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Viral Determinants of Simian Immunodeficiency Virus (SIV) Virulence in Rhesus Macaques Assessed by Using Attenuated and Pathogenic Molecular Clones of SIVmac

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To identify viral determinants of simian immunodeficiency virus (SIV) virulence, two pairs of reciprocal recombinants constructed from a pathogenic (SIVmac239) and a nonpathogenic (SIVmac1A11) molecular clone of SIV were tested in rhesus macaques. A large 6.2-kb fragment containing *gag*, *pol*, *env*, and the regulatory genes from each of the cloned (parental) viruses was exchanged to produce one pair of recombinant viruses (designated SIVmac1A11/239gag-env/1A11 and SIVmac239/1A11gag-env/239 to indicate the genetic origins of the 5′/internal/3′ regions, respectively, of the virus). A smaller 1.4-kb fragment containing the external *env* domain of each of the parental viruses was exchanged to create the second pair (SIVmac1A11/239env/1A11 and SIVmac239/1A11env/239) of recombinant viruses. Each of the two parental and four recombinant viruses was inoculated intravenously into four rhesus macaques, and all 24 animals were viremic by 4 weeks postinoculation (p.i.). Virus could not be isolated from peripheral blood mononuclear cells (PBMC) of any animals infected with SIVmac1A11 after 6 weeks p.i. but was consistently isolated from all macaques inoculated with SIVmac239 for 92 weeks p.i. Virus isolation was variable from animals infected with recombinant viruses; SIVmac1A11/239gag-env/1A11 and SIVmac239/1A11env/239 were isolated most frequently. Animals inoculated with SIVmac239 had 10 to 100 times more virus-infected PBMC than those infected with recombinant viruses. Three animals infected with SIVmac239 died with simian AIDS (SAIDS) during the 2-year observation period after inoculation, and the fourth SIVmac239-infected animal had clinical signs of SAIDS. Two animals infected with recombinant viruses died with SAIDS; one was infected with SIVmac239/1A11gag-env/239, and the other was infected with SIVmac1A11/239gag-env/1A11. The remaining 18 macaques remained healthy by 2 years p.i., and 13 were aviremic. One year after inoculation, peripheral lymph nodes of some of these healthy, aviremic animals harbored infected cells. All animals seroconverted within the first few weeks of infection, and the magnitude of antibody response to SIV was proportional to the levels and duration of viremia. Virus-suppressive PBMC were detected within 2 to 4 weeks p.i. in all animals but tended to decline as viremia disappeared. There was no association of levels of cell-mediated virus-suppressive activity and either virus load or disease progression. Taken together, these results indicate that differences in more than one region of the viral genome are responsible for the lack of virulence of SIVmac1A11.

Viral virulence, the ability of a virus to cause disease in an infected host, is determined by viral genes and by genetic as well as nongenetic host factors (25, 42). Identification of viral virulence factors has been facilitated by isolating infectious molecular clones of virulent and avirulent viruses and testing recombinants between such cloned variants in animals (e.g., reoviruses [42]). Recombinant retroviruses constructed from molecular clones of avian leukosis viruses, murine leukemia viruses, and feline leukemia viruses have been used to identify viral genes and regulatory elements that control viral latency, load, tissue tropism, and disease specificity (3, 5, 7, 8, 28, 32, 35, 37, 38, 40, 47). Some virulence factors (e.g., onset of disease and tissue tropism) are influenced by multiple, separate regions of the genome (3, 48). These earlier studies support the usefulness of recombinant viruses to study viral genetic determinants of simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) virulence in outbred species such as nonhuman primates.

SIV infection of rhesus macaques is a well-established animal model for HIV infection of humans (9, 12). Genetic sequence comparisons demonstrate that SIV and HIV types 1 and 2 are evolutionarily related (29). Several variants of SIV isolated from rhesus macaques (SIVmac) have been molecularly cloned and sequenced, and their biological properties have been characterized in vitro as well as in experimentally infected macaques. SIVmac239 is a molecularly cloned virus which replicates and causes cytopathology in cultures of macaque T lymphocytes but not macrophages (1, 2, 30). The molecular clone SIVmac1A11 produces virus that replicates and causes cytopathology in both T-lymphoid cells and cultured macaque macrophages (1, 2, 20, 44). In rhesus macaques, SIVmac239 establishes a persistent infec-

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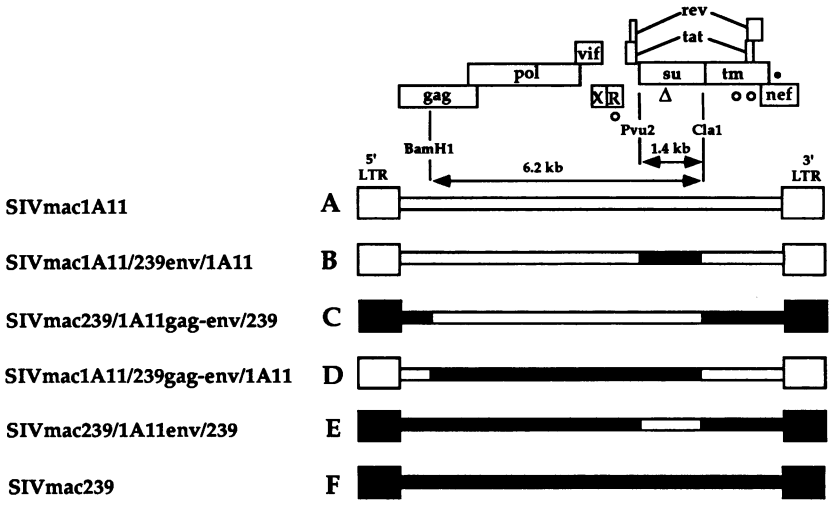

FIG. 1. Genetic organization of parental and recombinant viruses. Open reading frames and the 5′ and 3′ long terminal repeats (LTRs) are shown for the parental clones, SIVmac1A11 and SIVmac239. The SU and TM domains of the *env* gene are noted. Open circles indicate locations of premature stop codons in *vpr* and *env*-TM of SIVmac1A11, and the solid circle shows the premature stop codon in the *nef* genes of SIVmac239. Reciprocal recombinants were constructed by exchanging the regions between the *Pvu*II and *Cla*I sites (SIVmac239/1A11env/SIVmac239 and SIVmac1A11/239env/SIVmac1A11) and the *Bam*HI and *Cla*I sites (SIVmac239/1A11gag-env/SIVmac239 and SIVmac1A11/239gag-env/SIVmac1A11). Details of the construction of recombinant viruses and the analysis of replication properties have been described by Banapour et al. (1).

tion and causes fatal immunodeficiency disease similar to AIDS, whereas SIVmac1A11 produces a transient cell-associated viremia and is not pathogenic (14, 20, 22). Although the two viruses are very similar genetically (about 98% sequence identity), they differ in the length of open reading frames for three genes: the *nef* gene of SIVmac239 is prematurely truncated with a translational stop codon, and both the *vpr* gene and the transmembrane domain of the *env* gene (*env*-TM) of SIVmac1A11 are prematurely truncated (19, 34). Molecular clones of the virulent SIVmac239 and avirulent SIVmac1A11 have been used to produce reciprocal recombinant viruses for studying biologic properties of SIV in tissue culture systems (1, 2).

The goal of this study was to extend the previous in vitro experiments assessing SIV cell tropism (1, 2) by testing recombinants between SIVmac239 and SIVmac1A11 in macaques to identify regions of the SIV genome that affect phenotypic differences in pathogenicity. In this study, infection of rhesus macaques with recombinant viruses showed a spectrum of virulence between the parent viruses, SIVmac1A11 and SIVmac239; thus, molecular determinants of SIVmac virulence involve several regions of the viral genome.

MATERIALS AND METHODS

Animals. All animals used in this study were colony-bred juvenile rhesus macaques (*Macaca mulatta*) from the type D retrovirus-free and SIV-free colony at the California Regional Primate Research Center. The animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards. When necessary, the animals were immobilized with 10 mg of ketamine HCl (Parke-Davis, Morris Plains, N.J.) per kg, injected intramuscularly. We adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Resource Council.

Viruses. The isolation and characterization of all molecularly cloned viruses have been previously described (2, 20, 30). The molecularly cloned isolates SIVmac1A11 and SIVmac239 are designated parental viruses. Viruses obtained from molecular clones containing portions of both the parental virus genomes are designated recombinant viruses. All virus stocks were prepared and titered by endpoint dilution in CEMX174 cells as described previously (2), stored frozen at −135°C, and then thawed immediately prior to inoculation of macaques.

Construction of recombinant SIV clones. Two pairs of reciprocal recombinant genomes were constructed by combining portions of the two molecular clones SIVmac239 and SIVmac1A11 cleaved at restriction endonuclease sites common to both clones (2) (Fig. 1). Recombinants between the 6.2-kb *Bam*HI-*Cla*I fragment, which contains much of the *gag*, *pol*, and *env* as well as regulatory genes, and long terminal repeat regions are designated SIVmac1A11/239gag-env/1A11 and SIVmac239/1A11gag-env/239 to indicate the genetic origins of the 5′/internal/3′ regions, respectively, of the virus. A smaller region, 1.4 kb from the *Pvu*II site near the initiation of the external envelope glycoprotein and extending to the *Cla*I site, has been exchanged in the second pair of recombinants to assess the contribution of the external envelope protein to pathogenicity. These recombinants are designated SIVmac1A11/239env/1A11 and SIVmac239/1A11env/239.

Animal inoculation. Twenty-four macaques were assigned to six groups of four animals per group for inoculation with one of the two parental or with one of the four recombinant viruses (Table 1). Each macaque was inoculated intravenously with 10,000 50% tissue culture infectious doses ($TCID_{50}$) of one of the six viruses.

Virus isolation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll gradient separation (Lymphocyte Separation Medium; Organon Teknika, West Chester, Pa.). Whole, heparinized peripheral blood or PBMC were cocultured with CEMX174

TABLE 1. Experimental design for inoculation of rhesus macaques with parental and recombinant viruses[a]

Virus inoculated	Group	Animal			
		Text #	Tatoo #	Age (mos)	Gender
1A11	A	1	24293	28	M
		2	24943	16	M
		3	24971	16	M
		4	25000	15	M
	B	1	24996	19	M
		2	24999	19	M
		3	25066	18	M
		4	25074	18	M
	C	1	24699	17	M
		2	24958	16	M
		3	24962	16	M
		4	24963	16	M
	D	1	23489	42	M
		2	24268	28	F
		3	24728	17	M
		4	24942	16	M
	E	1	24933	19	M
		2	24994	19	M
		3	25043	18	M
		4	25113	17	M
239	F	1	24783	17	M
		2	24848	17	M
		3	24864	16	M
		4	24911	16	M

[a] Four rhesus macaques in each of six groups (A to F) were inoculated with cell-free preparations of a parental or a recombinant virus grown in CEMX174 cells. The structures and designations of these viruses (as indicated in Fig. 1) are shown at the left. The animal (text) number, California Regional Primate Research Center tattoo number, age at time of inoculation, and gender (M, male; F, female) are given for each macaque.

cells (13) (provided by James A. Hoxie, University of Pennsylvania, Philadelphia) to isolate infectious virus at 1, 2, 4, and 6 weeks and then monthly after inoculation with parental and recombinant viruses as previously described (17). A final blood sample was cultured to detect virus at the time of death. To detect virus in lymphoid organs, peripheral lymph nodes were recovered by transcutaneous biopsy and aseptically teased into single-cell suspensions of mononuclear cells (LNMC) in 60-mm-diameter tissue culture dishes. LNMC were then cocultured with CEMX174 cells to recover virus. Five million PBMC or LNMC were analyzed in each culture; cell numbers were determined with a Neubauer hemocytometer. Culture media were assayed regularly for the presence of SIV major core protein (p27) by antigen capture enzyme-linked immunosorbent assay (ELISA) (17). Cultures were considered positive if culture supernatants yielded 10 ng or more of SIVp27 antigen per ml at two consecutive time points. All cultures were maintained for 8 weeks and tested for SIV p27 by ELISA before being scored as virus negative. Although the sensitivity of the SIV antigen capture ELISA used here (~1 ng/ml) is not as great as that of some commercial ELISAs (e.g., the cutoff for the Coulter SIV ELISA is ~75 to 100 pg/ml), this system is suitable for detecting replicating SIV in the coculture assays described in this report and in other studies by us (21, 45). This assay could detect one SIV-infected cell per 10^6 PBMC. CD8-depleted PBMC from animals which were transiently viremic were also cocultivated to recover virus (see below).

Depletion of the CD8 T-cell subset in rhesus PBMC. T cells expressing the CD8 surface antigen were removed from 6×10^6 to 10×10^6 rhesus PBMC (purified by Ficoll gradient centrifugation) by immunomagnetic separation with anti-CD8 Dynabeads (Dynal Inc., Great Neck, N.Y.) as described previously (21). Cell populations recovered after using anti-CD8 Dynabead depletion techniques routinely had fewer than 5% $CD8^+$ cells by flow cytometric analysis. CD8-depleted PBMC (3×10^6 to 5×10^6) were pelleted by low-speed centrifugation, resuspended in complete RPMI, and cocultured with CEMX174 cells to recover SIV as described above for unfractionated PBMC.

Quantitation of cell-associated virus load. Cell-associated virus load in peripheral blood was determined by a limiting dilution assay (45). Serial 10-fold PBMC dilutions were made in complete RPMI, and four replicates of 10^2 to 10^6 PBMC were cultured with 2×10^5 CEMX174 cells in 24-well plates (Fisher, Santa Clara, Calif.); 10^7 cells were cultured with 2×10^6 CEMX174 cells in T75 flasks. Cultures were maintained and tested as described above for cocultivation except that negative cultures were discarded after 4 weeks. Cell-associated virus levels were calculated according to the method of Reed and Muench (33) and expressed as $TCID_{50}$ per 10^7 PBMC. The minimal level of cell-associated infectious virus that could be detected reliably was 1 $TCID_{50}$ per 10^7 PBMC (45).

Preparation of tissue for PCR amplification. PBMC were isolated from heparinized blood by Ficoll gradient separation. Peripheral lymph nodes were biopsied, and LNMC were prepared as described above. PBMC and LNMC were pelleted, resuspended at 10^7 cells per ml in polymerase chain reaction (PCR) lysis buffer (10 mM Tris-HCl [pH 8.3], 0.1% Nonidet P-40, 0.1% Triton X-100, 100 μg of proteinase K [Boehringer Mannheim] per ml). These lysates were incubated for 60 min at 55°C to digest cellular protein, and proteinase K was inactivated by incubation at 95°C for 10 min. Cell lysates were stored at −70°C until used for PCR amplification. Ten to 20 μl of each lysate, equivalent to DNA from 1×10^5 to 2×10^5 mononuclear cells, was used for PCR amplification.

PCR amplification. Nested PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus, Emeryville, Calif.). Two rounds of 30 cycles of amplification were performed on aliquots of plasmid DNA containing the complete genome of SIVmac1A11 (20) (positive control) or aliquots of crude cell lysates, using SIVmac-specific *gag* primers and conditions described elsewhere (43). DNA from uninfected CEMX174 cells was amplified as a negative control in all assays to monitor potential reagent contamination. This nested PCR amplification procedure allows visual detection of a single copy of SIV *gag* sequences in as many as 200,000 PBMC (43). PCR amplification (30 cycles) of β-globin DNA sequences from all PBMC lysates with primers PC03 and KM38 (39) was also performed to demonstrate the presence of cellular DNA and to detect potential inhibitors of *Taq* polymerase in cell lysates.

Antiviral serological responses. Titers of SIV-specific antibodies in sera, diluted at 1:100 to 1:100,000, were measured by ELISA, using sucrose gradient-purified SIVmac grown in HUT 78 cells as the antibody capture antigen as previously described (21). All dilutions of each sample serum and the positive control serum (from an SIV-infected animal) were assayed in duplicate, and mean values of optical density were calculated. Data are presented as the inverse of the highest sample dilution that was greater than twice the mean optical density of serum from each animal sam-

pled prior to inoculation. Immunoblots were also performed to detect specific SIV proteins as described previously (41).

Cell-mediated suppression of viral replication. The ability of rhesus PBMC to suppress production of SIV in vitro was determined in assays using whole blood (17) or populations of $CD4^+$ and $CD8^+$ lymphocytes purified from peripheral blood by immunomagnetic separation as described by Walker et al. (46). For the whole-blood assay, heparinized blood samples were centrifuged and cell pellets were washed twice with phosphate-buffered saline (PBS). Five hundred microliters of washed blood cells, containing approximately 10^6 PBMCs, was incubated at 37°C for 2 h with 10^3 $TCID_{50}$ of SIVmac1A11. After incubation with virus, blood cells were washed twice in PBS and resuspended in 5 ml of complete medium supplemented with *Staphylococcus* enterotoxin A (0.5 μg/ml; Toxin Technology, Madison, Wis.) and recombinant human interleukin-2 (50 U/ml; a gift of Cetus Corp., Emeryville, Calif.). For assays using enriched populations of lymphocytes, 5×10^6 PBMC were depleted of $CD4^+$ or $CD8^+$ lymphocytes by negative selection with immunomagnetic Dynabeads (Dynal) as instructed by the manufacturer. One million CD8-depleted (CD4-enriched) lymphocytes were incubated at 37°C for 2 h with 10^3 $TCID_{50}$ of SIVmac1A11. Cells were then washed and suspended in media as described above for whole blood. Infected CD4-enriched cells were cultured either alone or in the presence of an equal number of CD4-depleted (CD8-enriched) lymphocytes. For all assays, cultures were maintained 12 to 14 days after infection. Culture supernatant was removed twice weekly and replaced with fresh medium supplemented with interleukin-2. Supernatant samples were stored at −20°C until the assay was complete. The amount of SIV p27 antigen in culture supernatants was determined by a quantitative antigen capture ELISA as described above (see also reference 17). The percentage of SIV suppression by $CD8^+$ lymphocytes was determined by the formula $100 \times [(a-b)/a]$, where a is the amount of SIV p27 detected in day 12 to 14 supernatants of SIV-infected $CD4^+$ lymphocytes cultured alone and b is the amount of SIV p27 detected in day 12 to 14 supernatants of SIV-infected $CD4^+$ lymphocytes cultured with an equal number of $CD8^+$ lymphocytes from the same macaque.

The whole blood assay was developed to enable screening of a large number of samples at multiple time points. A positive control for this assay was selected by testing the level of SIV replication in whole blood from each of seven normal, uninfected rhesus macaques drawn at weekly intervals for 4 weeks (17). One uninfected macaque which had the most consistent, high production of SIV antigen was chosen as the positive control blood donor for all suppression assays performed for this study. Antigen production of replicate cultures of whole blood infected with SIV varied by 10% or less (data not shown). The percentage of SIV suppression was determined by the formula $100 \times [(a'-b')/a']$, where a′ is the amount of SIV p27 detected in day 12 to 14 supernatants of PBMC cultures from the uninfected macaque and b′ is the amount of SIV p27 detected in day 12 to 14 supernatants of PBMC cultures from an individual SIV-infected macaque. Levels of virus suppression measured by the rapid, whole-blood assay are in a range equivalent to that of levels obtained from assays using purified lymphocyte populations from PBMC (18).

RESULTS

Outcome of inoculations. The virulence of each of the two parental and four recombinant viruses was assessed by inoculating 10^4 $TCID_{50}$ of each virus into four rhesus macaques by the intravenous route. Virus was isolated from the PBMC of all 24 animals for 4 weeks postinoculation (p.i.), thus demonstrating that the parental and recombinant viruses were all infectious (Fig. 2). The pattern of subsequent cell-associated viremia varied between the six groups, although variation among animals within several groups was also observed. As reported in previous studies (14, 23), the parental viruses induced either a persistent viremia (SIVmac239) or a transient viremia lasting 4 to 6 weeks (SIVmac1A11) (Fig. 2). Three of four SIVmac239 infected animals died between weeks 32 and 93 weeks p.i., while all four animals infected with SIVmac1A11 remained healthy for more than 2 years.

The pattern of cell-associated viremia for animals infected with the recombinant viruses varied according to the relative proportions of SIVmac239 and SIVmac1A11 genomes that the recombinants contained. The SIVmac1A11/239env/1A11 recombinant persisted slightly longer in PBMC than did the parental SIVmac1A11: the initial periods of cell-associated viremia were similar (6 weeks for three of four animals); however, virus was intermittently isolated from two of four animals until 37 to 62 weeks p.i. (Fig. 2).

The SIVmac239/1A11gag-env/239 and SIVmac1A11/239gag-env/1A11 recombinants were more virulent than SIVmac1A11 or the SIVmac1A11/239env/1A11 recombinant (Fig. 2). Persistent viremia was observed in two of four, and death with simian AIDS was observed in one of four, animals in each recombinant group in which the 6.2-kb *gag-env* fragment was exchanged. Macaques infected with the SIVmac239/1A11env/239 virus tended to be persistently viremic for the first 27 weeks of infection and intermittently viremic or aviremic thereafter; all of the latter animals were still healthy at the completion of the study.

Virus isolations were performed from PBMC and LNMC at 12 months p.i.; SIV proviral DNA in lymph nodes was also assayed (Fig. 3). Infectious virus from PBMC and LNMC as well as viral DNA from LNMC were detected from the remaining three animals infected with the parental SIVmac239. Virus was not recovered from the PBMC of any of the animals infected with SIVmac1A11 but was isolated from PBMC of one animal infected with SIVmac1A11/239env/1A11 (Fig. 3, B1); however, LNMC from four of eight of these animals yielded infectious virus (Fig. 3, A1, A3, B1, and B2). None of these eight monkeys contained detectable levels of SIV DNA in LNMC. Similarly, virus was recovered from LNMC of all four animals infected with SIVmac239/1A11env/239 but from PBMC of only one of these animals (Fig. 3, E1); viral DNA was also detected in LNMC from only one animal (Fig. 3, E4) infected with this recombinant. Virus was recovered from the PBMC of two animals infected with SIVmac239/1A11gag-env/239 (Fig. 3, C1 and C2) and from two infected with SIVmac1A11/239gag-env/1A11 (Fig. 3, D2 and D3); however, LNMC from all but one (Fig. 3, C4) of these eight animals yielded infectious virus. Six of these eight monkeys contained detectable levels of SIV DNA in LNMC. Thus, virus was isolated from PBMC of 9 of 23 animals and from LNMC of 18 of 23 surviving animals that were infected with parental or recombinant viruses; 10 of 23 animals were positive by for SIV DNA in lymph node cells. The discordance between results obtained by culture and PCR methods may reflect differ-

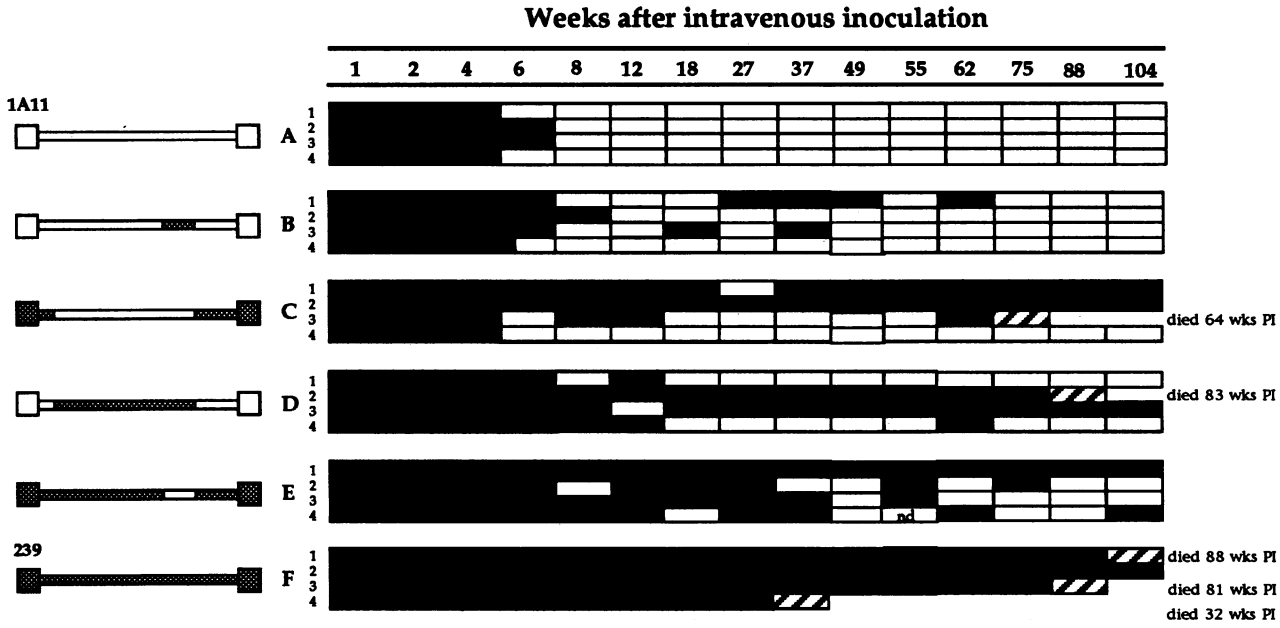

FIG. 2. Recovery of parental and recombinant viruses from PBMC cultures of infected macaques. Structures and designations of these viruses (as indicated in Fig. 1) are shown at the left. At various times after inoculation, 5×10^6 PBMC were cocultivated with CEMX174 cells and culture supernatants were assayed for virus by measuring SIV core antigen in an ELISA (17). Each row of boxes represent one macaque; a solid box in each row indicates that SIV was detected in culture supernatants, and an open box indicates that no virus was detected. Five macaques developed fatal AIDS-like disease and were euthanized (hatched boxes) at the times shown.

ences in the maximum number of cells used for each assay; 10^6 to 10^7 cells are cultured for infectious virus, whereas DNA from 10^5 cells is analyzed by PCR.

Quantitative cell-associated viremia. Cell-associated viremia was determined for all 24 animals in blood samples collected at about 1 year following infection (Fig. 4). Monkeys infected with SIVmac1A11 had no detectable virus-infected cells per 10^6 PBMC, while all four SIVmac239-infected animals had more than 100 infected cells per 10^6 PBMC. The virus load of recombinant viruses in PBMC was intermediate between the parental types. The low virus load in monkeys infected with recombinant viruses that contained

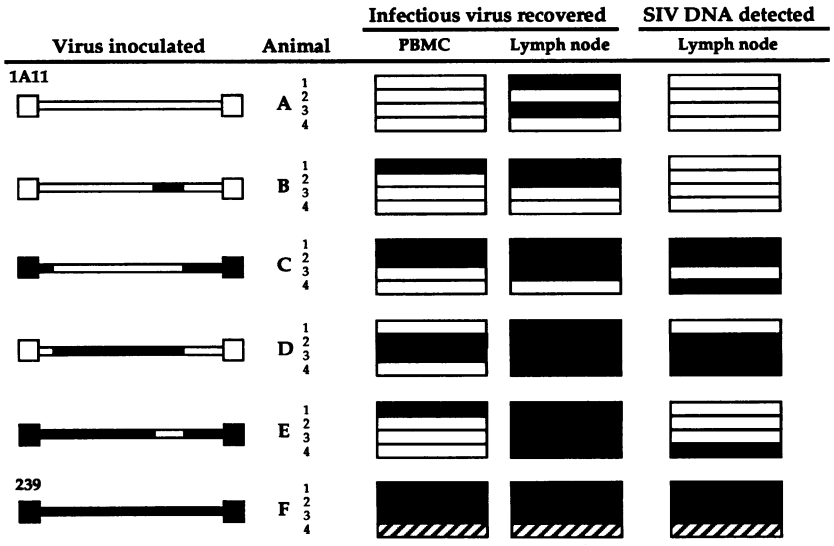

FIG. 3. Detection of parental and recombinant viruses in PBMC and lymph nodes of infected macaques. Structures and designations of these viruses (as indicated in Fig. 1) are shown at the left. Twelve months after inoculation, 5×10^6 PBMC or LNMC were cocultivated with CEMX174 cells and culture supernatants were assayed for virus by measuring SIV core (p27) antigen in an ELISA (17). Each row of boxes represents one macaque; a solid box in each row indicates that SIV core antigen was detected in culture supernatants or SIV DNA was detected in LNMC lysates by PCR, and an open box indicates that no virus core antigen or DNA was detected. One SIVmac239-infected macaque developed fatal AIDS-like disease and was euthanized (hatched boxes) at 8 months p.i.

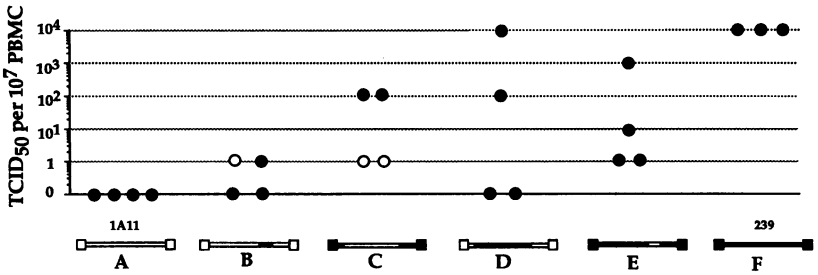

FIG. 4. Virus load in PBMC of rhesus macaques 9 months after inoculation with parental or recombinant viruses. Structures and designations of these viruses (as indicated in Fig. 1) are shown along the x axis; the $TCID_{50}$ per 10^7 PBMC is shown on the y axis. Nine months p.i., the TCID per 10^7 PBMC from each animal was determined by measuring SIV core (p27) antigen from supernatants of limiting dilutions cultures of either unfractionated or CD8-depleted PBMC (45) in an ELISA (17). Each circle represents one macaque. Filled circles indicate that virus was detected in culture supernatants of unfractionated PBMC, and open circles indicate that virus was detected in culture supernatants of CD8-depleted PBMC.

predominantly SIVmac1A11 genes resembled that of animals infected with SIVmac1A11; the virus load of monkeys infected with recombinant viruses made up of predominantly SIVmac239 genes was similar to that of animals infected with SIVmac239.

To enhance recovery of virus from animals that were aviremic or intermittently viremic at 9 to 11 months following infection, PBMC were depleted of $CD8^+$ lymphocytes prior to coculture with CEMX174 cells. The levels of infected cells detected by depletion of $CD8^+$ T cells were still at the level of one infected cell per 10^6 PBMC. Virus was isolated at several additional time points by this method from three monkeys infected with the SIVmac1A11/239env/1A11 recombinant and two monkeys infected with the SIVmac239/1A11gag-env/239 virus (Fig. 4). Repeated attempts to isolate virus from SIVmac1A11-infected animals by coculture of PBMC depleted of $CD8^+$ T cells were unsuccessful.

Antiviral antibody responses. SIV antibodies were detected in sera of all 24 animals by 6 weeks p.i., (data not shown). The SIV-specific antibody titers at 8 and 25 weeks p.i. varied according to the genotype of the infecting virus and the duration of viremia (Fig. 5). The lowest levels of antibodies were observed for animals infected with SIVmac1A11, and the highest levels were measured in animals infected with SIVmac239. Animals infected with recombinant viruses consisting mainly of SIVmac1A11 genome had low antibody responses similar to those of animals infected with SIVmac1A11, while animals infected with recombinant viruses consisting mainly of SIVmac239 genes had high antibody levels similar to those of monkeys infected with SIVmac239 (Fig. 5). The relative differences in antibody responses between the various groups were also evident on immunoblots (data not shown).

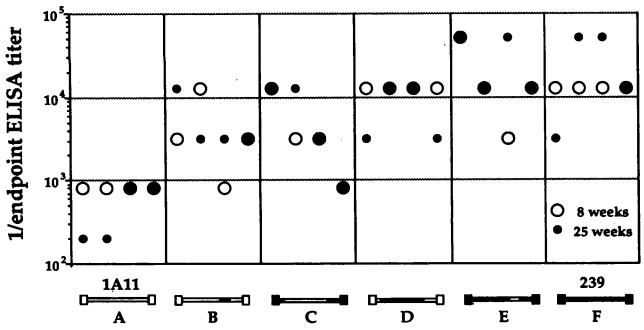

FIG. 5. SIV-specific serum antibody titers for macaques 25 weeks after inoculation with parental or recombinant viruses. Structures and designations of these viruses (as indicated in Fig. 1) are shown along the x axis; the reciprocal of the SIV-specific antibody titer is shown on the y axis. At 8 and 25 weeks p.i., serum from each animal was assayed for SIV-specific antibodies in a whole-virus ELISA (21). Data are presented as the inverse of the highest sample dilution that was greater than twice the mean optical density of serum from each animal sampled prior to inoculation. Each circle represents one macaque.

Cell-mediated suppression of virus production. The relative ability of PBMC from all 24 animals to suppress virus production was assessed by an in vitro assay before and after infection. Data from 0, 2, and 49 weeks p.i. demonstrate the trend in suppression responses (Table 2). Five of twenty-four macaques had detectable levels of suppressive activity prior to inoculation. There was no association of the level of virus suppression prior to inoculation with the pattern of virus persistence, virus load, or the onset of disease in any infected group. Virus-suppressive activity appeared within 2 weeks p.i. in the remaining 19 animals, and higher levels of virus suppression were observed for those animals from which virus was consistently isolated (i.e., animals infected with SIVmac239, SIVmac239/1A11gag-env/239, or SIVmac239/1A11env/239; Fig. 2 and Table 2, week 49). In addition, virus could be recovered only from CD8-depleted (CD4-enriched) lymphocytes from some animals (Fig. 4, groups B and C). However, the levels of virus suppression after infection were not related to cell-associated virus load or to the onset of disease in any of the animals. The discordance between virus load and cell-mediated suppression of virus replication is consistent with the hypothesis that the mechanism of suppression does not involve killing of virus-infected cells in vivo as has been demonstrated in vitro (46).

DISCUSSION

In this study, large portions of the molecularly cloned genomes of two closely related but biologically distinct viruses, SIVmac239 and SIVmac1A11, were exchanged to produce recombinant viruses to analyze viral and host factors which affect virulence. Animals infected with the parental and two pairs of reciprocal recombinant viruses were compared with respect to three parameters: the levels of virus in blood and lymph nodes, levels of antiviral immune responses, and clinical signs of disease. As observed for

TABLE 2. Cell-mediated suppression of SIV production by PBMC and purified lymphocytes of rhesus macaques before and after infection with parental viruses[a]

Virus inoculated	Group	Animal #	% reduction SIV p27 antigen: Weeks after inoculation 0	2	49	49[b]
1A11	A	1	0	25	55	nd[c]
		2	0	19	0	4
		3	70	95	100	100
		4	0	73	48	0
	B	1	0	90	25	11
		2	0	82	15	12
		3	0	90	0	13
		4	0	95	94	98
	C	1	56	98	97	nd
		2	0	0	54	nd
		3	58	41	100	100
		4	0	82	95	30
	D	1	60	93	98	nd
		2	0	59	63	nd
		3	0	99	90	nd
		4	39	93	97	88
	E	1	0	94	44	nd
		2	0	96	80	100
		3	0	87	78	nd
		4	0	95	22	33
239	F	1	0	100	99	nd
		2	0	95	95	nd
		3	0	98	95	nd
		4	0	50	nd[d]	nd[d]

[a] Structures and designations of the viruses (as indicated in Fig. 1) are shown at the left. The percentage of cell-mediated suppression of SIV production by PBMC was determined for each animal on the day of inoculation (week 0) and at 2 and 49 weeks p.i. by using the formula $100 \times [(a'-b')/a']$, where a' is the amount of SIV p27 detected in day 12 to 14 supernatants of PBMC cultures from the uninfected macaque and b' is the amount of SIV p27 detected in day 12 to 14 supernatants of PBMC cultures from an individual SIV-infected macaque. The percentage of SIV suppression by $CD8^+$ lymphocytes was determined for selected animals 49 weeks p.i. by using the formula $100 \times [(a-b)/a]$, where a is the amount of SIV p27 detected in day 12 to 14 supernatants of SIV-infected $CD4^+$ lymphocytes cultured alone and b is the amount of SIV p27 detected in day 12 to 14 supernatants of SIV-infected $CD4^+$ lymphocytes cultured with an equal number of $CD8^+$ lymphocytes from the same macaque.

[b] CD8-mediated suppression of SIV in CD4-enriched lymphocytes.

[c] nd, not determined.

[d] One SIVmac239-infected animal (F4) died with clinical signs of simian AIDS 32 weeks p.i.; cell-mediated virus suppression at the time of death was 0%.

genetic crosses between variants for other multigenic traits, the phenotypes of recombinant viruses were intermediate between the parent viruses. In general, the more of one parental genome that the recombinant virus contained, the more similar it was to that parent virus with respect to virus load, host immune response, and disease in infected animals. Thus, two or more regions of the SIVmac genome influence the phenotype of each of these three parameters. In addition, analysis of the pair of recombinant viruses, in which only the external domain of *env* was exchanged, demonstrated that this portion of the viral genome is not the sole determinant of levels of virus load, titers of antiviral immune responses, or pathogenic potential. These results are consistent with those from studies on other retroviruses in which virulence was also found to be influenced by multiple, separate regions of the viral genome (4, 48).

Viral determinants of pathogenesis have been investigated for the acutely lethal variant SIVsmmPBj; pigtail macaques (*M. nemestrina*) infected with this virus die of severe gastrointestinal dysfunction in 5 to 14 days p.i. (11). Recombinant viruses were constructed from a molecular clone of SIVmmPBj and a molecular clone of a closely related virus, SIVsmmH4, which causes late onset of immunodeficiency disease in pigtail macaques (31). Acute disease was shown to be governed by more than one region of the viral genome exclusive of the long terminal repeat. Because this study used a virus that causes acute fatal disease (SIVsmmPBj), it is not clear how these findings relate to those in our study of recombinant viruses constructed from a virus that establishes chronic infection accompanied by fatal immunodeficiency (SIVmac239) and a nonpathogenic variant that produces a transient viremia (SIVmac1A11).

The results of this study have important implications for the evaluation of in vitro correlates of HIV disease, for experiments to identify HIV gene function, and for the development of live-attenuated viral vaccines to prevent AIDS. Although the relationship of virus load, antiviral immune responses, and disease in SIV-infected macaques remains to be determined, the simplest explanation is that virus load directly effects levels of antiviral immune responses and disease in chronically infected animals. This hypothesis is generally supported by the results of this study and of other investigations of mutants of SIVmac239 with deletions in accessory genes; SIVmac239 variants with mutations in either *vpr* or *nef* infect macaques but establish low virus load and do not produce disease (15, 16). However, it is also possible that the precise nature of the change(s) in the SIVmac genome which results in reduced virus load may be critical in determining decreased viral virulence as well as low levels of virus per se.

The differences in virus load and virulence between parental and recombinant viruses may involve a combination of two or more regions of the SIVmac genome that contain significant genetic differences. The SIVmac1A11 envelope contains two 3-bp deletions within the first variable region in the *env* surface (*env*-SU) domain and a premature stop codon in both the *vpr* gene and the *env*-TM domain (19). The *nef* gene of SIVmac239 contains a premature stop codon, but this stop codon reverts in vivo (15). Although there are relatively few sequence differences in the genomes of SIVmac1A11 and SIVmac239 (2%, or about 200 bp), these differences have profound phenotypic effects in vitro and in vivo. SIVmac1A11 has an in vitro cell tropism for both T cells and monocytes/macrophages, whereas SIVmac239 grows in T cells but not in monocytes/macrophages in vitro (1, 26). SIVmac1A11 induces only a transient cell-associated viremia, no plasma viremia, a persistent low-level humoral antibody response, and no disease signs following observation periods as long as 5 years (21a, 23). The SIVmac1A11 genome appears to persist at very low levels in the body; infectious virus can be identified only intermittently and in only a proportion of individuals by animal-to-animal passage of blood by transfusion or tissue culture isolation (23). In contrast, SIVmac239 causes a persistent viremia in all infected rhesus macaques, and 50% or more of these animals die with AIDS-like disease within the first year (14). The experiments in this study confirm and extend these previous studies concerning the relative immunogenicity and virulence of both SIVmac1A11 and SIVmac239.

A second finding of the present study, which is especially relevant to HIV infection, is that changes in in vitro growth characteristics of SIVmac recombinants did not correlate well with the differences in virulence that were observed in

vivo. The ability of SIVmac1A11 to grow in monocytes/macrophages was previously shown to be associated with both the *env*-SU domain and other regions of the viral genome (2). Substituting the SIVmac239 *env*-SU domain in the genome of SIVmac1A11 produced a virus with increased persistence in monkeys (relative to the parental SIVmac1A11) but which had lost its ability to grow in monocytes/macrophages in vitro. Substituting the SIVmac1 A11 *env*-SU domain in the genome of SIVmac239 did not enhance the ability of this recombinant to grow in monocytes/macrophages, and virus load in macaques infected with SIVmac239/1A11env/239 was lower than that of SIVmac239. In contrast, exchanging the larger internal *gag-env* portions of the genomes either added or deleted monocyte/macrophage tropism in the expected reciprocal fashion (2); these recombinant viruses behaved similarly in vivo. Thus, as reported previously (10, 27, 36) for other SIVmac isolates, in vitro monocyte/macrophage tropism per se was not associated with virulence in macaques in this study. The latter result suggests that the proposed relationship between in vitro monocyte/macrophage tropism of HIV and virulence may not be valid. Thus, observations from experiments with SIV deletion mutants and recombinants emphasize the need to evaluate potential in vitro correlates of HIV virulence in an animal model in which infection produces disease.

Finally, results of this study provide the basis for a model for evaluating a potential virulence-attenuated live SIV or HIV vaccine; i.e., a virus that would produce an infection sufficient to induce persistent, protective antiviral immune responses but not to cause disease. We propose that a threshold level of viral attenuation is a critical determinant of live vaccine-induced protection from challenge with virulent virus. Previous studies of virulence-attenuated molecular clones of SIVmac as live-virus vaccines support this hypothesis (6, 23). Macaques infected with SIVmac1A11 resist challenge with low levels (1 to 10 100% animal infectious doses [AID_{100}]) of virulent uncloned SIVmac (24). However, these animals will become persistently viremic if challenged with high doses (100 to 1,000 AID_{100}) of virulent virus (23). In contrast to animals vaccinated with SIVmac1A11, macaques immunized with SIVmac239Δnef resist challenge with up to 1,000 AID_{50} of virulent uncloned SIVmac251 (6). One explanation for the greater immunizing potential of SIVmac239Δnef is that it induces a longer-lasting primary viremia, a persistent, low-level secondary viremia, and a higher level of antiviral immune responses than does SIVmac1A11 (15) (Fig. 2, 4, and 5). Virus strains that behaved similarly to SIVmac239Δnef were produced in this study by exchanging portions of the avirulent SIVmac1A11 and virulent SIVmac239 genomes. One recombinant, SIVmac1A11/239env/1A11, was particularly promising as a vaccine candidate because it induced a persistent viremia for 4 to 6 weeks followed by an intermittent low-level viremia lasting for nearly 1 year; no signs of disease have been observed in macaques infected with this recombinant for more than 2 years p.i., and the antibody levels in these animals are much higher than those in macaques infected with SIVmac1A11. The hypothesis of a critical threshold for viral attenuation can be tested by evaluating protection from challenge in macaques inoculated (immunized) with viruses that produce different levels of virus load or antiviral immune responses but no disease. Studies comparing the outcome of challenge of such virulence-attenuated recombinant viruses will provide an opportunity to identify both viral and host immune parameters which correlate with protection against infection and disease.

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