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Older Rhesus Macaque Infants Are More Susceptible to Oral Infection with Simian-Human Immunodeficiency Virus 89.6P than Neonates

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Earlier primate studies revealed that oral transmission of immunodeficiency viruses can occur at all ages [R. M. Ruprecht et al., J. Infect. Dis. 179(Suppl. 3):S408–S412, 1999]. Using a stock of pathogenic simian-human immunodeficiency virus, SHIV89.6P, we compared the 50% animal infectious dose needed to achieve systemic infection after oral challenge in newborn and older infant or juvenile rhesus macaques. Unexpectedly, the older monkeys required a 150-fold-lower virus challenge dose than the neonates ($P = 3.3 \times 10^{-5}$). In addition, at least 60,000 times more virus was needed to achieve systemic infection in neonates by the oral route than by the intravenous route ($P < 1 \times 10^{-5}$). Thus, route of inoculation and age are important determinants of SHIV89.6P infectivity in rhesus macaques.

Oral transmission of simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus (SHIV) strains in rhesus monkeys has been used as a model system (1, 2) to study oral HIV transmission in humans. In human infants, HIV may be transmitted orally during delivery (17) and through breastfeeding (18); in adults, oral HIV transmission through sexual contact has been described (27).

We have previously established a rhesus monkey model to mimic postnatal HIV transmission through breastfeeding (1); this model uses nontraumatic oral challenge with cell-free viruses and allows evaluation of preventive strategies against milk-borne virus transmission (3, 11, 11a, 13, 15). Several SIV and SHIV strains with different tropism have been titrated orally in newborn monkeys to establish reproducible oral challenge systems (3, 15, 21).

We hypothesized that neonates are more susceptible to infection than older infants, given the neonates' smaller size and more immature immune system. Using SHIV89.6P as test virus, we sought to measure age-related differences in oral virus transmissibility. SHIV89.6P was derived from the nonpathogenic SHIV89.6, which expresses the envelope glycoproteins of primary, dual tropic HIV89.6 (7). After passage of SHIV89.6 through several rhesus monkeys, the resulting SHIV89.6P became acutely pathogenic, causing profound $CD4^+$ T-cell depletion within 2 weeks postchallenge (20). Our SHIV89.6P stock had been generated in rhesus monkey peripheral blood mononuclear cells (PBMC) and contained 2.6×10^4 50% tissue culture infectious doses (15).

To determine the 50% animal infectious dose (AID_{50}) in neonatal rhesus monkeys, infants born by normal vaginal delivery were exposed orally during the first 3 days of life to various dilutions of the SHIV89.6P stock. After all oral intake was withheld for 4 h, 3 ml of the diluted virus was placed nontraumatically at the back of the tongue. Blood was collected and real-time reverse-transcriptase PCR, as described previously (14), was used to analyze viral loads in plasma at weeks 0, 1, 2, and 4 postinoculation. In addition, PBMC of the virus-exposed monkeys were monitored repeatedly for 6 or 7 weeks by cocultivation, as described previously (2). In monkeys challenged as neonates, a virus dilution of 1:40 resulted in systemic infection, whereas a dilution of 1:100 did not (Table 1). The statistical method of Spouge (23) was used to determine the AID_{50} of the SHIV89.6P stock in this group of animals. The AID_{50} for the oral route was approximately 3.3×10^{-2} (95% confidence interval [CI], 7.59×10^{-3} to 1.42×10^{-1}) (Table 2). The results of monkey PBMC cocultures confirmed these data (Table 3); no virus was detected for animals RCu-6 and RZt-6 (Table 3). As expected, infected infants demonstrated marked losses of $CD4^+$ T cells compared to the published values obtained from age-matched, uninfected rhesus monkeys (8) (Table 1).

To determine the susceptibility of older infants to orally inoculated SHIV89.6P, the same virus stock was used; the protocol included careful oral exams prior to oral inoculation. Animals between the ages of 11 and 23 months were enrolled (Table 1); no significant oral pathology was noted in any animal. A virus dilution of 1:6,000 resulted in systemic infection in both animals that had been given this dilution, whereas a dilution of 1:10,000 did not. The AID_{50} was 2.2×10^{-4} (95% CI, 5.8×10^{-5} to 9.6×10^{-4}) (Table 2). When this dose was compared with that required to infect half of the newborn infants by the oral route, we found that 150-fold more virus was needed to infect the latter ($P = 3.3 \times 10^{-5}$). Thus, our titra-

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TABLE 1. SHIV89.6P titrations in neonatal and older rhesus monkeys

Route of inoculation	Animal	Age	Dilution	Systemic infection present	Peak viral RNA load at week 2 (RNA copies/ml)	$CD4^+$ T cells (cells/μl)[a]	
						at week 2	at week 4
Oral	RGt-6	1 day	1	Yes	110,750	1,240	397
	RHt-6	1 day	1:10	Yes	5,094,275	82	115
	RJv-6	1 day	1:20	Yes	15,036,775	2,880	150
	RJu-6	3 days	1:20	Yes	1,237,798	0	0
	RUz-6	2 days	1:40[b]	Yes	164,852	ND[c]	63
	RCu-6	1 day	1:100	No	<200	3,726	2,435
	RZt-6	1 day	1:1,000	No	<50	ND[c]	ND[c]
	RSt-7	11 months	1:6	Yes	363,726,500	27	42
	RAu-7	11 months	1:10	Yes	23,319,530	1,489	1,176
	RSs-7	12 months	1:100	Yes	27,688,625	68	66
	RGn-7	16 months	1:1,000	Yes	1,922,178	180	110
	REu-7	18 months	1:3,000	Yes	14,342,610	382	578
	RAy-7	18 months	1:6,000	Yes	8,824,345	1,058	1,323
	RPj-7	23 months	1:6,000	Yes	12,564,025	562	483
	RRd-7	16 months	1:10,000	No	<50	3,421	3,504
Intravenous	RZv-6	1 day	1:2,000	Yes	44,761,565	14	ND[c]
	RVv-6	1 day	1:20,000	Yes	52,827,105	23	14
	RWz-6	2 days	1:200,000	Yes	25,133,855	54	ND[c]
	RTe-7	1 day	1:2,000,000	Yes	484,813	110	0

[a] The absolute number of $CD4^+$ T cells in naive infants at week 2 was 3,025; at week 3, 3155; and at week 8, 2,585 (8).
[b] The ratio of the minimal oral dose (1:40) to the assumed minimal i.v. dose ($1{:}2 \times 10^6$) in neonates was at least 50,000.
[c] ND, not determined.

tions revealed a highly significant age dependence in the rhesus monkeys' susceptibility to oral SHIV89.6P inoculation.

Next, we assessed the different doses of the same SHIV89.6P stock required to achieve systemic infection of neonatal rhesus monkeys after intravenous (i.v.) versus oral inoculation. For i.v. challenge, the injected volume was 1 ml of virus at various dilutions. The highest virus dilution (1:2,000,000) still resulted in systemic infection of the neonate (Table 1). Because no virus-challenged animal remained uninfected, the determination of the AID_{50} with i.v. challenge ($AID_{50\text{-iv}}$) according to the method of Spouge (23) was problematic. In a simulation, we assumed that the next animal inoculated with the same dilution of 1:2,000,000 would remain uninfected. Based on this worst-case scenario, we estimated an $AID_{50\text{-iv}}$ for neonates at 5×10^{-7} (95% CI, 9.14×10^{-8} to 4.5×10^{-6}) (Table 2); however, even less virus may have been required. This estimated $AID_{50\text{-iv}}$ was compared to the AID_{50} of 3.3×10^{-2} for the oral route in neonates (Table 2). According to these AID_{50} values, at least 60,000-fold more virus was needed to achieve infection by the oral route than by the i.v. route ($P < 1 \times 10^{-5}$). In a previous study (4), we had compared oral versus i.v. challenges in adult rhesus monkeys, using minimal infectious doses of SIVDeltaB670 as parameters. We found that the ratio of the minimal oral dose to the minimal i.v. dose ($r_{\text{min-adult}}$) was approximately 1,000 (4). In the present neonatal study, the estimated $r_{\text{min-neonate}}$ was at least 50,000 (Table 1), a value similar to that obtained when AID_{50} values were compared (Table 2). Together, the r_{min} values reflect a relative 50-fold resistance of the oral route in neonatal monkeys compared to that in adults. It would be interesting to perform oral and i.v. challenge studies in neonatal macaques with SIVDeltaB670 to confirm this notion, as this virus differs in its coreceptor usage from that of SHIV89.6P. SIVDeltaB670, a primary biological isolate (5), was shown to use CCR5 but not CXCR4 (9).

In addition, we sought to compare peak viral RNA loads at week 2 after SHIV89.6P challenge in our three cohorts (Table 1). Only animals with systemic infection were included in the analysis. No dose response between virus inocula and peak viral RNA levels was observed in any of the three cohorts. We then compared viral load distribution in the five neonates versus that of the seven older animals infected orally. The peak viral RNA load was 10-fold higher in the older animal cohort

TABLE 2. SHIV89.6P AID_{50} as a function of age and route of inoculation

Route of inoculation	Age of monkey	AID_{50}[a]	95% CI	*P* value[d]
Oral	1–3 days	3.3×10^{-2}	7.6×10^{-3}–1.4×10^{-1}	
	11–23 months	2.2×10^{-4}	5.8×10^{-5}–9.6×10^{-4}	3.3×10^{-5}[b]
Intravenous	1–2 days	5.0×10^{-7}	9.1×10^{-8}–4.5×10^{-6}	$<1.0 \times 10^{-5}$[c]

[a] The AID_{50} was determined by the statistical method of Spouge (23).
[b] The oral AID_{50} in neonates compared to the oral AID_{50} in older animals.
[c] The oral AID_{50} in neonates compared to the i.v. AID_{50} in neonates.
[d] The *P* values listed are significant with the Bonferroni correction.

TABLE 3. Results of the cocultivation of PBMC isolated from monkey infants after oral virus exposure[a]

No. of weeks postinoculation	Infected monkey PBMC/10^6 cells for indicated monkey:						
	RGt-6	RHt-6	RJv-6	RJu-6	RUz-6	RCu-6	RZt-6
0	0	0	ND[b]	0	0	0	ND[b]
1	1,640	10	ND[b]	1,040	ND[b]	0	0
(1.5)/2	102	26	>1	410	ND[b]	0	(0)
3/4	6	6	ND[b]	6	(16)/64	0	(0)/0
5	ND[b]	ND[b]	ND[b]	6	ND[b]	ND[b]	0
(6)/7	26	ND[b]	>64	ND[b]	0	0	(0)

[a] Cocultivation was performed as described (2).
[b] ND, not determined.

than in neonates (two-sided P value = 0.048 by the Wilcoxon rank-sum test) (16). In addition, we compared the viral RNA load distribution of the five orally infected neonates versus that of the four i.v.-infected neonates. No statistically significant differences were seen when the same nonparametric test was used. Thus, neither route of inoculation nor virus dose influenced peak viral RNA loads once systemic infection had been achieved in neonatal monkeys. In contrast, peak viral RNA loads differed significantly as a function of age after oral inoculation.

To summarize, our data showed for the first time that monkeys of different ages differ significantly in their susceptibility to oral SHIV89.6P challenge; in newborns, an oral virus dose more than 2 logs higher than that for older monkeys was required to achieve systemic infection. The underlying mechanisms for the differential susceptibility could involve a number of factors.

Saliva contains a number of nonspecific factors with antiviral activity, such as lactoferrin, secretory leukocyte protease inhibitor (SLPI), mucins, proline-rich proteins, and cystatins (22). Possibly, in our experiments age-related differences in saliva composition led to different levels of viral inactivation in the oral cavity. Interestingly, a recent study found that human neonates had higher concentrations of salivary SLPI than older infants; in a group of uninfected infants breast-fed by HIV-positive mothers for 1 month, a significant association was seen between higher SLPI levels in saliva and a decrease in HIV transmission (10). The site(s) of viral entry after oral inoculation is unknown; the virus could enter the submucosal tissues in either the oral cavity, esophagus, stomach, or intestine (4, 19, 24, 25). When highly concentrated SIV was applied directly to tonsils in rhesus monkeys, virus entry through these lymphoid tissues and subsequent rapid spread to local and regional lymph nodes were demonstrated (24). These results, however, do not rule out that the virus can also pass across intact mucosal surfaces at other levels in the gastrointestinal tract. It is possible that age-related differences in mucosal permeability influenced SHIV89.6P transmission after oral inoculation.

Age-related differences in the virus target cell population, such as higher levels of chemokine coreceptor expression and/or the state of cellular activation, may account for the significantly higher peak viral RNA loads we observed in neonatal monkeys versus those in older monkeys with systemic infection after oral challenge. The latter animals were not only more susceptible to the oral challenge but also seemed to replicate SHIV89.6P to higher levels once systemic infection had been established. We postulate that systemic infection is achieved once the virus inoculum exceeds a predetermined threshold, which differs for the various routes of inoculation and is age dependent, according to our new data. However, once this threshold has been surpassed and systemic infection is established, there is no association between the inoculum size and peak viral RNA; this lack of a dose response has been observed previously (6) and is not surprising for an outbred population of primates.

The levels of lentiviral receptor and coreceptor expression in tissues are key determinants for virus entry into target cells and subsequent spread to distant sites (26). In the present study, we inoculated neonatal and older monkeys with dual tropic SHIV89.6P, a virus that utilizes either CCR5 or CXCR4 in vitro. A previous study sought to correlate the coreceptor usage of three variants of HIV89.6, all of which were dual tropic when tested in vitro, with their coreceptor usage when assayed ex vivo in human tonsillar explants (12). In this tissue microenvironment, one HIV89.6 variant surprisingly preferred CXCR4, another used CXCR4 and CCR5 equally, and the third exhibited a preference for CCR5; the two X4 strains were more cytopathic than the one preferring CCR5 in tonsillar explants. In our rhesus monkey study, the actual coreceptor(s) used by SHIV89.6P to initiate replication after oral inoculation is unknown, but given its pronounced pathogenicity in this species, preferred usage of CXCR4 would not be unexpected. Age-related variations in CXCR4 or CCR5 expression levels in gastrointestinal tissues could have influenced the transmissibility of SHIV89.6P in our animals. Thus far, no data have been published regarding chemokine receptor expression in mucosal tissues as a function of age in rhesus macaques. However, the pattern of chemokine receptor expression in the central nervous system of rhesus monkey neonates differed from that of older infants; CCR5 and CXCR4 expression increased significantly from birth to 9 months of age on neurons and glia (26). Similar age-related increases in chemokine expression could account for the age-related differences in oral SHIV89.6P transmission in our study.

In summary, our primate studies have revealed a significant difference in the susceptibility of neonatal and older rhesus macaques to oral SHIV89.6P transmission. It will be important to test whether virus strains with different tropism, especially SHIV strains encoding R5 HIV envelope genes, exhibit similar age dependence for transmission through the oral route or other mucosal routes.

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