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Multiple Viral Determinants Contribute to Pathogenicity of the Acutely Lethal Simian Immunodeficiency Virus SIVsmmPBj Variant

FRANCIS J. NOVEMBRE,[1]† PHILIP R. JOHNSON,[1,2]‡ MARK G. LEWIS,[3] DANIEL C. ANDERSON,[4] SHERRY KLUMPP,[4] HAROLD M. McCLURE,[2,4] AND VANESSA M. HIRSCH[1]*

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Rockville, Maryland 20852[1]; Yerkes Regional Primate Research Center, Atlanta, Georgia 30322[2]; Henry M. Jackson Foundation, Research Laboratory, Rockville, Maryland[3]; and Department of Pathology, Emory University, Atlanta, Georgia 30322[4]

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Simian immunodeficiency virus (SIV) induces an immunodeficiency syndrome similar to human AIDS. Although the disease course of SIV-induced immunodeficiency is generally measured in months to years, a disease syndrome that results in death in 5 to 14 days has been described in pig-tailed macaques infected with the SIVsmmPBj (PBj) strain. The purpose of this study was to derive an acutely lethal PBj molecular clone in order to study viral genes involved in pathogenesis. Six infectious molecular clones were generated; acutely fatal disease was induced by experimental inoculation of pig-tailed macaques with virus stocks derived from either of two clones, PBj6.6 or PBj14.6. Molecular chimeras were constructed by exchange of regions of the genome of PBj6.6 and a nonlethal, related clone, SIVsmH4. Only a chimera expressing the PBj genome under the control of a SIVsmH4 long terminal repeat induced death soon after inoculation. These studies suggest that multiple viral genes of PBj are critical for development of acute disease. More specifically, the *env* gene but not the long terminal repeat PBj was required for acute disease induction; however *env* must act in concert with another gene(s) of the PBj genome.

Simian immunodeficiency virus (SIV) isolated from sooty mangabeys (SIVsmm) or macaques (SIVmac) induces an immunodeficiency syndrome in infected macaques that is similar to human AIDS. Experimental infection in macaques is characterized by a progressive loss of circulating CD4 lymphocytes and infections due to opportunistic agents such as mycobacteria, cytomegalovirus, and pneumocystis (1, 7, 25, 28, 38). Additional similarities to human AIDS include a wasting syndrome and central nervous system disease that may result from direct SIV invasion (1, 8, 25, 27, 38). These similarities make the SIV/macaque model ideal for the study of AIDS pathogenesis. The disease associated with SIV and human immunodeficiency virus type 1 (HIV-1), like that of other lentiviruses, is generally slow, progressive, and chronic. However, similar to many other viral infections, HIV-1 infection also induces an acute viral syndrome during a period prior to seroconversion, coinciding with peak antigenemia and viremia (34–36). Symptoms observed include fever, malaise, flu-like symptoms, rash, diarrhea, meningitis, lymphadenopathy, and oral thrush (34–36). Patients then enter an asymptomatic phase of infection that is variable in duration. The acute phase of SIV infection of macaques has not been intensively studied; however, SIV-infected macaques develop antigenemia, rash, and lymphadenopathy within 2 weeks of infection, suggesting a comparable acute syndrome (25, 38). The acute phase of HIV-1 infection of humans and experimental SIV infection of macaques is apparently self-limiting, and the symptoms are generally not severe.

One SIVsmm strain (PBj14) induces an acutely lethal syndrome following intravenous inoculation of pig-tailed macaques (*Macaca nemestrina*). This strain was isolated from peripheral blood mononuclear cells (PBMC) of a pig-tailed macaque (PBj) that was chronically infected with the SIVsmm9 strain for 14 months (14). Whereas infection of macaques with the parental SIVsmm9 resulted in immunodeficiency (13, 28), this new isolate consistently caused severe diarrhea, acidosis, and volume depletion, culminating in death of inoculated pig-tailed macaques within 5 to 14 days after infection (14, 26, 37). In addition to having altered pathogenesis, SIVsmmPBj (PBj), unlike other SIVsm and SIVmac isolates, infects chimpanzee PBMC and mitogenically stimulates resting macaque PBMC (13, 14). Recently, molecular clones that reproduce the lethal syndrome (PBj4.41, PBj1.9, and PBj4.9 [9]) were generated. Sequence analysis of PBj and comparison with other SIVsm molecular clones revealed minor differences in the envelope, primarily within the first variable region, and duplication of an enhancer element, the NF-κB motif within the long terminal repeat (LTR) (9). The same duplication was also observed in LTRs of PBj derived by the polymerase chain reaction (PCR) in two other studies (6, 31), whereas the LTRs of the parental SIVsmm9 had a single NF-κB element (6). Since NF-κB is an important regulatory element controlling HIV-1 transcription in activated lymphocytes (15), it is tempting to speculate that duplication of the NF-κB motif might be responsible for the enhanced acute pathogenesis of PBj. However, circumstantial evidence and previous experiments suggest otherwise. First, other minimally pathogenic primate lentiviruses such as SIVagm and SIVmnd also have a duplicated NF-κB site within their LTRs (21, 29). In addi-

* Corresponding author.
† Present address: Yerkes Regional Primate Research Center, Atlanta, GA 30322.
‡ Present address: Department of Pediatrics, Children's Hospital, The Ohio State University, Columbus, OH 43210.

tion, a molecular clone in part derived from a cognate variant of PBj (SIVsmmPGg) that contains the duplicated NF-κB motif does not induce the acutely lethal syndrome in inoculated macaques (31). Therefore, duplication of this element appears not to be sufficient to cause the acutely lethal syndrome. A more precise definition of viral genes and biologic properties associated with pathogenicity would aid in understanding the pathogenic mechanism of PBj-induced acute disease.

We therefore derived molecular clones of PBj that reproduced the acute lethal syndrome upon macaque inoculation. Regions of the PBj genome and a related SIVsmm clone (SIVsmH4 [smH4]) were exchanged to dissect the genetic determinants of pathogenicity. Our results suggested the following conclusions. First, duplication of the core enhancer within the LTR does not appear to be critical for development of this syndrome. Second, although specific determinants within *env* are crucial for the development of the acute disease, the *env* gene of PBj is not the sole determinant of the acute disease. Finally, it appears that the genetic determinants of PBj are complex and involve genes in both the 5′ (*gag-pol*) and 3′ (regulatory and *env* genes) ends of the viral genome.

MATERIALS AND METHODS

Virus, cells, and DNA. PBj14bcl3, a biological clone of PBj14 (14), was used to infect CEMx174 cells. Infected cells were harvested at peak reverse transcriptase activity, and genomic DNA was prepared. Virus stocks were prepared from cloned DNAs by transfection of full-length molecular clones into CEMx174 cells by the DEAE-dextran method. At peak reverse transcriptase activity, supernatant fluids were harvested, filtered (0.45-μm-pore-size filter), aliquoted, and stored under liquid nitrogen. Pig-tailed macaque (*M. nemestrina*) and sooty mangabey (*Cercocebus atys*) PBMC were obtained by density centrifugation in lymphocyte separation medium (Organon Teknika Corp., Durham, N.C.) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0), glutamine, 10 μg of phytohemaglutinin (PHA) per ml, and 1% interleukin-2 for the first 4 days. Macaque and mangabey PBMC were infected by incubating cells with cell-free virus for 2 h at 37°C with intermittent mixing. At the end of the adsorption period, cells were washed and resuspended in medium as described above without PHA, and the culture supernatant was monitored for reverse transcriptase activity.

Cloning strategy. Genomic DNA prepared from PBjbcl3-infected CEMx174 cells was used as the template for PCR amplification of SIV-specific sequences as described previously (30, 31). The strategy for producing full-length molecular clones by generating 5′ and 3′ halves using PCR and then combining them has previously been described in detail (9, 31). Primers for amplification of 5′ and 3′ halves of PBjbcl3 were synthesized on an Applied Biosystems 380B DNA synthesizer (Applied Biosystem, Inc., Foster City, Calif.) and were derived from the smH4 nucleotide sequence (18). For each primer listed below, the smH4 nucleotide sequence coordinates are given following the 3′ base, and underlined nucleotides represent restriction enzyme sites included to facilitate cloning. Primers used for amplification of 5′ and 3′ halves were as follows: 5′ forward 895, 5′CGCTAAGCTTCCAGATTGGCAAAATTACACAG CAGGG3′-101; 5′ reverse 896, 5′CCTCCTCGAGTGTTG GAATTGTATTTCTTGTTCTGTG3′-5130; 3′ forward 694, 5′ATGCAAGCTTAGGGGATATGACTCCAGCAGA3′-5108; and 3′ reverse 700, 5′AATACTCGAGGCAGAAAGG GTCCTAACAGAC3′-10223. PCR-amplified products were digested with the appropriate restriction enzymes and cloned into pGEM-7ZF (Promega, Madison, Wis.). Ligations containing half- or full-length proviral genomes were transformed into *Escherichia coli* JM109 by electroporation (Cell-Porator; Bethesda Research Laboratories, Gaithersburg, Md.). When digestion with the methylation-sensitive enzyme *Bcl*I was required, plasmids were passed through *dam E. coli* 2474 (supplied by Joel Jessee, Bethesda Research Laboratories).

Double-stranded plasmid DNA was sequenced by the dideoxynucleotide chain termination method with T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, Ohio). Sequence analyses were performed with the PCGene programs and Genalign, (Intelligenetics, Mountain View, Calif.).

PCR mutagenesis. PCR mutagenesis was performed on a *Cla*I-*Xho*I subclone of 3′PBj6 as shown in Fig. 1B and as previously described (32). Individual clones were sequenced to confirm the introduced mutation and the *Cla*I-*Xho*I fragment introduced into the full-length PBj6.6 clone. PCR primers used for mutagenesis were derived from the PBj6.6 molecular clone nucleotide sequence. Primer sequences were as follows (the nucleotide in bold type is the mutation introduced to generate an *Nde*I site [underlined]): forward 1607, 5′ACATATGGGAGACTCTGGGAAGGGTTG3′; and reverse 1608, 5′GTCTCCACGCGCTTGCAAGAGTCTC TC3′.

Animal inoculations. Pig-tailed macaques were inoculated with 2 ml of virus intravenously (50% tissue culture infective dose; Tables 1 and 4). Animals were monitored daily for clinical signs of the acute syndrome, such as diarrhea, rash, fever, dehydration, loss of appetite, or lethargy, and for clinicopathologic changes by determination of complete blood counts and lymphocyte subset analysis on days 3, 7, 10, and 14 and less frequently after the first 2 weeks postinoculation. Infection was confirmed by cocultivation of 5×10^6 PHA-stimulated PBMC with CEMx174 cells as described previously (31). Plasma was tested for the presence of antigen or free virus by SIV p27 antigen capture enzyme-linked immunosorbent assay (ELISA) (Coulter, Hialeah, Fla.).

PBMC proliferation experiments. PBMC were isolated from a donor pig-tailed macaque on lymphocyte separation medium. This macaque had been demonstrated to be free of infection with foamy virus, simian retrovirus, and simian T-lymphotropic virus type 1. PBMC were diluted to 10^6 cells per ml in RPMI 1640 containing 10% human AB serum and seeded into 96-well round-bottom plates at 0.1 ml per well. The amount of input virus was equilibrated by determining the amount of Gag antigen, p27, in the virus stocks by antigen capture assay (Coulter). Filtered virus stocks (generated by transfection of CEMx174 cells) were added to the PBMC in triplicate at either 0.01, 0.1, 1.0, or 10 ng of SIV p27 per well. Some viral stocks were not sufficiently concentrated to allow use of 10 ng of p27. Negative controls were medium alone and supernatant from uninfected CEMx174 cells incubated with macaque PBMC; 5 μg of PHA served as the positive control. The cell-virus suspensions were incubated at 37°C for 6 days. On day 6, each well was pulsed with 1 μCi of [^{3}H]thymidine overnight. Cells were washed and harvested the following day, and uptake of radioactivity was determined as counts per minute per well.

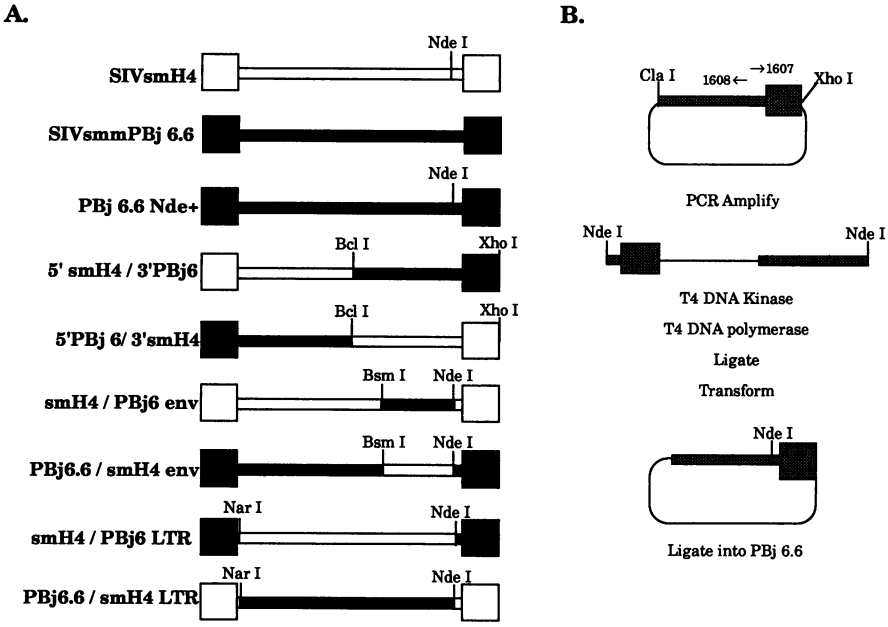

FIG. 1. Schematic of the various SIV chimeras constructed by exchange of smH4 and PBj6.6 sequences. (A) The two parental DNA clones are shown at the top; white boxes represent smH4, and black boxes represent PBj6.6. The constructions of the chimeric clones are shown below with relevant restriction sites. These schematics are representative of the genetic composition of the molecular clones, not of the viral RNA that would result from transfection into susceptible cell lines. As a result of reverse transcription, the RNA genome that would result from 5'smH4/3'PBj6 and 5'PBj6/3'smH4 would contain R and U5 at the 5' end and U3 and R at the 3' end; the U3 and R regions would be derived from the 3' LTR, and U5 would be derived from the 5' LTR, of the transfected clones. (B) Inverse PCR technique to introduce an *Nde*I site into PBj6.6. Oligonucleotide primers 1608 and 1607 (see Materials and Methods) were used to amplify a linear copy of the *Cla*I-*Xho*I subclone of PBj6.6. The resulting PCR fragment was treated with T4 DNA kinase and then with T4 DNA polymerase, ligated to recircularize, and transformed into *E. coli*. The resulting clones were screened for introduction of the unique *Nde*I site, and the sequence was determined in its entirety.

A mean was derived for each triplicate set, and a stimulation index (SI) was calculated for each concentration of virus.

Nucleotide sequence accession number. The nucleotide sequences of PBj clones PBj6.6, PBj6.9, and PBj6.12 have been submitted to GenBank under accession numbers L09212, L09213, and L09211, respectively.

TABLE 1. Outcome of pig-tailed macaques following inoculation with PBj clones[a]

Virus inoculum	Log $TCID_{50}$ of inoculum	Macaque designation	Plasma antigen[b]	Disease outcome
PBj6.6	3.9	PBs	+++	Dead (6 days)
		PIo	+++	Dead (6 days)
PBj14.6	3.4	PAp	+++	Dead (6 days)
		PNn	+++	Dead (7 days)
PBj6.9	3.6	PSn	+	Dead (10 days)
		PVn	−	Alive
PBj14.9	2.2	PDs	−	Alive
		PRs	−	Alive
PBj6.12	3.9	PJn	−	Alive
		PYn	−	Alive
PBj14.12	3.4	PWn	−	Alive
		PZo	−	Alive

[a] Macaques were inoculated intravenously with cell-free culture supernatants and monitored for clinical signs such as diarrhea, and dehydration, viremia, and alterations in lymphocyte subsets. Animals were humanely sacrificed if they developed severe diarrhea, dehydration, and collapse. Macaque PSn was euthanized because of neurologic signs; an autopsy revealed SIV-related meningitis and myocarditis. $TCID_{50}$, 50% tissue culture infective dose.

[b] The presence (+) or absence (−) of SIV antigen in the plasma was assessed at 7 days postinoculation. The number of + signs indicates the relative level of antigen; +++ indicates that the level of antigen was greater than the linear range of the ELISA reader, and + indicates a low level of antigen.

RESULTS

Generation of infectious molecular clones of PBj. Genomic DNA isolated from the biological clone of PBj (PBjbcl3) was used as a template for PCR amplification of 5' and 3' segments of the PBj genome. Two 5' genome halves (5'6 and 5'14) and three 3' genomes (3'6, 3'9, and 3'12) were generated, and six full-length molecular clones were thus derived. These clones were designated according to the numbers of the 5' and 3' halves used to generate the clone. For example, clone PBj6.9 was constructed from 5'PBj6 and 3'PBj9. All clones were infectious, as determined by transfection into CEMx174 cells; pronounced cytopathic effects were evident by 2 days after transfection. Similar to the parental virus, all six viruses replicated efficiently in CEMx174 cells, pig-tailed macaque PBMC, and mangabey PBMC.

A molecular clone of PBj that reproduces the acute disease. Each viral stock was inoculated into two pig-tailed macaques. The infectious titers of these viral stocks were similar with the exception of PBj14.9, for which the in vitro infectious titer was about 10-fold lower. Macaques inoculated with PBj clones sharing the 3'PBj6 sequences (PBj6.6 and PBj14.6) developed the acute lethal syndrome. Severe diarrhea accompanied by dehydration, lethargy, rash, fever, and severe lymphopenia (a mean of 972/μl) were observed and necessitated humane sacrifice of these four macaques by 7 days postinoculation. Virus could be isolated from PBMC by 3 days postinoculation, and high levels of viral antigen, as

TABLE 2. Comparison of acutely lethal PBj6 with related SIV clones

PBj6.6 vs	% Amino acid and nucleotide identity[a]									
	Gag	Pol	Vif	Vpx	Vpr	Tat	Rev	Env	Nef	LTR
smH4	95	98	95	94	93	86	92	90	70	94
SIVmac251	90	88	82	90	86	77	73	82	66	87
SIVmne clone 8	92	89	81	91	89	76	73	83	75	87
PBj4.41	99	99	99	99	100	100	98	99	99	99
PBj6.9				99	100	100	100	99	99	99
PBj6.12[b]				98	100	100	100	99	99	99

[a] Sequences were obtained from reference 29. The numbers shown represent percent amino acid identities of various gene products and percent nucleotide identity of the LTRs. PBj6.6, PBj6.9, and PBj6.12 share identical 5′ ends, so comparisons of Gag and Pol are not shown.

[b] Has a premature stop codon in the transmembrane glycoprotein coding region; percentages were calculated by comparison of Env sequences prior to the stop codon.

measured by a p27 antigen capture assay, were detected in the plasma by 7 days postchallenge. Pathologic lesions were characteristic of those seen in macaques inoculated with the parental, uncloned PBj virus stocks (data not shown).

Although all of the macaques used in this study became persistently viremic, the characteristic, acute diarrheal disease was not observed in macaques inoculated with the other four virus stocks. One macaque that was inoculated with PBj6.9 was sacrificed at 9 days as a result of severe neurologic signs (blindness and disorientation). Pathologic examination revealed meningitis, but intestinal lesions typical of PBj were absent. Immunohistochemical detection of SIV antigen in the brain (36a) and isolation of SIV from the cerebral spinal fluid suggested a direct link between the pathology and replication of SIV within the meninges. Low levels of viral antigen were detected in the plasma of this macaque prior to death. Transient fever, a moderate lymphopenia (a mean of 2,325/μl), and lymphocyte-associated viremia without plasma viremia were noted in the remaining nine macaques. Although persistently viremic, with high levels of SIV-specific antibodies in the plasma, these animals have remained healthy for 1 year postchallenge. Therefore, it was possible to compare the structures and biologic activities of lethal and nonlethal clones to identify viral factors involved in pathogenesis.

Lethal and nonlethal PBj molecular clones are highly related. The two clones that induced acute disease shared a common 3′ half, strongly implicating genes within this half of the genome in viral pathogenesis. The sequences of each of the three 3′ ends (3′PBj6, 3′PBj9, and 3′PBj12) and one of the 5′ ends (5′PBj6) were determined, and the predicted protein products of the lethal clone, PBj6.6, were compared with those of other SIV clones. The predicted Gag and Pol protein sequences of these clones were identical to those of the previously reported lethal clone, PBj4.41 (Table 2). All of the PBj clones, both lethal and nonlethal, were closely related (96 to 100% amino acid identity).

All three clones contained duplicated NF-κB sites in their 3′ LTRs. Interestingly, three tandem NF-κB sites were observed in the 5′ LTR of 5′PBj6, compared with two in that of PBj4.41 (9). Gene products encoded within the 3′ ends of the lethal and nonlethal clones were virtually identical in terms of predicted amino acid sequence. A detailed comparison revealed minimal differences (Table 3). The acutely lethal PBj6.6 differed from the nonlethal clones PBj6.9 and PBj6.12 in seven and five amino acids, respectively. Both differed in one or two amino acids in Vpx, two or four amino acids in Env, and one amino acid in Nef. One of the amino acid alterations in PBj6.12 was an in-frame stop codon at a glutamine residue in gp41, which would result in a prematurely truncated transmembrane glycoprotein. Premature stop codons have been observed previously in this location in SIVsmm and SIVmac clones (17, 22). The amino acid substitution within Nef of the two nonlethal clones relative to PBj6.6 was shared with the previously reported lethal PBj4.41 clone. Thus, alterations in Env and/or Vpx rather than in Nef are likely to explain the differences in disease potential between these highly related clones.

Viral determinants of the acutely lethal PBj6.6. To determine whether specific genes of the lethal PBj6.6 clone could confer a similar pathogenicity to a minimally pathogenic AIDS-inducing SIVsmm clone, various regions of the PBj6.6 clone were exchanged. These studies utilized the highly related smH4 molecular clone (19), since infectious clones representing the parent virus, SIVsmm9, have not been derived. The two PBj clones that reproducibly induced disease shared a common 3′ end, thereby implicating genes within this region of the genome in disease pathogenesis. In addition, others have theorized that the PBj envelope in combination with duplication of the NF-κB motif might be critical regions of the genome responsible for pathogenicity (6, 9). A series of PBj6.6/smH4 molecular chimeras was therefore constructed by exchanging three regions of the viral genome: (i) the 5′ and 3′ segments, (ii) the LTRs, and (iii) envelope genes.

The 5′ and 3′ segments were exchanged by using a conserved *Bcl*I site within the terminal end of *vif*, as shown in Fig. 1. A conserved *Nar*I site within the primer binding site was used to exchange the LTR regions, in addition to an *Nde*I site in near the C terminus of the envelope gene (and N terminus of the *nef* gene) of smH4. The PBj6.6 clone lacked this *Nde*I site as a result of a single base substitution relative to smH4; thus, an *Nde*I site was introduced into PBj6.6 by PCR mutagenesis (Fig. 1B). Four errors were introduced in the resulting clone presumably as a result of *Taq* polymerase; the resulting changes in amino acid sequence of Env and Nef are detailed in Table 3. Finally, the envelope genes were similarly exchanged by using a conserved *Bsm*I site in the amino terminus of *env* and the *Nde*I site described above to generate two clones (smH4/PBj *env* and PBj6.6/smH4 *env*). Envelope genes were not exchanged precisely as a result of the positions of the *Bsm*I site, 16 amino acids upstream of the predicted N terminus of gp120, and the *Nde*I site, 28 amino acids prior to the end of Env. Since PBj6.6 and smH4 have identical Env C termini and their N-terminal sequences differ by only one amino acid substitution (Ile: Val), the consequence of the position of these sites within *env* are considered to be minimal. All constructed clones were biologically active following transfection of CEMx174 cells.

TABLE 3. Comparisons of proteins and LTRs of PBj clones

Protein or region	Amino acid[a]	PBj6.6 vs[b]:			
		PBj6.9	PBj6.12	PBj4.41	PBj6.6 *Nde*+
Gag	306	—	—	Leu:Ser	—
	317	—	—	Ser:Asn	—
Pol	386	—	—	Thr:Ser	—
	652	—	—	Tyr:Ser	—
Vif	214	—	—	Ala:Val	—
Vpx	57	—	—	Tyr:His	—
	79	—	MetΔ	—	—
	80	—	PheΔ	—	—
	89	Cys:Arg	—	—	—
Rev	5	—	—	Glu:Gly	—
Env					
gp120	119	Asp:Gly	—	—	—
	288	—	Ala:Val	—	—
	355	—	—	Glu:Lys	—
	516	—	—	Gly:Ala	—
	655	—	—	—	Asn:Ser
gp40	743	—	Glu:Stop	—	—
	745	—	—	Ile:Val	—
	850	—	—	Phe:Leu	—
	861	—	—	Asp:His	Asp:His
	862	—	—	—	Leu:Ile
	871	Arg:Gly	—	—	—
	872	Gly:Arg	—	—	—
	874	Leu:Pro	—	—	—
Nef	26	—	—	Glu:Asp	Glu:Asp
	252	Phe:Leu	Phe:Leu	Phe:Leu	Phe:Leu
LTR	377	T:A	T:A	T:A	—
	455	A:G	A:G	A:G	—
	550	A:G	A:G	A:G	—
	744	—	—	C:T	—
	772	—	C:T	—	—

[a] Numbers other than those for the LTR indicate amino acid position from the initiating methionine. Numbers for the LTR indicate nucleotide position from the 5′ end of the provirus. Comparisons are shown only for proteins that differed in amino acid sequence from the lethal clone PBj6.6; no differences were observed in Vpr or Tat.

[b] Dashes indicate identity at that position with PBj6.6. Substitutions are indicated by the PBj6.6 amino acid shown first, separated by a colon from the amino acid in the compared clone. Δ indicates a deletion.

Pathogenicity of progeny virions of these six chimeras was tested by intravenous inoculation of two macaques per construct. The reconstructed PBj clone with the engineered *Nde*I site (PBj6.6 *Nde*+) was included as a positive control to verify that nucleotide substitutions introduced during PCR mutagenesis had not altered its pathogenicity. As shown in Table 4, despite the PCR errors in PBj6.6 *Nde*+, this virus maintained the lethal phenotype.

Macaques inoculated with each of the chimeras became persistently viremic with the exception of PBo, an animal inoculated with a chimera containing the smH4 genome with PBj6.6 LTRs. This animal has shown no evidence of SIV infection by virus isolation, Western immunoblot analysis, and PCR amplification from PBMC. Only virus derived from a clone that expressed the entire PBj genome under the control of the smH4 LTRs induced acute disease. One of the two macaques inoculated with this chimera developed typical fulminant PBj disease. Lethargy, rash, and lymphopenia were the only signs observed in the other macaque of this pair. Plasma from either of these macaques contained p27 viral antigen; however, the level of antigenemia was much higher in the macaque that succumbed to disease (Table 4). As observed previously, macaques sacrificed with acute disease had high levels of viral antigen in their plasma at 7 days; an apparent exception was a macaque inoculated with PBj6.6 *Nde*+ that died acutely at 4 days postinoculation. Presumably, this time point was too early for virus dissemination to the circulation. In addition, macaques inoculated with PBj6.6/smH4 *env* were antigenemic at 7 days but did not succumb to the acute disease. Lack of disease in macaques inoculated with five chimeras was an unexpected result. One potential reason for the lack of pathogenicity of these viruses could be instability of the NF-κB duplication within the LTR following transfection, as noted in a study using related clones (10). Therefore, PCR amplification and sequence analysis of SIV-specific sequences (a portion of *env* and the LTR) from PBMC of four animals was used as a confirmatory measure. The expected characteristic sequences of the V1 region of *env* and the LTR were observed in macaques inoculated with the chimeras 5′PBj6/3′smH4 and 5′smH4/3′PBj6.

In summary, the pathogenic determinants of the acute disease phenotype of PBj appear to be complex and multideterminant. Viral genes within the 5′ and 3′ halves of the genome appear to contribute significantly to pathogenesis, since neither chimera that exchanged 5′ and 3′ halves induced acute disease. Which genes within the 5′ half contribute to pathogenicity were not defined by this study. However, the PBj envelope gene is clearly one of the 3′ genes that contributes to disease expression, since exchange

TABLE 4. Experimental infection of pig-tailed macaques with PBj chimeras

Virus inoculum[a]	Virus titer (log)	Macaque designation[b]	Virus isolation	Plasma SIV antigen[c]	Disease outcome
5'smH4/3'PBj6	4.3	PBp	Yes	−	Alive
		PCs	Yes	−	Alive
5'PBj6/3'smH4	4.4	PCp	Yes	−	Alive
		PUo	Yes	−	Dead (45 days)
PBj6.6 *Nde*+	4.2	POl	Yes	NT	Dead (4 days)
		PPl	Yes	+++	Dead (6 days)
PBj6.6/smH4 LTR	4.2	PBi	Yes	+++	Dead (6 days)
		PVr	Yes	+	Alive
smH4/PBj6 LTR	3.4	PBo	No	−	Alive
		PWs	Yes	−	Alive
smH4/PBj6 *env*	4.4	POs	Yes	−	Alive
		PVg	Yes	−	Alive
PBj6.6/smH4 *env*	4.4	PNo	Yes	+++	Alive
		PTs	Yes	+++	Alive

[a] For structures, see Fig. 1A.
[b] Macaque PUo died at 45 days postinoculation with myocardial disease unrelated to SIV infection. Macaque PBo did not become infected following virus inoculation, as evidenced by negative virus isolation, Western blot analysis for SIV-specific antibodies, and inability to amplify SIV-specific sequences from PBMC DNA.
[c] Relative levels of plasma SIV antigen at 7 days postinoculation. +++, high level above the linear range of the ELISA reader; +, low level of antigen (barely above the negative cutoff); NT, not tested; −, no detectable antigen.

of the PBj envelope for that of smH4 eliminated the acutely pathogenic phenotype. In contrast, the duplicated NF-κB motif within the PBj LTR does not appear to be a critical disease determinant; a chimera in which the PBj LTR was replaced with an smH4 LTR was still capable of inducing full expression of the acute disease in 50% of inoculated macaques. Further inoculations would be required to determine whether this chimera is somewhat attenuated with respect to wild-type PBj.

Mitogenic activity of PBj as an in vitro correlate of pathogenicity. The results of macaque inoculations with chimeric viruses suggested the involvement in multiple genes in pathogenicity. An in vitro assay to screen candidate constructs would minimize the number of macaques required for in vivo studies. PBj has a number of unique biologic properties, such as the ability to stimulate proliferation of macaque PBMC and the ability to infect chimpanzee PBMC, that could be used as in vitro correlates of pathogenicity. Since the ability to stimulate proliferation of resting macaque PBMC could be a potential basis for the acute disease induced by PBj, the PBj viruses and chimeras generated in this study were analyzed for their effects on PBMC of an uninfected pig-tailed macaque. The mitogenic effects of virus supernatants derived from CEMx174 cell transfections were assessed after incubation of resting macaque PBMC with different concentrations of SIV p27 (Table 5). Triplicate wells were assessed for thymidine incorporation, a mean was derived for each virus, and thymidine incorporation was compared with that in a medium-alone control to derive an SI for each virus (Fig. 2). Incubation of the acutely pathogenic PBj6.6 virus resulted in a significant proliferative response (SI of 80), whereas incubation with smH4 did not induce a proliferative response (SI of 3.8). As shown in Fig. 2, the three viruses that induced the characteristic PBj disease (PBj6.6, PBj14.6, and PBj6.6 *Nde*+) induced proliferation of macaque PBMC in this assay, whereas those that did not induce disease induced minimal to no proliferation. A few of the virus supernatants induced a moderate degree of proliferation, with SIs of 10 to 15 with 1 ng of the viral antigen. Interestingly, these viruses, PBj6.9, PBj6.12, 5'PBj6/3'smH4, and PBj6.6/smH4 LTR, included the latter chimera, which induced 50% mortality of inoculated macaques. A gradation of intermediate effects was observed with this assay, and no single gene was associated with the mitogenic property. The general correlation of mitogenic effect with pathogenicity suggests that the underlying pathogenesis of acute PBj disease is linked to the ability of PBj to stimulate resting PBMC in vivo.

DISCUSSION

Six infectious molecular clones of the SIVsmm variant PBj were generated, and their pathogenicity for pig-tailed macaques was evaluated. Two clones induced the acutely lethal disease in inoculated pig-tailed macaques. In contrast to the genetic heterogeneity of clones derived in a preliminary study in which the virus isolate used for cloning was derived from a terminally ill PBj-infected macaque (PGg)

TABLE 5. Proliferative responses of PBMC from an uninfected pig-tailed macaque to PBj6.6 and smH4

Virus inoculum	SIV p27 antigen (ng)	Thymidine incorporation [cpm mean (SD)][a]	SI[b]
Medium		198 (48)	
5 μg of PHA		6,068 (2,062)	30.6
CEMx174 medium	Undiluted	698 (320)	3.5
	1:10	309 (228)	1.6
	1:100	136 (58)	0.7
	1:1,000	161 (60)	0.8
PBj6.6	10	31,044 (6,422)	156.8
	1	16,090 (5,140)	81.3
	0.1	4,253 (5,176)	21.5
	0.01	918 (1,232)	4.6
smH4	10	757 (692)	3.82
	1	206 (133)	1.04
	0.1	191 (86)	0.96
	0.01	197 (71)	1.0

[a] Values are means of triplicate wells pulsed for 16 h with [^{3}H]thymidine after incubation of the cultures for 6 days.
[b] Calculated from thymidine incorporation.

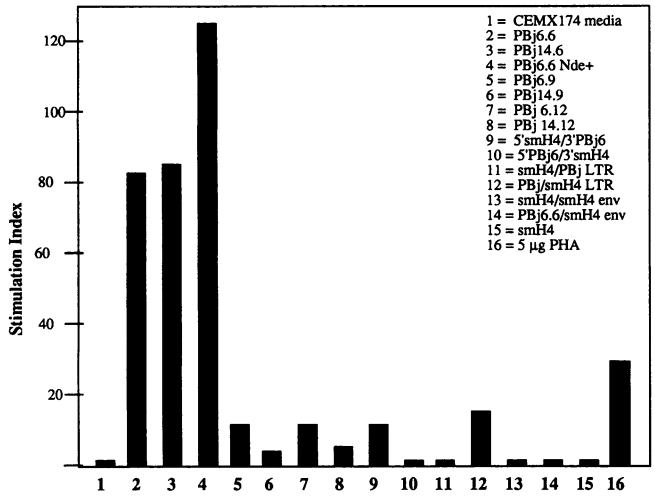

FIG. 2. Stimulation of lymphocyte proliferation by PBj viruses. The SIs for the PBj viruses and smH4/PBj chimeras are shown in histogram form. Unstimulated PBMC from a pig-tailed macaque were incubated with medium, supernatant from CEMx174 cells, supernatant from virus-infected cells containing 1 ng of SIV p27, or medium supplemented with 5 µg of PHA. To obtain equivalent amounts of viral antigen in all assays, the volumes of virus supernatant used were adjusted on the basis of SIV p27 content (Coulter). Uptake of [^{3}H]thymidine was measured from triplicate cultures in counts per minute, and an SI was calculated for each virus as described in Materials and Methods.

(31), the clones used in this study were similar in sequence to one another and to PBj4.41 (9). Despite this genetic homogeneity, only two of the six clones induced the acute disease syndrome in experimentally inoculated pig-tailed macaques. Fine genetic differences (amino acid substitutions and deletions) in *vpx*, *env*, the LTR, and *nef* were observed among these clones. Substitutions in *nef* were also shared with the lethal PBj4.41 clone; hence, *nef* is unlikely to be critical gene responsible for the differing pathogenicity of these clones. Therefore, *vpx*, *env*, and the LTR appear to be critical genes in determining the pathogenicity of these highly related clones. It is intriguing that PCR-induced substitutions in *env* and *nef* of PBj6.6 *Nde*+ did not abrogate the pathogenicity of this clone in vivo.

The viral determinants of PBj disease are complex. We attempted to characterize the genetic determinants responsible for the acute pathogenicity of PBj by exchanging defined regions of the genome (5′ and 3′ halves, LTR, and *env* genes) of the lethal clone with those of a highly related, minimally pathogenic SIVsmm clone. Surprisingly, only one chimera (PBj6.6/smH4 LTR) induced acutely lethal disease, and this virus did not appear to reproduce the full disease potential of the parental PBj clone; one of the two macaques inoculated with this chimeric virus died with the acute disease, compared with 100% mortality of macaques inoculated with parental PBj6.6. This difference suggested that this chimera was attenuated with respect to the parental virus; however, the number of animals studied (two) was too limited to permit definitive conclusions regarding degrees of pathogenicity. smH4 chimeras expressing the 5′ half of PBj6.6, the 3′ half of PBj6.6, or the PBj6 envelope, and PBj6.6 chimeras expressing the smH4 envelope, did not cause acute disease in inoculated macaques. These data suggest that (i) genes within both the 5′ and 3′ halves of the genome contribute to pathogenicity, (ii) duplication of the NF-κB motif within the LTR is not a critical determinant for disease, (iii) the PBj envelope is a critical determinant of pathogenesis, and (iv) the PBj envelope must act in concert with an additional PBj gene within the 5′ half of the genome (*gag*, *pol*, and the N terminus of *vif*).

This study demonstrates that the pathogenic effects of PBj cannot be conferred to a related minimally pathogenic clone by simple genetic exchanges. Thus, the pathogenicity of the PBj variant is probably the result of long-term accumulation of mutations in multiple genes. It is reassuring that the mutations necessary to reproduce this acute disease are complex, since this finding suggests that similar variants of HIV-1 are less likely to arise spontaneously. The complexity of the genetic determinants of PBj has precedence in the avian leukosis viruses, for which both the envelope and LTR have been implicated in pathogenicity (4, 12, 33). The determinants of the feline leukemia virus FAIDS strain (11) and the murine leukemia virus MAIDS strain (2) are less complex, involving one major viral gene, *env* for FAIDS and *gag* for MAIDS. The complexity of viral determinants of virulence is not surprising for the PBj variant, considering the complexity of viral transcriptional control of lentiviruses such as SIV.

In vitro mitogenic effects of acutely lethal PBj clones. Unraveling the mechanisms underlying PBj-induced disease requires a dissection of both the genetic and biologic properties of the virus. In this study, the previously reported mitogenic effect of PBj on resting macaque PBMC (13, 14) was analyzed as a possible in vitro correlate of disease. The ability of virus stocks to stimulate proliferation of resting PBMC in vitro correlated with pathogenic effects in vivo. Thus, virus stocks that stimulated resting macaque PBMC to proliferate were acutely lethal following inoculation of macaques. The chimeric virus containing the PBj genome with the smH4 LTRs that caused 50% mortality induced only moderate proliferation of PBMC. The numbers of animals were too small to permit definitive conclusions to be drawn from these differences. If these differences in vivo and in vitro were consistent in larger studies, the PBj LTR could be a minor disease determinant. However, it is evident from this study that the PBj LTR and the duplicated NF-κB binding site are not essential for induction of acute PBj disease. Therefore, as suggested previously, the mitogenic effects of PBj are associated with pathogenicity (13). However, this study did not delineate a specific viral protein in the proliferative phenotype of PBj. Similar to the pathogenic determinants, the genes responsible for inducing resting PBMC to proliferate appear to be complex.

The underlying mechanism of PBj-induced proliferation of resting macaque PBMC is unknown. This study strongly linked this in vitro effect to virulence but did not elucidate the underlying mechanism. The complex pattern of viral determinants for this phenotype suggests that two or more mechanisms may be acting simultaneously. Some possible mechanisms of action are (i) the direct effect of viral protein(s), (ii) cytokines secreted in response to infection or binding of PBj virus to PBMC, or (iii) cytokines or lymphokines secreted in culture supernatants of virus-infected CEMx174 cells. The proliferative assay in this study did not use purified virus preparations and thus would not distinguish between the direct effects of viral proteins and the effects of cytokines or lymphokines in the culture supernatant. In previous studies, proliferation was observed with purified, heat-inactivated PBj, suggesting a direct effect of viral proteins (13). In the present study, heat-inactivated PBj

did not induce proliferation in this assay system (data not shown). Such a direct effect could be nonspecific, such as caused by mitogens like PHA, or specific, such as a superantigen effect. Antigens of the latter type are thought to react with a specific T-cell receptor Vβ-expressing subset of T lymphocytes, inducing selective proliferation, and have been associated with the pathogenesis of mouse mammary tumor virus (16) and the MAIDS strain of murine leukemia virus (19). Preliminary studies have investigated a potential role for superantigens in HIV-1 infection, but so far these studies have been inconclusive (20, 23). Distinguishing between these possible mechanisms of PBj-induced proliferation is beyond the scope of this study. More detailed studies of the in vitro effects of PBj on PBMC will be necessary to address this issue. Since the in vitro effect correlates with pathogenicity, a better understanding of PBj-induced proliferation may aid in delineating the pathogenic mechanism of PBj-induced gastroenteritis.

The disease syndrome induce by PBj is not unlike the acute phase of HIV infection in humans. Thus, as in the acute viral syndrome observed in humans shortly after infection by HIV-1, PBj-infected macaques develop a fever, rash, lymphadenopathy, lymphopenia, and diarrhea (26, 34–37). However, whereas the acute phase of HIV infection is a transient nonlethal syndrome, the symptoms of PBj-induced disease are rapid, severe, and life threatening. Since PBj induces proliferation of resting macaque PBMC in culture, it is feasible that a similar effect in the infected animal results in widespread activation of lymphocytes/monocytes and the concurrent release of cytokines such as tumor necrosis factor α and interleukin-6. Indeed, the pathologic features of PBj-induced gastroenteritis, diffuse infiltration of SIV-infected lymphoid cells into the lamina propria of the intestinal tract with blunting of villi, are consistent with a cytokine-mediated enteritis (26, 27, 37). Lymphocyte activation would amplify the number of cellular targets for virus infection and allow rapid dissemination of virus throughout the lymphoid system, as suggested by the early plasma-associated viremia (7 days postinoculation) observed in PBj-infected macaques. Further molecular, virologic, and pathologic studies will be necessary to more clearly define the events that lead to the rapid death of these macaques, the viral genes responsible, and the critical interactions with the immune system of their host.

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