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Intrarectal Transmission of Simian Immunodeficiency Virus in Rhesus Macaques: Selective Amplification and Host Responses to Transient or Persistent Viremia†

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Intrarectal simian immunodeficiency virus (SIV) infection in rhesus macaques is a model for sexual transmission of primate retroviruses. Phylogenetic studies on envelope gene sequences that were present in blood following intrarectal SIV inoculation provided evidence for selective amplification of a subset of viruses present in the inoculum and defined one amino acid sequence uniquely associated with intrarectal infection. Both persistent and transient viremia states were observed after intrarectal infection. Immune responses in persistently infected animals accounted for slower rates of disease progression despite the presence of highly pathogenic viruses that were documented by transfusion studies. Transient viremia elicited protective immunity against subsequent intrarectal virus challenge but did not protect against intravenous virus challenge. Transient viremia usually but not always led to self-limiting infection. In one animal, we documented a relapse to active viremia long after the initial transient viremia. SIV transmission across mucosal barriers affects pathogenesis in the short term by limiting the types of viruses established in the host and in the longer term by establishing host responses that slow disease progression despite the presence of highly pathogenic viruses in blood.

Sexual transmission is the predominant mode for epidemic spread of human immunodeficiency virus type 1 (HIV-1). Viruses in genital secretions contact mucosal surfaces, gain access to the underlying blood or lymphoid tissues, and then spread systemically. Transmission across mucosae may also be important for intrapartum infection of newborns (6) or for oral infection as a consequence of breast feeding (21). Animal studies on mucosal simian immunodeficiency virus (SIV) inoculation in macaques (11, 16, 18) and HIV-1 inoculation in chimpanzees (9) and analyses of natural HIV-1 transmission in humans (1, 10, 25, 28), including recent reports that some HIV-1 strains are transmitted preferentially by heterosexual contact (20), depicted mucosal barriers as complex biological filters that restrict the population of viruses gaining access to the host. We studied intrarectal SIV infection in the rhesus macaque to understand selective pressures that control primary infection, the virus-host interactions within mucosal tissues, and how these mechanisms affect disease progression.

Intrarectal SIV infection in rhesus is characterized by selective amplification of a subset of viruses present in the inoculum (23). Sexual transmission of HIV-1 in humans also resulted in amplification of a subset of viruses present in the donor, although common envelope gene sequence motifs that might be associated with mucosal transmission were not observed among recently infected individuals (28). We returned to questions about selective amplification in the macaque model. Multiple animals were inoculated intrarectally or intravenously with a single virus stock to eliminate the type of complexity encountered when natural HIV-1 transmission is studied in humans. Analysis of envelope gene sequences present in peripheral blood mononuclear cells (PBMC) soon after intravenous or intrarectal transmission provided additional information about selective amplification mechanisms. Moreover, these studies revealed a correlation between virus dose, route of inoculation, development of protective immunity, and disease progression, all pointing to the unique features of mucosal virus transmission in primates.

We and others noted an unusual infection condition termed transient viremia after mucosal inoculation of rhesus macaques with low doses of SIV. Transient viremia was recognized as a possible outcome that is distinct from the more familiar state of persistent infection with evident disease progression (12). Transient viremia was noted after intrarectal (16) or intravaginal inoculation (12). Using virus challenge experiments coupled with envelope gene sequencing studies, we evaluated transient viremia in the context of mechanisms responsible for sequence selection across the mucosal barrier and the role for mucosal inoculation in eliciting regional antiviral immune responses. Our studies also document a relapse of active viremia, suggesting that virus replication can continue in an animal long after the interval of transient viremia.

MATERIALS AND METHODS

Virus stock and animal inoculation. Two- to three-year-old rhesus monkeys were selected from the Wisconsin Regional Primate Research Center colony. They were determined to be negative for type D retroviruses and for SIV by serological testing and by in vitro virus isolation assays. The SIV-negative diagnosis was confirmed by PCR assays for SIV long terminal repeat (LTR) sequences (16). The SIVmac251 virus stock (7) was obtained from Ronald C. Desrosiers (Harvard University and New England Regional Primate Research Center), and then titers were determined in macaques and in cell culture as described previously (19). Virus doses from 0.1 to 1,000 animal infective doses (ID) were used in our studies. Viral inocula were prepared in 1 ml of RPMI 1640 medium without serum supplement. Intravenous inoculation was by the saphenous vein. Intrarectal inoculation was performed by inserting a French 18 soft

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TABLE 1. Plasma antigenemia (p27) during the first 139 days after intrarectal or intravenous inoculation

Day	Antigenia (ng of p27/ml of plasma)[a]											
	90090, 10 i.v.	90100, 10 i.v.	90047, 1,000 i.r.	90051, 1,000 i.r.	90036, 100 i.r.	90019, 100 i.r.	90061, 10 i.r.	90113, 10 i.r.	90089, 1 i.r.	90054, 1 i.r.	90072, 0.1 i.r.	90046, 0.1 i.r.
Preinfection	0	0	0	0	0	0	0	0	0	0	0	0
Postinfection	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
13	2.22	0.36	0	0	0	0	0	0	0	0	0	0
20	0.32	0	0	0	0	0	0	0	0	0	0	0
27	3.74	0	0	0	0	0	0	0	0	0	0	0
34	0.06	0.09	0	0	0.06	0	0.11	0.06	0	0.06	0.04	0.06
41	0	0.08	0.06	0	0	0	0.11	0.06	0	0.09	0.07	0.09
55	0	0	0.73	0	0	0	0.06	0.07	0.54	0	1.2	0
69	0.69	0.04	0.78	0.112	0.19	0	0.06	0.07	0.09	0.72	2.6	0
83	0.17	0	0.04	0.08	0	0.05	0	0.4	0.1	0.11	0.15	0
97	0.13	0	0	0.06	0.04	0.17	0	0	0.09	0.01	0.07	0
111	0.69	0.04	0.41	0.01	0.33	0.31	0	0	0	0	0	0
125	1.6	0.51	0.85	0.61	0.67	0	0	0	0	0	0	
139	2.2	2.5	1.7	0	3.1	0.89	0	0	0	0	0	0

[a] Determined by using the Dupont p27 detection kit for animals given the indicated ID intravenously (i.v.) or intrarectally (i.r.).

pediatric nasogastric catheter approximately 10 cm into the rectum. The virus inoculum was delivered in 1 ml, and the catheter was flushed with an additional 5 ml of medium. Animals were tested weekly or biweekly for 2 months and then at approximately monthly intervals. Peripheral blood samples were collected, body weights were determined, and physical examinations were performed. Animal 90051 was individually housed beginning 1 year after inoculation until the time of study.

Monitoring of plasma p27 antigen levels. Assays for SIV p27 protein in plasma samples were performed by using commercial reagents (Coulter p27 antigen detection kit).

Cytotoxicity assay. Effector cells were PBMC isolated by density gradient centrifugation and cryopreserved in 10% dimethyl sulfoxide (tissue culture grade; Sigma, St. Louis, Mo.)–20% fetal calf serum (Harlan Bioproducts, Indianapolis, Ind.)–RPMI 1640 (Gibco, Grand Island, N.Y.). In preparation for cytotoxic T-lymphocyte (CTL) assays, effector cells were thawed, washed three times in RPMI 1640, cultured for 3 days at 5×10^6 cells per ml in RPMI 1640 with 5 μg of concanavalin A (Sigma) per ml washed, and supplemented for 4 days with 20 U of recombinant interleukin-2 (a gift from John Detrich, Biological Response Modifiers Program, National Cancer Institute, Vienna, Va.) per ml. Target cells were autologous or allogeneic B-lymphoblastoid lines made by transformation with herpesvirus papio (a gift from N. Letvin, Beth Israel Hospital and Harvard Medical School). Titers of recombinant vaccinia virus stocks (a gift from Therion Biologics, Boston, Mass.) were determined on HeLaT4+ cells. $Na_2{}^{51}CrO_4$ (NEZ-030; NEN/Dupont, Boston, Mass.) was incorporated at 100 μCi/10^6 target cells. Spontaneous release varied from 10 to 20%. Specific release was calculated as (experimental release − spontaneous release)/(release in 1% Nonidet P-40 − spontaneous release) × 100. Specific release values greater than 10% were considered positive.

Virus isolation assays. Virus isolation assays were performed with density gradient-purified PBMC isolated from infected animals. A total of 10^6 to 10^7 phytohemagglutinin-stimulated PBMC were plated with 10^6 CEM×174 cells and incubated for up to 30 days. Virus isolations were confirmed by the presence of cytopathic effect in the culture, by reverse transcriptase assay (Boehringer Mannheim), and by p27 antigen assay.

Cloning and sequence analysis of the envelope gene. Amplification and cloning of viral DNA sequences was performed as described previously (23), using primers specific for envelope regions. Primary amplifications were done with the sense primer beginning at position 7020 (5′CCACTCAGTGCCTACC3′) and an antisense primer beginning at position 8561 (5′GCTGTTGCTGTTGCTGCAC 3′). Secondary or nested amplifications used a sense primer beginning at position 7114 and including CAU sequences for incorporation in the Gibco pAMP plasmid system (5′CAUCAUCAUCAUGGCAATAGAGGATGTATGGC3′); the antisense primer began at position 8228 and also included sequences for vector insertion (5′CUACUACUACUAGGTCTCCCTCTCTTGGAGGC3′). The amplified and cloned fragment was 1,114 bp in length. At least five separate amplification reactions were pooled before fragments were cloned. Several independent cloning reactions were also performed for each template. Plasmid sequences were selected and sequenced; in most cases, both DNA strands were sequenced for each plasmid, and some regions were sequenced multiple times. Sequence data were compiled and analyzed by using MacVector 4.5.3 software.

Phylogenetic analysis of envelope sequences. Maximum parsimony analysis of SIV envelope gene clones was performed with PAUP 3.1.1 software (22); analyses used amino acid sequences translated from primary nucleotide sequence data. A weighted assumptions block matrix was used to convert amino acid differences into the minimum number of nucleotide substitutions in each codon. A heuristic search was done for 73 replicates with a Maxtrees Saved option of 5 at each step. Phylogenetic trees were created with a monophyletic sister outgroup containing SIVmac239, SIVmac251, 46*140 (consensus sequence for envelope genes from animal 90046 at 140 weeks after original inoculation), and in vitro sequences. Data are reported as the scored distance between branch points of the phylogram based on amino acid sequences.

Nucleotide sequence accession number. The complete envelope sequences are available from GenBank under accession numbers U62333 through U62383.

RESULTS

Transient viremia after low-dose intrarectal inoculation. Rhesus macaques were inoculated intrarectally with 0.1 to 1,000 animal ID of pathogenic SIVmac (16). The animal ID is defined as the minimum intravenous inoculation required to establish persistent infection with active viremia and evident disease progression in age-matched rhesus macaques (17). We examined SIV p27 antigenemia following intrarectal inoculation in 10 animals and in two control animals given 10 ID by intravenous inoculation.

The response to virus exposure depended on the dose and route of inoculation. The control animals with intravenous exposure developed an established infection with stable positive virus isolation and viral DNA in PBMC (16). Plasma p27 antigen was detected in all animals receiving SIV inoculation but was not detected in any of the preinoculation samples (Table 1). Serum antibody responses were detected in one of two animals after intravenous infection (16).

Four animals received 1,000 or 100 ID by intrarectal inoculation. Three developed a stable infection with positive virus isolation, viral DNA in PBMC, and serum antibody responses to SIV (16). The fourth animal (90051) developed a transient viremia that was characterized by an inability to isolate virus from PBMC during 3.5 years after inoculation despite having detectable viral DNA in PBMC during the first year after inoculation (16) and the transient appearance of p27 antigen in blood (Table 1). No serum antibody response to virus was detected in 90051. Six of six animals inoculated intrarectally with 10 to 0.1 ID developed transient viremia that was similar to the pattern observed in 90051 (Table 4).

Envelope gene sequences in intrarectally infected animals. We reported previously a selective amplification of LTR sequences during mucosal transmission in this same cohort of SIVmac-infected animals (23). On the basis of the distribution

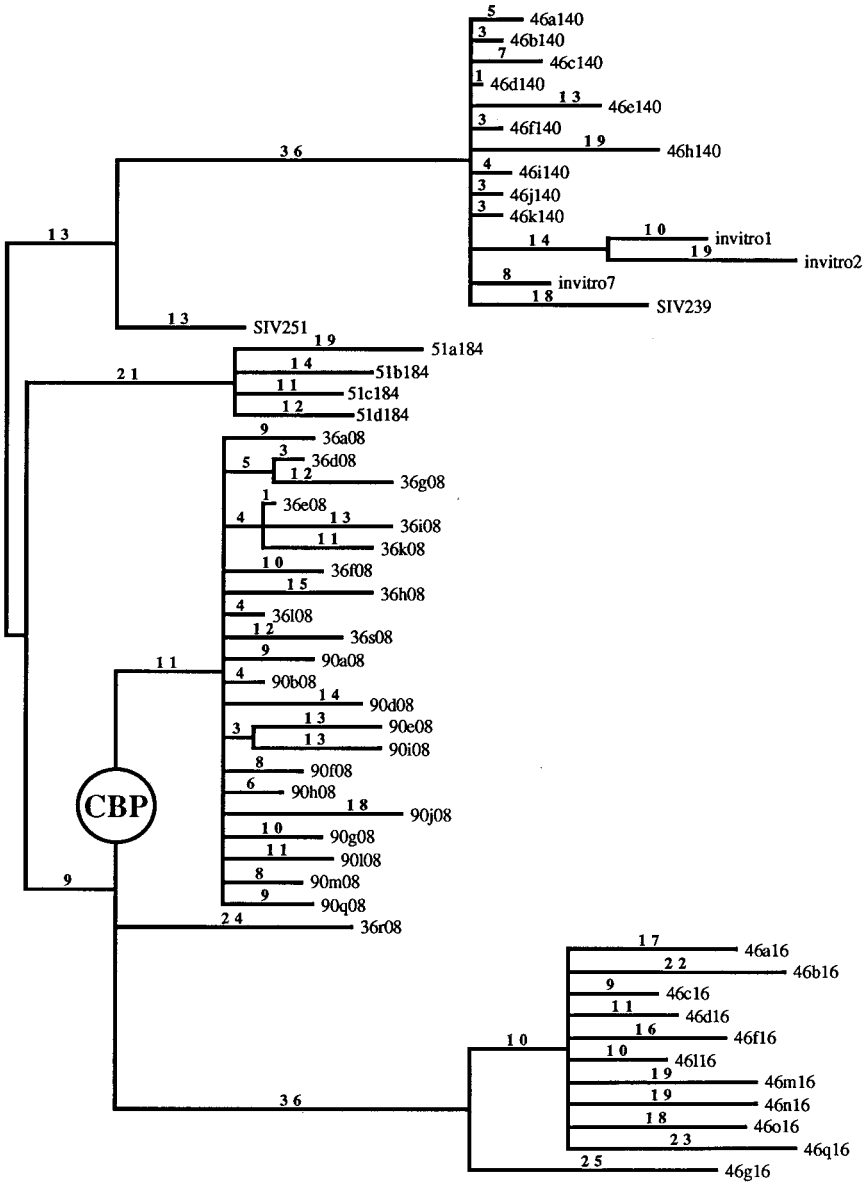

FIG. 1. Phylogenetic analysis of envelope gene sequences, using PAUP version 3.1.1. Sequences are numbered as indicated in the text. The CBP represents the most likely precursor to 36*08 and 90*08 groups. Numerical distances reflect sequence divergence from the nearest branching point.

of LTR sequences, we selected three animals for analysis of envelope gene sequences. Envelope genes including nucleotides 7114 to 8228 were amplified from PBMC DNA templates; this portion of envelope includes variable region 1 (V1) at one end and stretches to the CD4 binding region at the other end. We selected this portion because of extensive variation reported previously for the V1, V2, and V4 regions of envelope and relatively conserved sequences in the constant and CD4 binding site regions (2, 3, 15). DNA sequences were converted to amino acid sequences and analyzed for phylogenetic relationships. A total of 34 envelope sequences were analyzed from animals 90090 (10 ID intravenously), 90036 (100 ID intrarectally), and 90046 (0.1 ID intrarectally). 90090 and 90036 had stable infections with evident disease progression, and 90046 showed transient viremia (16).

Phylogenetic analysis using the PAUP software for bootstrap analysis with tree bisection-reconnection branch-swapping showed the relatedness of envelope sequences after 73 calculation replicates. Envelope genes from animals 90090 (designated 90*08, where 90 is the animal identification number, * designates this as a group or consensus sequence, and 08 shows that samples were collected 8 weeks after inoculation) and most examples from animal 90036 (36*08, indicating consensus sequence at 8 weeks after inoculation from animal 90036) comprised a single large group of related sequences. Only the sequence 36r08 diverged from this group and comprised a distinct phylogenetic branch with a single member. A common branch point (CBP) was defined as the progenitor sequence for the largest single group (90*08 plus 36*08). On average, 36*08 and 90*08 sequences differed from the CBP by a score of 22 (excluding the 36r08 sequence), and the 36*08 sequences were indistinguishable from the 90*08 sequences by phylogenetic analysis. Molecular clone sequences were included for comparison; the SIVmac251 sequence had a score

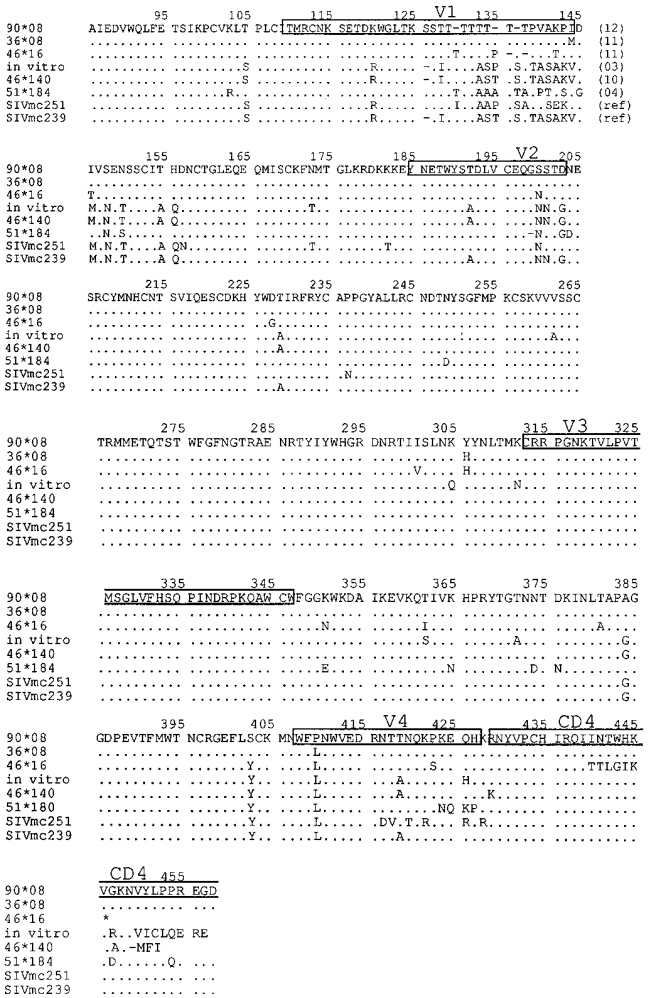

FIG. 2. Aligned consensus sequences for intrarectal (36*08 and 46*16), intravenous (90*08), intrarectal challenge (46*140), in vitro (in vitro 1, 2, and 7), and relapse (51*184) infections with reference sequences (SIVmac251 and SIV mac239). Predicted amino acid sequences are aligned and compared with the 90*08 consensus sequence. Numbers in parentheses to the right of the first line indicate the number of sequences used to generate the consensus or reference (ref) sequence. Amino acid identities are indicated by dots, deletions are indicated by dashes, and new amino acids are represented. Unique histidine residues at position 306 in 36*08 and 46*16 sequences are shown in boldface. The stop codon at position 446 in 46*16 is shown by an asterisk. Sequences identified as V1, V2, V3, V4, and CD4 binding sites are indicated by boxes and labels. The CD4 binding site is incomplete in our sequence data.

of 35 from the CBP, and the SIVmac239 sequence had a score of 76 from the CBP. Envelope genes from the 90*08 or 36*08 group were 98.0% identical to 46*16 envelope sequences (indicating consensus sequence from animal 90046 at 16 weeks after inoculation) and 96.3% identical to the SIVmac239 sequence at the nucleotide level.

Envelope sequences from the low-dose intrarectal inoculation animal 90046 (transient viremia) are also compared in this phylogenetic analysis (Fig. 1). Envelope sequences labeled 46*16 comprised a distinct phylogenetic group with individual sequences differing from the CBP by a average score of 62. Variation in the V1 region distinguished 46*16 sequences from those of the 90*08 and 36*08 groups. The 46*16 group was relatively homogeneous with the exception of 46g16 and was different from the pathogenic molecular clone sequence SIVmac239. The 46*16 group was on average 94.9% identical to SIVmac239 at the nucleotide sequence level. A important feature of 46*16 sequences was the presence of a stop codon in the CD4 binding region (Fig. 2). Even though this sequence appears near the end of our envelope gene clones, it was observed in all clones from samples that included multiple independent PCR amplifications and independent cloning experiments. Despite the stop codon, viral DNA sequences were present in PBMC from 90046 immediately after infection (16) and were detectable for 30 weeks after inoculation (not shown).

We compared envelope sequences obtained after in vitro or in vivo infection with the same virus stock. Sequences labeled "in vitro 1, 2, and 7" were included in the phylogenetic analysis and were distinct from sequences identified in the original animal inoculation studies. Template DNA samples were collected from rhesus PBMC cultures 8 hours after infection to prevent subsequent virus spread or selection of viral variants (23). The in vitro envelope sequences were closely related to the SIVmac239 envelope sequence and were distinct from envelope sequences in the 90*08, 36*08, and 46*16 groups. Oligonucleotide primers were designed to be specific for V1 and V4 region signature sequences present in the 36*08, 90*08, and 46*16 sequences and not present among in vitro sequences or the SIVmac239 and SIVmac251 sequence. These primers detected very low levels of in vivo-type DNA sequences after in vitro infections with the primary SIVmac stock (not shown), indicating that viruses established after intrarectal inoculation were present in the original stock but were near the limit of PCR detection.

Transiently viremic animals were resistant to intrarectal but not intravenous SIVmac challenge. Transiently viremic animals had unique outcomes after infection. We tested whether transient viremia after intrarectal inoculation could engender a specific immune response to SIV. Beginning 1 year after the original intrarectal virus exposure, transiently viremic animals were challenged with intrarectal doses of virus. On the basis of the initial titration data, we selected 200 ID for the challenge dose. All challenge experiments were performed with the same virus stocks as used in the original infection study. In vitro virus titration and inoculation of control animals confirmed the stability of virus stocks.

The results of virus challenge experiments (Table 5) showed that 200 ID intrarectally was sufficient to establish a stable infection in both control animals (91039 and 91041). At the same time, four transiently viremic animals were exposed to intrarectal challenge with 200 ID of SIVmac, and three transiently viremic animals remained as controls. Animals 90051, 90061, and 90046 were exposed to 200 ID intrarectally. Only 90046 developed a persistent infection after challenge, and the others remained negative for virus isolation. A second challenge experiment at 30 months involved 90113, 90089, and 90072 receiving 200 ID intrarectally; all three resisted the challenge (Table 6).

Six of the intrarectally inoculated animals (three with persistent infection and three with transient viremia) were assayed for CTL activity. Five of six displayed very low but reproducible CTL activity against SIV antigens. The assay shown in Table 3 occurred 3.5 years after the initial infection, though the data are similar to those for assays done before and after challenge inoculations. Four of six animals, including 90054 with transient viremia, had CTL activity against *gag*-expressing targets; two of six had CTL activity against *nef*-expressing targets. Anti-*env* CTL was also detected in some uninfected animals (Table 3 and references 26 and 27); however, *gag*- and *nef*-specific CTL activities were detected only in infected animals.

TABLE 2. Virus transmission by whole-blood transfusion in rhesus macaques transiently or persistently infected by SIV

Original dose/animal no.	Time (wk) when transfusion was performed	Donor status	Survival (wk) after transfusion for:	
			Donors	Recipients
100 ID/90036	40	Viremic	30	22
0.1 ID/90072	40	Negative[a]	112[b]	Not infected (sacrificed at 22 wk)
100 ID/90019	63	Viremic	86	23
1,000 ID/90047	63	Viremic	133	28
1,000 ID/90051	195[c]	Viremic	30 (surviving)	11
1,000 ID/90051	195[c]	Viremic	30 (surviving)	28
1,000 ID/90051	195[c]	Viremic	30 (surviving)	30 (surviving)

[a] No detectable viral DNA in PBMC, viral antigen in plasma, infectious cells in PBMC, or serum antibody to virus at 40 weeks after inoculation. The animal was originally described as having transient viremia.
[b] Animal was enrolled in an unrelated terminal study.
[c] Animal 90051 developed transient viremia after original inoculation and had a relapse of active viremia 3.5 years later.

Three transiently viremic animals that resisted high-dose intrarectal challenge experiment were subsequently inoculated intravenously with 10 ID of SIVmac (Table 6). All three animals developed a persistent infection with positive virus isolation from PBMC, serum antibody responses to virus, and evident disease progression defined by weight loss and decline in absolute numbers of CD4 cells. At 130 weeks, 90061 and 90054 were challenged with 20 ID intravenously; both developed persistent infection. Animals 90113, 90089, and 90072 were susceptible to transfusion-associated infection at 185 weeks, using 90051 (relapse) as the blood donor (Table 6). These data exclude the possibility that animals were generally resistant to SIVmac challenge, and they support the contention that transient viremia provided resistance only to intrarectal virus challenge. In a total of seven intrarectal virus challenge experiments using transiently viremic animals, only one persistent infection was established. Protection against mucosal challenge after transient intrarectal infection was significant ($P = 0.03$).

Envelope gene sequences were obtained for animal 90046 4 weeks after the second intrarectal virus inoculation (this was the only example of persistent infection following intrarectal challenge of a transiently viremic animal). The 10 sequences (46*140, indicating 140 weeks after the original intrarectal inoculation and 4 weeks after the intrarectal challenge) were different from envelope sequences detected in this animal after the original intrarectal virus inoculation (Fig. 2). Viruses present after the successful challenge were most similar to in vitro envelope sequences and the published SIVmac239 envelope sequence. On average, 46*140 sequences were a score of 64 away from the CBP and a score of 126 different from the 46*16 sequences. The 46*140 sequences were on average 94.8% identical at the nucleotide sequence level to the 46*16 sequences. The 46*140 sequences were 99.8% identical at the nucleotide sequence level to the envelope gene from SIVmac239.

Relapse after transient viremia. At 3.5 years after the original virus inoculation, we detected the first positive virus isolation in animal 90051. This macaque was exposed originally to 1,000 ID intrarectally, resulting in transient viremia, and was challenged intrarectally with 200 ID. No positive virus isolations were detected after the original or challenge inoculation. The initial positive virus isolation was recorded more than 1 year after the intrarectal challenge experiment. We used phylogenetic analysis in an attempt to determine whether virus in 90051 was a relapse from the original infection or whether persistent infection occurred as a result of the challenge. Four envelope gene sequences from 90051 were compared with all other sequences by phylogenetic analysis (Fig. 1). These sequences were an average score of 44 from the CBP, compared with distances of 22 for the 36*08 and 90*08 group, 35 for SIVmac251, 76 for SIVmac239, 62 for 46*16 sequences, and 64 for the 46*140 sequences. Inspection of 51*184 sequences within the V1 region (Fig. 2) showed that they are more related to the 36*08 and 90*08 group than to the inoculum (in vitro 1, 2, 7). Apparently, envelope sequences in 51*184 are the product of a relapsed infection in which low and undetectable levels of virus replication continued long enough after transient viremia to permit sequence divergence and evolution of a pathogenic virus. Virus arising in 90051 replicated well in vitro and established pathogenic infection in three naive recipients that were inoculated with 10 ml of whole blood via intravenous transfusion (Tables 2 and 6).

Comparisons among consensus sequences. Consensus sequences represent the most likely common precursor for each phylogenetic branch in the tree of envelope sequences. Consensus envelope sequences for 46*16, 36*08, 90*08, 51*184, 46*140, and in vitro sequences were compared with SIVmac239 and SIVmac251 reference sequences. Our goal in these comparisons was to identify amino acids uniquely present in 36*08 and 46*16 sequences that might affect intrarectal transmission efficiency. The 46*140 sequences were excluded because they appeared only after challenge of a transiently viremic animal and may be a consequence of immune selection for envelope variants.

Differences between the 90*08, 36*08, 46*16 group of consensus sequences and the reference group SIVmac239, SIVmac251, in vitro infection consensus sequences included threonine in place of serine at amino acid position 105, lysine for arginine at position 120, proline for lysine at position 143, histidine for glutamine at position 156, and alanine for glycine at position 384. A tyrosine at position 306 was conserved in 90*08, in vitro, SIVmac251, and SIVmac239 sequences but was changed to histidine in 36*08 and 46*16 sequences. The effect of this amino acid change on mucosal transmission efficiency is being tested by constructing and characterizing recombinant viruses. Despite reports (28) that viruses present after primary mucosal inoculation had a monocytotropic phenotype, we were unable to relate any amino acid sequence variations in our samples to the envelope changes described previously (13) that convert SIVmac239 to a monocytotropic phenotype.

The 46*16 sequences contained (in 10 of 11 sequences) a deletion of nucleotide T at base 8170 that created a amber stop codon in place of amino acid 446. We cannot rigorously ascribe the transient viremia status of these animals to a defective envelope gene; however, this finding may account for the inability to isolate virus from the same PBMC samples used to obtain 46*16 sequences. We do not yet understand how viruses with envelope stop codons can remain in peripheral blood for

TABLE 3. CTL activity among intrarectally infected animals with persistent or transient viremia[a]

Effector (ID)	Target[b]	% Specific lysis at effector/target ratio of:		
		25:1	50:1	100:1
90047 (1,000 i.r.), persistent viremia	Uninfected	−0.1	1.0	−1.5
	VVwt	0.5	−1.7	0.6
	VVenv	15.5	22.5	29.3
	VVgag	5.2	8.3	11.5
	VVnef	1.2	2.3	0.5
90019 (100 i.r.), persistent viremia	Uninfected	0.2	0.2	−1.0
	VVwt	1.8	−1.5	−0.5
	VVenv	10.6	12.8	18.4
	VVgag	4.4	7.8	13.5
	VVnef	2.5	3.6	4.2
90061 (10 i.r.), transient viremia	Uninfected	−1.3	−1.8	−1.3
	VVwt	−0.5	−0.2	−0.4
	VVenv	10.5	12.5	20.3
	VVgag	4.4	5.9	7.1
	VVnef	6.5	8.0	10.9
90054 (1 i.r.), transient viremia	Uninfected	−1.3	−1.0	−1.1
	VVwt	−0.7	−0.8	−1.4
	VVenv	9.1	11.0	18.6
	VVgag	4.3	7.6	12.9
	VVnef	2.8	5.2	6.0
90072 (0.1 i.r.), transient viremia	Uninfected	−1.0	−0.6	−0.2
	VVwt	−1.7	1.2	1.0
	VVenv	11.3	16.0	19.2
	VVgag	3.2	2.1	2.4
	VVnef	3.6	3.8	2.5
90046 (200 i.r.), persistent viremia after rechallenge	Uninfected	−1.5	−1.6	−1.3
	VVwt	−1.8	−1.8	−2.0
	VVenv	7.7	15.6	21.8
	VVgag	4.9	6.9	14.5
	VVnef	4.4	7.1	11.3
91070, uninfected	VVwt	−0.2	−1.0	−1.0
	VVenv	9.2	12.0	17.3
	VVgag	0.0	−1.1	−1.0
92093, uninfected	VVwt	−0.6	−0.3	−0.8
	VVenv	3.4	3.3	1.8
	VVgag	−0.4	0.7	

[a] Data are from one CTL assay (1 January 1995, 3.5 years after initial infection) that is representative of other assays, performed at least 4 times and as many as 10 times per animal, that yielded similar results. CTL activity in a 5 h ^{51}Cr release assay using 5×10^3 target cells per well is expressed as percent specific lysis (percentage of total release). Lysis of VVwt-infected targets and uninfected targets was always below 2% specific lysis. Specific lysis ≥10% was considered positive. i.r., intrarectally.

[b] B-lymphoblastoid cell targets were infected with vaccinia virus construct NYCBH (VVwt) or vaccinia virus constructs expressing SIVmac251 *gag*, *env*, and *nef* (VVenv, VVgag, and VVnef) at a multiplicity of 3 PFU per cell at 16 h before the assay.

up to 30 weeks after transient viremia, though it is possible that translation reads through the stop codon at some low efficiency as was shown recently for Ebola virus glycoprotein (24).

Transfusion studies to evaluate determinants of disease progression. Sequence analysis and phylogeny studies helped to identify amino acid changes associated with intrarectal transmission to evaluate infection after challenge among transiently viremic animals and to examine relapse long after transient viremia. We also used blood transfusions from persistently infected donors to naive or transiently viremic macaques to assess the virulence of viruses present in animals with persistent infection and slow disease progression (Tables 2 and 6).

Peripheral blood from three persistently infected animals was transfused into three separate, naive recipients (Table 2).

TABLE 4. Summary of original inoculation and outcomes[a]

Animal no.	Original dose (ID) (August 1991)	Status (90 wks)
90090	10 intravenously	Dead at 4 mo
90100	10 intravenously	Dead at 11 mo
90047	1,000 rectally	Viremic
90051[b]	1,000 rectally	Transient
90036	100 rectally	Dead at 13 mo
90019	100 rectally	Viremic
90061	10 rectally	Transient
90113	10 rectally	Transient
90089	1 rectally	Transient
90054	1 rectally	Transient
90072	0.1 rectally	Transient
90046	0.1 rectally	Transient

[a] Data from reference 17.

[b] Animal 90051 had a relapse of active viremia at 180 weeks after original inoculation. Animal 90051 was the blood donor for transfusions conducted at 185 weeks.

As a control, peripheral blood from 90072 (transiently viremic) was also transfused to a naive recipient. The transiently viremic animal failed to transfer infection to the transfusion recipient. When persistently infected animals (originally infected by high-dose intrarectal inoculation) were used as transfusion donors, the recipient animals experienced a rapid disease leading to death within 28 weeks. Animal 90051 with relapse viremia was also used as blood donor into animals with transient viremia states (Table 6). All of these animals became infected, and one died by 11 weeks whereas two others were surviving but had reduced CD4 cell counts at 15 weeks. These data confirm the presence of highly pathogenic viruses in intrarectally infected animals.

DISCUSSION

Intrarectal SIVmac inoculation provided the first example of an unusual infection status (16) that is now designated transient viremia (12). In addition, persistent SIVmac infection after intrarectal inoculation led to slower disease progression than in intravenously infected animals. Using our standard virus stocks, juvenile macaques infected by intravenous inoculation showed an average time of 173 days until CD4 cells were not detected in blood, and 189 days was the average time to death. We include both values because death is generally by euthanasia following a period of general morbidity. Among animals with persistent viremia after intrarectal infection with the same virus stock, disease progression was much slower. For three animals described in this study, one died 13 months after inoculation and 2 others were surviving as along as 3 years after

TABLE 5. Evidence that animals with prior transient viremia resist high-dose rectal challenge (200 ID) at 95 weeks

Animal no.	Status (90 wk)	Outcome (125 wk)
90051[a]	Negative after transient[b]	Negative
90061	Negative after transient	Negative
90054	Negative after transient	Negative
90046	Negative after transient	Viremic
91039	Uninfected control	Viremic
91041	Uninfected control	Viremic

[a] See Table 4, footnote *b*.

[b] No detectable viral DNA in PBMC, viral antigen in plasma, infectious cells in PBMC, or serum antibody to virus in animals known to have previously had transient viremia.

TABLE 6. Evidence that animals with prior transient viremia resist high-dose rectal challenge but are susceptible to intravenous challenge

Animal no.	Status[a]	Challenge (ID)[b]	Outcome (156 wk)
Expt 1			
90061	Negative after challenge	20 intravenously	Viremic
90054	Negative after challenge	20 intravenously	Viremic
90089	Negative after transient	200 rectally	Negative
90113	Negative after transient	200 rectally	Negative
90072	Negative after transient	200 rectally	Negative
Expt 2			
90113	Negative after challenge	Transfusion from viremic	Viremic
90089	Negative after challenge	Transfusion from viremic	Viremic
90072	Negative after challenge	Transfusion from viremic	Viremic

[a] At 125 or 180 weeks for experiments 1 and 2, respectively.
[b] At 130 or 185 weeks for experiments 1 and 2, respectively.

inoculation before being enrolled in other terminal studies. These data are similar to what we observed in seven other intrarectally infected animals that are not discussed here. To understand transient viremia and to determine whether slowed disease progression was another aspect of immune responses uniquely triggered by rectal inoculation, we studied the biological consequences of this mucosal infection route, including development of specific immunity and the possible role for unique virus sequences selected as a result of the transmission mechanism.

Envelope sequences similar to that of the molecular clone SIVmac239 (14) were recovered after in vitro infection with our standard virus stock. These SIVmac239-related envelope sequences were not present in animals infected by intravenous or intrarectal exposure except for one case in which intrarectal virus challenge was successful in an animal previously characterized as having transient viremia. Consensus sequences for each of the major groups (intravenous, high-dose intrarectal, and low-dose intrarectal) were compared with those of molecular clones SIVmac239 and SIVmac251 to search for patterns that might be required for efficient mucosal transmission. One sequence change (histidine for tyrosine at position 306) was associated with intrarectal transmission. It is known that biological properties of HIV envelope can be changed by a single amino acid substitution (5). We must extend our analysis to additional animals and develop molecular cloned viruses to establish a firmer correlation between the sequence at position 306 in SIV envelope and the capacity for mucosal transmission.

When LTR sequences were analyzed for these same animals, we noted that 90*08 viruses all contained LTRs with three Sp1 sites and that 36*08 viruses were a mixed group with 7 of 16 clones containing three Sp1 sites and 9 of 16 containing two Sp1 sites. LTR sequences from the 46*16 sample all (20 of 20) contained 2 Sp1 sites (23). Envelope sequence analysis showed a high degree of similarity among clones from the 90*08 and 36*08 samples. We expected that the 36*08 sample would contain a mixture of envelope sequences with some similar to 90*08 and others similar to 46*16 consensus sequences. The relative homogeneity of this group and the high degree of similarity to 90*08 sequences after intravenous transmission suggests that a single envelope with very high mucosal transmission frequency was not amplified after high-dose intrarectal inoculation but that other properties of these viruses ensured their amplification after in vivo inoculation. Unique amino acid sequences present in the 46*16 sample may influence transmission efficiency; final proof awaits construction and testing of relevant molecular chimeras.

It is important to note the significant differences between viruses from the 90*08, 36*08, 46*16 group and the sequences obtained after in vitro infection. The virus sequence obtained after in vitro infection was not detected in vivo after a single inoculation despite its similarity to SIVmac239 in the envelope V1 to CD4 binding site regions and its nearly identical LTR (23). That this virus was indeed present in the stock was confirmed in the case of 46*140 samples obtained after intrarectal challenge of an animal previously shown to have transient viremia. Thus, a envelope sequence similar to that of SIVmac239 is a component of our virus stock that is efficiently replicated during in vitro infection but does not compete well via intravenous or intrarectal routes of inoculation in virus-naive animals.

Transient viremia was noted in six of six animals after low-dose intrarectal virus inoculation and in one of four animals after high-dose intrarectal virus inoculation (16). Animals exposed mucosally to lower virus doses were positive for viral antigen and viral DNA in PBMC and were occasionally positive for virus isolation in vitro during the weeks immediately after inoculation (12). These low-dose intrarectal inoculations did not result in persistent infection and did not elicit serum antibodies against SIV (16). Animals with transient viremia showed lymphoproliferative responses to SIV antigen that were increased after intrarectal virus challenge (19). Lymphoproliferative responses often correlate with subsequent resistance to virus challenge in macaques (4), and we observed that a total of seven high-dose intrarectal virus challenges resulted in only one case of persistent infection. Resistance to subsequent high-dose intrarectal virus challenge and the pattern of lymphoproliferative responses argue for a host mechanism that limits virus replication after initial infection and constitutes a regional protective immunity. We did not detect antibody responses in transiently viremic animals (16). Two of three transiently viremic animals had low but reproducible CTL responses to SIV; one had responses to *nef*, and the other had responses to *gag*. CTL responses to *env* are frequently observed among uninfected animals and are not related to infection status (26). Overall, studies of immune responses after transient viremia showed lymphoproliferation as the only specific response that was associated with virus exposure. Immunity against virus challenge was also specific for route of inoculation, and transiently viremic animals were readily infected by intravenous inoculation. These data may explain the finding that low-dose exposure did not protect against intravenous virus challenge in another study (8).

Positive virus isolation appeared 3.5 years after the original virus inoculation and more than 1 year after a unsuccessful virus challenge in animal 90051. Envelope sequences from this macaque were more similar to those found after primary inoculation than those found after challenge in animal 90046. We concluded that virus in 90051 at 184 weeks after the original inoculation represented a relapse of the original virus and was not due to the subsequent intrarectal challenge. The many sequence changes unique to 90051 and the unusual patterns of sequence duplication just upstream of the V1 region imply that this virus continued to replicate and diverge during the time from transient viremia to relapse. More than 3 years were required to evolve a rapidly replicating strain after the transient viremia. Viruses present in 90051 after relapse were pathogenic (induced rapid disease in transfusion recipients) although this animal showed the familiar pattern of slow disease progression that is common to mucosal inoculation.

Transient viremia and resistance to subsequent virus challenge may reflect underlying properties that control disease progression after intrarectal inoculation. Animals with persistent infection after intrarectal inoculation maintained high levels of infectious lymphocytes in peripheral blood with sporadic plasma viremia until late stages of infection (not shown). Transfusion studies showed that slower disease progression was not due to lack of pathogenic virus. Intrarectally infected donors survived longer than recipients of their blood transfusions, showing that highly virulent viruses were indeed present in these slowly progressing animals but that host responses contained their disease potential. SIV-specific major histocompatibility complex-restricted CTL responses and serum antibodies were detected in animals after persistent infection, but these responses were similar to those in animals after intravenous inoculation. Host immunity mechanisms specific for mucosae can limit virus replication and provide protective immunity against subsequent challenge in the case of transient viremia. These same mechanisms influence disease progression even after persistent infection by intrarectal inoculation and show the potential for host immunity to overcome the pathogenic potential of circulating viruses.

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