectal transmission. The two IVAG transmitted founder variants were relatively more closely related, differing by only 5 nucleotides. Additional work is needed to determine whether this is a chance occurrence or whether the two variants shared properties that made them more prone to IVAG transmission.

Mucosal SIVagm transmission depends on the availability of target cells. Our previous work suggested that the efficiency of SIV transmission depends on the number of susceptible target cells present at mucosal sites (49, 54). To test this hypothesis directly, we determined the abundance of $CD4^+$ T cells expressing CCR5 in the rectum of all IR inoculated animals (Fig. 4a). The IVAG infected females were excluded from this analysis since cell separation from the vagina necessitates large amounts of tissues, which would have inflicted considerable trauma (72). FACS analysis of the mononuclear cells isolated from the rectal mucosa revealed a significantly smaller fraction of $CD4^+$ T cells expressing CCR5 in AGMs than in PTMs (5% and 45%, respectively) (Fig. 4a). Moreover, juvenile AGMs exhibited a significantly ($P = 0.0277$) smaller fraction of $CD4^+$ T cells expressing CCR5 than adult monkeys (3% and 7%, respectively), with the lowest values (2%) being observed in the two juvenile AGMs that did not become infected (Fig. 4a). Similar results were also observed for blood samples from the same animals (Fig. 4b). Finally, there was a significant correlation between the percentage of $CD4^+$ T cells expressing CCR5 in the preexposure rectal biopsy specimen and the number

of transmitted founder viruses (Spearman's correlation = 0.9449, $P = 0.001$). Taken together, these data strongly suggest that mucosal target cell availability determines susceptibility to infection.

To examine whether the lower level of CD4⁺ T cells expressing CCR5 in juveniles relative to adults is a general feature of AGMs, we analyzed the age-related levels of this T cell subset in the blood (Fig. 4c) and jejunal mucosa (Fig. 4d) of a larger cohort of uninfected *C. sabaues* monkeys housed at the Wake Forest University NHP facility. We observed a significant increase in the levels of CD4⁺ T cells expressing CCR5 at the time of sexual maturity in both the peripheral blood (Fig. 4c) and the jejunum (Fig. 4d). When infant and juvenile AGMs were grouped together, their levels of CD4⁺ T cells expressing CCR5 in the jejunum were significantly lower than those in adult animals (>5 years old) ($P < 0.0001$). We also examined the abundance of CCR5-expressing CD4⁺ T cells in a subset of infant and juvenile AGMs for which esophageal mucosa samples were available. These infant and juvenile (Fig. 4d) animals all showed very low levels of CCR5-expressing CD4⁺ T cells, raising the possibility that these low levels contribute to the lack of breast-feeding transmission in AGMs (46).

Natural history of mucosal SIVagm infection. All previously reported studies in natural hosts of SIVs (AGMs, mandrills, sooty mangabeys) involved i.v. inoculation of large amounts of virus (22, 23, 44, 45, 48, 51, 52, 54, 55, 65), and it is well-known that many variants are transmitted when this infection strategy is used (reference 21 and others). Conversely, IR and IVAG administration of SIVagm resulted in only a few transmitted viruses, indicating a significant mucosal bottleneck. To examine whether the natural history of SIVagm infection differed following mucosal and intravenous transmission, we compared VLs and CD4⁺ T cell depletion in IR and i.v. infected animals, limiting this analysis to adult animals. First, as shown in Fig. 5a, the pattern of viral replication was similar between mucosally and i.v. infected animals. However, as previously seen for SIVmac (59), there was a slight delay in viral ramp-up in the mucosally infected animals, with peak viremia occurring at days 15 p.i., 12 p.i., and 8 to 10 p.i. in IVAG, IR, and i.v. infected animals, respectively. Second, peak viremia levels were lower in mucosally infected AGMs, but this difference was not statistically significant. The small number of IVAG infected AGMs precluded a statistical evaluation. With the resolution of acute infection and passage to the chronic stage, VLs became similar between IR and i.v. infected AGMs, resulting in equivalent set-point VLs during chronic infection.

The delay in peak viremia in IR versus i.v. challenged AGMs was also reflected in the number of mucosal CD4⁺ T cells, which were more rapidly depleted in i.v. infected monkeys (Fig. 5b). During chronic infection, mucosal CD4⁺ T cells were partially restored, with no statistically significant differences between the two groups. In the periphery, only a transient CD4⁺ T cell depletion occurred and was similar between the two groups. CD4⁺ T cell counts returned to preinfection levels during the chronic phase in both IR and i.v. challenged AGMs (data not shown). No

significant differences with regard to changes in other major lymphocyte populations were noted between the two groups (data not shown). Finally, a comparison of T cell activation and proliferation levels between IR and i.v. infected AGMs (Fig. 5c) showed a delay in peak T cell activation in the i.v. infected animals, although the magnitude of immune activation during the acute infection was similar between IR challenged monkeys and those i.v. inoculated. Thus, with the exception of a slight delay in peak viremia, there were no significant differences in the natural history of SIVagm infection in IR versus i.v. infected monkeys.

DISCUSSION

In this study, we have investigated mucosal transmission in African green monkeys, which represent a natural host of SIV. We found that (i) SIVagm can be readily transmitted by both intrarectal and vaginal routes, (ii) the susceptibility to SIVagm infection is proportional to the availability of target cells at mucosal sites, and (iii) the likelihood of SIVagm acquisition increases with age.

All experimental infections carried out thus far in natural SIV hosts, such as AGMs, mandrills, and sooty mangabeys, have involved i.v. inoculations of large quantities of virus (44, 45, 48, 50, 51, 54, 55, 65). We used sequential administrations of increasing amounts of virus that had not previously been passaged *in vitro*, followed by an enumeration of the transmitted founder viruses to characterize the extent of the mucosal bottleneck. Thus, our transmission design is more physiologically relevant and may thus be more appropriate to compare and contrast the initial virus-host interaction of pathogenic and nonpathogenic SIV infections.

Macaques are not a natural host of SIV, and mucosal transmission of SIVmac does not naturally occur in this species. Most previous studies have involved SIVmac strains that were heavily passaged (9). We describe here mucosal infections using a plasma virus stock that was collected at the peak of viral replication from an i.v.-infected *C. sabaues* monkey (21, 48). There are three advantages of using an acute infection plasma stock for mucosal transmission studies. First, it represents a naturally occurring SIVagm strain that was never adapted in tissue culture. Second, it has a higher quasispecies complexity than is typically seen in stocks of SIVmac (21). Finally, it is highly infectious, having been collected at the end of the ramp-up stage of viral replication. In macaques, the number of infectious SIV particles is significantly higher in ramp-up plasma than in set-point-stage plasma (40).

Mucosal transmission studies in AGMs revealed several interesting features. First, the dose of virus necessary to initiate infection in AGMs appears to be 1 order of magnitude higher than the dose required to infect PTMs. Interestingly, this does not seem to impact SIVagm spread in the wild, where prevalence rates are extremely high, with up to 80% of adult multiparous AGM females being infected (58). The reason for this may be very high steady-state levels of SIVagm replication. In chronically HIV-1-infected humans, average levels of plasma VLs during the chronic phase of infection are 3×10^4 RNA copies/ml (75). In a study of over 100 wild-living AGMs from South Africa, the average VLs

FIG 2 Enumeration of transmitted founder viruses. Single-genome amplification was used to generate SIVagm *env* sequences from the plasma of four IR infected adult AGM males (A to D), two IR infected juveniles (E and F), and two IVAG infected adult females (G and H). Sequences were compared by Highlighter plot analysis as described previously (32), with the sequence length (in bp) indicated on the x axis and number of transmitted founder viruses (V) indicated on the y axis. REC marks one recombinant virus in animal FV83, which was most likely generated postinfection. Tick marks indicate differences compared to the top sequence (red, T; green, A; blue, C; orange, G; gray, gap).

FIG 3 Evolutionary relationships of mucosally transmitted founder SIVagm. The phylogenetic relationship of mucosally transmitted SIVagm *env* sequences is shown in relation to sequences from the plasma virus stock (red). Sequences from the same animal are color coded. Each newly infected animal harbors one or two low-diversity lineages, indicative of transmitted founder viruses. REC marks one recombinant virus in animal FV83, which was likely generated postinfection. The tree was constructed using the neighbor-joining method (63). Asterisks on nodes indicate bootstrap values of $\geq 80\%$; the scale bar represents one nucleotide.

FIG 4 Susceptibility to SIVagm infection depends on target cell availability. Abundance of CCR5-expressing CD4⁺ T cells in the rectum (a) and peripheral blood (b) in intrarectally exposed AGMs and PTMs, as determined by flow cytometry. The x axis shows individual animals; the y axis denotes the percentage of CCR5-positive T cells. Blue bars, PTMs; orange bars, AGMs that did not become infected after intrarectal exposure; red bars, AGMs that became infected after intrarectal exposure; violet bars, AGMs that became infected after intravaginal exposure. Juvenile AGMs had lower levels of target cells than adult AGMs. In the juvenile group, the lowest target cell levels were observed in those monkeys that did not become infected. (Lower panels) Cross-sectional assessment of CCR5⁺ CD4⁺ T target cells both in peripheral blood (c) and at mucosal sites (d) in a cohort of uninfected AGMs. (c) In the peripheral blood, the levels of CD4⁺ T cells expressing CCR5 is very low in monkeys aged less than 4 years and dramatically increase after this age, which corresponds to sexual maturity. This increase is highly significant and parallels increases in SIV prevalence in the wild. The linear regression line and 95% confidence intervals (dashed lines) are shown. The x axis represents the age of the animals; the y axis represents the fraction of CD4⁺ T cells expressing CCR5, as determined by flow cytometry. (d) Similar results were observed at two mucosal sites; thus, CD4⁺ T cells from both the esophageal and jejunal mucosae from uninfected juvenile AGMs express very low levels of the CCR5 molecule. Significantly higher levels of CCR5 expression by jejunal CD4⁺ T cells are observed in adult AGMs ($P < 0.0001$).

were up to 2 log units higher (C. Apetrei, unpublished data). Assuming similar mechanisms of mucosal shedding, the higher set-point viral load levels in AGMs are probably facilitating SIVagm transmission through sexual contact. Nonetheless, the higher infection threshold in AGMs may explain the absence of vertical transmission. Indeed, RNA viral loads in milk of chronically infected AGMs and acutely infected mandrills (10^4 and 10^5 copies/ml, respectively) are similar to those reported in macaques (54, 76), while the doses needed to initiate mucosal infection are 1 log unit higher in AGMs than in RMs.

A second unexpected finding of our study is that IR and IVAG infections with SIVagm seem to require similar amounts of virus. In humans, the probability of infection through anal intercourse is much higher than that through vaginal or penile exposure (25). This was also observed in macaques, where smaller amounts of virus are needed for intrarectal transmission than for intravaginal transmission (11). The greater vulnerability of the gastrointestinal tract is likely due to the fact that the colorectal mucosa is comprised of a single layer of columnar epithelium, which is fragile and may be easily damaged during intercourse. In contrast, the vaginal stratified malpighian epithelium or the keratinized fore-

skin epithelium may represent a more robust barrier (25). Lymphocytes in the colorectal tissue are also constitutively activated, providing the cell targets that support virus replication (24, 73). Finally, the female genital mucosa is impacted by the menstrual cycle, which affects both cervical tissue and mucus characteristics (78). It will be important to determine whether IVAG and IR infection doses will differ in larger numbers of AGMs.

The strongest correlate to explain the difference in susceptibility between PTMs and AGMs, as well as between juvenile and adult AGMs, was the relative abundance of CCR5-expressing CD4⁺ T cells at mucosal sites. Similar to other African NHP species that are natural hosts of SIVs, AGMs have very low levels of CCR5⁺ CD4⁺ target cells at the mucosal surfaces (49). It has been proposed that the restriction of CCR5 expression to more activated effector CD4⁺ T cells may confine SIV replication to a subset of more expendable cells, thus preserving central memory CD4⁺ T cells, despite high levels of viremia (47). It has also been proposed that low CCR5 expression may reduce the homing of activated CD4⁺ T cells to the gut (49, 69). Finally, we have hypothesized that a paucity of CD4⁺ T cells expressing CCR5 in mucosal tissues may influence SIV transmission (49). Our current results

FIG 5 Natural history of mucosal SIVagm infection. Comparative assessment of plasma viral loads (a), duodenal CD4⁺ T cells (b), and immune activation, as assessed by expression of Ki-67 on the CD8⁺ T cells from the blood (c), in intrarectally infected versus intravenously infected AGMs. A slight delay in the peak of viral replication was observed in intrarectally infected AGMs (red solid lines) compared to intravenously infected AGMs (black solid lines). No differences in the levels of chronic viral replication were observed between the two groups. No significant differences in the levels or timing of mucosal CD4⁺ T cell depletion existed between the two groups (b). Increased immune activation during acute SIVagm infection paralleled viral replication, being slightly delayed in intrarectally infected AGMs.

provide direct evidence in support of this hypothesis. Moreover, at least in mandrills and AGMs, the reported low CCR5 expression levels on CD4⁺ T cells may explain the near absence of SIV transmission via breast-feeding (54). A caveat of our study is the relatively small number of animals. Research on additional wild and captive monkeys is needed to confirm whether the availability of target cells at mucosal sites is a major determinant of transmission.

In conclusion, we report here the development of a mucosal SIV transmission model in a natural SIV host. This new model may be employed to assess the earliest events of persistent non-progressive natural SIV infection in order to establish their impact on the benign outcome of this infection. Studies in macaques

demonstrated that during these early stages of infection, a window of opportunity exists when the virus is highly vulnerable and can be controlled (26, 27). Comparing and contrasting the sequence of these early events in natural and nonnatural hosts may be informative for designing strategies for controlling HIV infection. Finally, our study provides proof of concept that strategies aimed at reducing the number of target cells may have a major impact on HIV transmission and may be useful as a tool to control pandemic spread.

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