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Loss of the SIVsmmPBj14 Phenotype and *nef* Genotype during Long-Term

REBECCA S. SCHWIEBERT,* BINLI TAO, and PATRICIA N. FULTZ[1]

*Departments of Microbiology and *Comparative Medicine, University of Alabama School of Medicine, Birmingham, Alabama 35294*

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The ability of the simian immunodeficiency virus SIVsmmPBj14 (SIV-PBj14) to activate and induce proliferation of quiescent peripheral blood lymphocytes from macaques is an *in vitro* correlate of its acutely lethal *in vivo* phenotype. SIV-PBj14 differs from other SIV strains by encoding tyrosine at amino acid 17 (Y17) in Nef, which generates an activation motif important for signal transduction. Although intravenous inoculation of pig-tailed macaques with SIV-PBj14 uniformly leads to death within 2 weeks, inoculation by mucosal routes results in persistent infections that progress to AIDS. In the present study, we determined whether viruses in long-term survivors retained not only the Nef Y17 residue but also the biologic properties associated with rapid disease and death. Viruses reisolated at early and late times after mucosal infection of macaques with SIV-PBj14 were tested *in vivo* for acute lethality and *in vitro* for the ability to replicate in and induce activation and proliferation of quiescent macaque lymphocytes. In addition, the coding sequence for the first 55 amino acids in Nef was amplified from proviral DNA or plasma virion RNA by PCR or RT-PCR, respectively, and nucleotide sequences were obtained. The results showed that the majority of the quasispecies that persisted as disease progressed not only lost biological properties unique to SIV-PBj14, but also lost through mutation either Y17 or Y28 in Nef, which together were part of the activation motif. In the case of Y17, these mutations were stepwise to histidine then arginine, the amino acid encoded in this position in other SIV strains. We conclude, therefore, that replicative properties of the acutely lethal virus provide no selective advantage during long-term infections with SIV-PBj14 and that disruption of the activation motif in Nef is associated with loss of the acutely lethal phenotype.

INTRODUCTION

The extensive genetic diversity of strains of the human immunodeficiency virus (HIV) is manifest as differences in biologic properties, leading to questions regarding the influence of viral genotypes and phenotypes on progression to disease. It has been proposed that as an infected person progresses to AIDS, HIV increases in pathogenicity as mutations accumulate in the quasispecies (Albert *et al.,* 1990; Cheng-Mayer *et al.,* 1988; Connor *et al.,* 1993; Schuitemaker *et al.,* 1992; Tersmette *et al.,* 1989). This hypothesis is supported by studies in which transitions in HIV phenotypes from nonsyncytium-inducing (NSI) to syncytium-inducing (SI) were identified in viruses isolated as disease progressed (Connor *et al.,* 1993; Roos *et al.,* 1992; Schuitemaker *et al.,* 1992; Tersmette *et al.,* 1989). That these transitions often were coincident with declines in numbers of circulating $CD4^+$ lymphocytes reinforced the idea that the SI strains, which are more cytopathic for T-cell lines, were also more pathogenic (Connor *et al.,* 1993; Richman and Bozzette, 1994; Schuitemaker *et al.,* 1992).

The simian immunodeficiency virus (SIV)-macaque model has been used extensively to gain insight into the pathogenesis of HIV. SIV and HIV are closely related viruses, and strains of SIV that elicit disease in macaques similar to that induced by HIV in humans have been identified (Desrosiers and Letvin, 1987; Murphey-Corb *et al.,* 1986). Furthermore, studies have shown that successful transmission of primate lentiviruses is a function of viral genotype and phenotype as well as route of exposure. During transmission from an infected to an uninfected individual, minor variants of both HIV and SIV appear to establish infections in recipient humans and macaques, respectively, especially when virus exposure is to mucosal surfaces (Ahmad *et al.,* 1995; Cornelissen *et al.,* 1995; Furuta *et al.,* 1994; Wolinsky *et al.,* 1992; Zhang *et al.,* 1993; Zhu *et al.,* 1993; Zhu *et al.,* 1996; Trivedi *et al.,* 1994). Direct evidence for selective transmission comes from a study with two molecularly cloned chimeric SIV/HIV (SHIV) strains that encode the *env* genes from HIV-1_{HXBc2} and HIV-$1_{89.6}$. $SHIV_{HXBc2}$ was shown to be infectious for macaques after intravenous exposure, but it did not establish infection by the vaginal route; however, $SHIV_{89.6}$ established infections by both routes (Lu *et al.,* 1996).

Among various SIV isolates characterized to date, the SIVsmmPBj14 (SIV-PBj14) strain is the most pathogenic in that it reproducibly leads to death of pig-tailed macaques (*Macaca nemestrina*) within 6 to 12 days after intravenous inoculation (Dewhurst *et al.,* 1990; Fultz *et*

[1] To whom correspondence and reprint requests should be addressed. Fax: (205) 975-6788.

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al., 1990; Fultz and Zack, 1994; Schwiebert and Fultz, 1994). The pathogenesis of this strain for pig-tailed macaques, however, can be altered if infection occurs across a mucosal surface. Although all macaques infected by mucosal routes developed the acute disease syndrome (diarrhea, anorexia, depression, and rash) characteristic of intravenous SIV-PBj14 infections (Fultz *et al.,* 1989; Fultz and Zack, 1994; Lewis *et al.,* 1992), more than 50% of the animals recovered, and long-term persistent infections that eventually progressed to AIDS were established (Fultz *et al.,* 1995; this report). These results demonstrated unequivocally that the route of infection by a primate lentivirus can influence its natural history, especially during the acute phase.

Use of the SIV-PBj14-macaque model provides an advantage over other SIV models because this virus strain has unique biologic properties that not only can be evaluated *in vitro* but also have been shown to correlate directly with rapid death *in vivo* (Fultz, 1991; Fultz *et al.,* 1990; Novembre *et al.,* 1993; Schwiebert and Fultz, 1994). Furthermore, one of these properties — the ability to replicate efficiently in resting peripheral blood mononuclear cells (PBMC) from normal macaques — was recently shown to be genetically linked to a specific mutation in the *nef* gene. Using site-directed mutagenesis of SIVmac239, Du *et al.* (1996) replaced the arginine (R) at amino acid residue 17 and glutamine (Q) at position 18 with tyrosine (Y) and glutamic acid (E), respectively, which are identical to those encoded by SIV-PBj14 *nef,* to generate a mutant virus, termed SIVmac239/YEnef. This virus, like SIV-PBj14, replicated in resting lymphocytes, induced T-cell activation, and caused an acute disease, but not death, in macaques. The presence of Y17 in SIV-PBj14's Nef, together with amino acids 18 through 31, creates an immunoreceptor tyrosine-based activation motif (ITAM), with the structure YXXL-X_7-YXXL (Chan *et al.,* 1995). Since both tyrosine residues in ITAM's have the potential to be phosphorylated, an event that occurs during signal transduction in lymphocytes (Pawson, 1995), it is possible, given SIV-PBj14's unique properties, that Nef interacts with such a pathway. While it is clear that one role of the Nef protein is to accelerate replication in primary lymphocytes (Baur *et al.,* 1994; de Ronde *et al.,* 1992; Miller *et al.,* 1994; Spina *et al.,* 1994; Zazopoulos and Haseltine, 1993), with the possible exception of SIV-PBj14, Nef does not eliminate the requirement for lymphocyte activation before replication of primate lentiviruses can occur (Polacino *et al.,* 1993).

That SIV-PBj14 evolved from a minimally pathogenic strain, SIVsmm9, as macaque PBj progressed to AIDS suggests that this phenomenon might occur during HIV infection. It is possible, however, that survival of a virus mandates that less pathogenic viruses be selected during persistent infections. Macaques that were infected by mucosal routes with SIV-PBj14 and survived the acute infection provide an opportunity to address such possibilities by asking the following questions. First, do the viral quasispecies isolated from macaques that either survive or die during the acute disease phase have the same biologic properties as SIV-PBj14? Second, irrespective of outcome, are viruses with the same phenotypes transmitted in both groups? Third, does the highly pathogenic lentivirus SIV-PBj14 become more or less pathogenic during long-term persistent infections?

We report here biological and molecular analyses of viruses recovered from macaques at early and late times after mucosal infection with SIV-PBj14. The results show that replicative properties required for the acutely lethal phenotype apparently confer no selective advantage to SIV-PBj14 during long-term infections. Furthermore, loss of these properties is associated with mutations that lead to loss of Y17 in Nef or, in at least one case, to an apparent compensatory loss of the second tyrosine (Y28) in the ITAM.

MATERIALS AND METHODS

Animals and virus

Adult female (retired breeders) and young adult male pig-tailed macaques, seronegative for antibodies to SIV, STLV-I, and SRV, were used in this study. All animals were housed and maintained at the University of Alabama at Birmingham according to Animal Welfare Act guidelines. Inoculation of macaques by mucosal routes was described previously (Fultz *et al.,* 1995). Briefly, young adult pig-tailed macaques were atraumatically inoculated via the rectal or vaginal mucosa with a biological clone derived from SIV-PBj14, designated bcl3 (Fultz *et al.,* 1989). In addition, one female and one male macaque were exposed vaginally or urethrally to virus derived from PBMC of a macaque (52-97) that was originally infected by the rectal route with SIV-PBj14-bcl3 and died 9 days after exposure to virus. Two macaques (100-103, 9216) were inoculated intravenously with 10^5 50% tissue culture infectious doses ($TCID_{50}$) of a virus stock derived at 5 months after infection from PBMC of a macaque (3033) that was infected vaginally with SIV-PBj14-bcl3.

Periodically during the first month after infection and approximately every 6 weeks thereafter, blood samples were obtained from long-term survivors for analysis of lymphocyte subsets and virus reisolation. Heparinized blood was separated into PBMC and plasma by density gradient centrifugation on lymphocyte separation medium (LSM; Organon Teknika). Lymph node biopsies were performed on some animals at 5 to 6 months after infection; tissues were obtained at necropsy from animals that were euthanized when moribund. Single-cell suspensions of lymph nodes were prepared by mincing the tissue and passing it through a 1-mm^2 stainless steel wire mesh. At the time of routine blood collections, vaginal washes were performed by flushing the vaginal vault with 2 ml of RPMI-1640 containing 1% gentamicin and 1%

penicillin/streptomycin. Cells in the vaginal wash were pelleted and washed twice in RPMI-1640 medium containing 10% fetal bovine serum (10%-RPMI) before being used in various assays.

Reisolation of virus and generation of virus stocks

The SIV-PBj14-bcl3(52-97) stock was generated by coculturing PBMC obtained from macaque 52-97 at death with PBMC, previously stimulated with Concanavalin-A (Con-A), from a normal pig-tailed macaque. Plasma, serum, PBMC, vaginal wash cells and fluid, or mononuclear cells from lymph nodes were cocultured with uninfected pig-tailed macaque PBMC to amplify viruses. Supernatants were harvested when the reverse transcriptase (RT) activity in culture medium was approximately 5×10^5 counts per minute or greater. Cells and debris were pelleted by centrifugation; the supernatant was filtered through an 0.45-μm filter and aliquoted for storage at $-80°$. The $TCID_{50}$ of virus stocks was determined by limiting dilution and infection of Con-A-stimulated macaque PBMC. Generation of SIVsmm9 and SIV-PBj14 stocks was described previously (Schwiebert and Fultz, 1994). Cocultured cells were washed, and cell pellets were stored at $-80°$ for subsequent proviral DNA extraction and sequence analysis.

Assays for SIV $p27^{gag}$ and cytokines

The concentration of SIV $p27^{gag}$ antigen in plasma was determined with an SIV-p27 enzyme-linked immunosorbent assay (ELISA) antigen capture kit (Coulter), according to the manufacturer's instructions. Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) concentrations in serum were assayed with commercial ELISA kits (Innotest hTNF-α, Innogenetics, Belgium, distributed by Biosource International; Cytoscreen Human IL-6 ELISA, Biosource International).

Infection of quiescent macaque PBMC

Quiescent macaque PBMC were obtained by separation of blood on LSM and were cultured immediately without mitogen stimulation in 10%-RPMI. Approximately 1.5×10^7 PBMC were infected with 10^4 $TCID_{50}$ of virus stocks in 2 ml of 10%-RPMI without IL-2; the virus was allowed to adsorb for 2 to 4 hr, after which time cultures were maintained in 10 ml of 10%-RPMI. Every 5 days, 3 ml of medium were replaced and cell-free RT activity was determined. Each set of experiments included mock-infected and SIVsmm9- and SIV-PBj14-infected cultures, as negative and positive controls, and were performed at least twice with PBMC from different normal macaques.

Activation of cultured PBMC populations was determined by measuring the percentage of cells that expressed CD25 (IL-2 receptor α chain). Analyses were performed with a FACS-STAR flow cytometer (Becton–Dickinson) using murine anti-human fluorescence-labeled monoclonal antibodies (Becton–Dickinson) and gating lymphocytes according to forward-scatter-versus-side-scatter characteristics. Induction of lymphocyte proliferation in these same cultures was measured by incorporation of [^{3}H]thymidine (DuPont NEN Research Products) into cellular DNA, as described (Schwiebert and Fultz, 1994). Stimulation indices were determined by dividing the cpm incorporated by lymphocytes infected with virus by the cpm of uninfected control lymphocytes.

PCR amplification, cloning, and sequence analysis

PBMC, single-cell suspensions of lymph node biopsy tissue, and cells from vaginal washes were cocultured with normal macaque PBMC. When virus replication was detected, cells from these cultures were pelleted and washed twice in 10 ml of phosphate-buffered saline. Genomic DNA was extracted using the QIAamp Blood kit (Qiagen Inc.), and 580-bp fragments covering approximately 75% of the 5′ end of *nef* were generated by PCR amplification with the following primers: Nef I, 5′-GTGCTCCAGAGCCTCTCAAGGACGCT-3′, and Nef IV, 5′-CGGTAAATGTCTCAGATGAAGCTCA-3′. After purification with the Geneclean kit (Bio 101), PCR amplification products were cloned into the TA cloning vector and then transfected into competent *Escherichia coli* cells (TA Cloning kit; Invitrogen). DNA from multiple clones, representing at least two independent PCR amplifications, was sequenced by the dideoxynucleotide chain termination method (Sequenase Version 2 kit; U.S. Biochemical Corp.), using the primer Nef III, 5′-TCATCAGAGTTCAGATAGCCTACCT-3′. Some samples for sequence analysis were generated by RT-PCR after ultracentrifugation of plasma to concentrate virions. Viral RNA was extracted from the pellet using phenol-chloroform, and cDNA was synthesized from the RNA template using Moloney Murine Leukemia Virus reverse transcriptase and the 1st-Strand cDNA Synthesis kit (Clontech Laboratories). Consensus sequences were generated using the Assembly Lign program (International Biotechnologies Inc.). The base designation threshold value for calculating a consensus sequence was 80%.

RESULTS

Clinical disease course following inoculation

The early disease course of six macaques infected after mucosal exposure to SIV-PBj14-bcl3 was described previously (Fultz *et al.,* 1995). Since that report, three of the macaques were euthanized after development of an AIDS-like disease (Table 1). Subsequently, two additional macaques were infected mucosally with virus reisolated 9 days after rectal infection of macaque 52-97; one macaque was exposed by the vaginal route (3006) and one by the penile urethra (118F). These two animals also developed acute disease characterized by diarrhea, de-

TABLE 1

Outcome of Infection of Macaques by Mucosal Routes with SIV-PBj14-bcl3

Animal	Route	Dose	Outcome
52-97	Rectal	10^4 $TCID_{50}$	Died Day 9
72-57	Rectal	10^3 $TCID_{50}$	Died Day 14
72-73	Rectal	10^3 $TCID_{50}$	Died 24 months p.i., AIDS
82-50	Rectal	10^4 $TCID_{50}$	Died 15 months p.i., AIDS, chronic malaria
3006	Vaginal[a]	10^4 $TCID_{50}$	Died Day 14
3033	Vaginal	10^3 $TCID_{50}$	Died 5 months p.i., AIDS
3097	Vaginal	10^4 $TCID_{50}$	Alive 30 months p.i., AIDS
118F	Urethral[a]	10^4 $TCID_{50}$	Died 6 months p.i., AIDS

[a] Inoculum: Virus reisolated from macaque 52-97 at time of death after rectal inoculation of SIV-PBj14-bcl3, with which the remaining animals were infected.

pression, and anorexia; the female macaque died on Day 14 after infection, but the male macaque survived, developed progressive disease, and was euthanized 6 months after infection. There was no correlation between virus dose in the inoculum and disease course. All of the animals that survived seroconverted within 4 to 6 weeks.

Although all macaques infected by the mucosal route developed an acute disease syndrome, virus replication and cytokine production were markedly decreased in animals that survived the primary viremia. Compared to those animals that died within 2 weeks, mean plasma SIV $p27^{gag}$ antigenemia was one to two $\log_{10}$ less in animals that survived initial infection (Fig. 1A); this difference was greater when the titers of infectious virus in plasma were determined. The mean titer of infectious virus in plasma of survivors was approximately 10^3 $TCID_{50}$, whereas that of animals that died acutely was 5×10^5 $TCID_{50}$. Either none or only minimal amounts of IL-6 and TNF-α were detected in serum from the surviving macaques (Figs. 1B and 1C). In contrast, all macaques that died during the acute disease phase after infection with SIV-PBj14-bcl3 by mucosal routes had virus burdens and cytokine levels comparable to those found in macaques that died early after intravenous inoculation, as reported previously (Schwiebert and Fultz, 1994). Consistent with the observation that virus dose did not correlate with disease outcome (Table 1), plasma levels of IL-6 and TNF-α also showed no correlation; macaque 72-57, that received the lower dose (10^3 $TCID_{50}$), had the highest levels of the two cytokines among all animals that died acutely (Fig. 1).

Biologic properties of viruses isolated during the acute disease phase after mucosal infection

The ability of SIV-PBj14 to replicate in and to activate and induce proliferation of resting macaque PBMC is directly correlated with the acutely lethal disease. Thus, it was of interest to determine whether these properties were retained by viruses circulating in animals that ultimately survived the acute disease syndrome. Virus isolates recovered from six macaques within the first 15 days after mucosal inoculation of SIV-PBj14-bcl3 were used to infect quiescent PBMC from normal macaques, and culture supernatants were monitored for production of virus. Regardless of an animal's survival outcome, viruses reisolated during primary viremia not only replicated to high levels, as measured by RT activity (data not shown), but also activated PBMC, as evidenced by increased percentages of cells expressing CD25 (Fig. 2A). Cells from these same cultures with high levels of $CD25^+$ lymphocytes proliferated and incorporated [^{3}H]-thymidine into cellular DNA (Fig. 2B). The extent of proliferation was directly related to virus production; stimulation indices for cultures infected with SIV-PBj14-bcl3-derived viruses increased as the RT activity of viruses in the culture supernatants increased (data not shown). These results showed that all viruses recovered early after mucosal infection retained *in vitro* biologic properties unique to SIV-PBj14. Furthermore, virus recovered from 52-97 on Day 9 was used to infect macaques 3006 and 118F; both animals developed the acute disease syndrome, indicating that the virulent *in vivo* phenotype was also retained.

Biologic properties of viruses isolated from long-term survivors of mucosal infection

Of more importance was whether viruses with the above properties persisted as the dominant phenotype of the quasispecies during long-term infections. Therefore, viruses isolated at 5 to 6 months after infection from lymph node cells or PBMC of those macaques that survived the acute disease syndrome were characterized. The isolates varied in their ability to replicate in and induce activation and proliferation of quiescent macaque PBMC (Figs. 3–5). Virus isolated from macaque 72-73 at 6 months retained the phenotype of SIV-PBj14, while that from animal 118F at 5 months appeared to retain some ability to replicate in and induce proliferation of resting PBMC. In contrast, isolates recovered from macaques 82-50 (6 months), 3033 (5 months), and 3097 (5 months

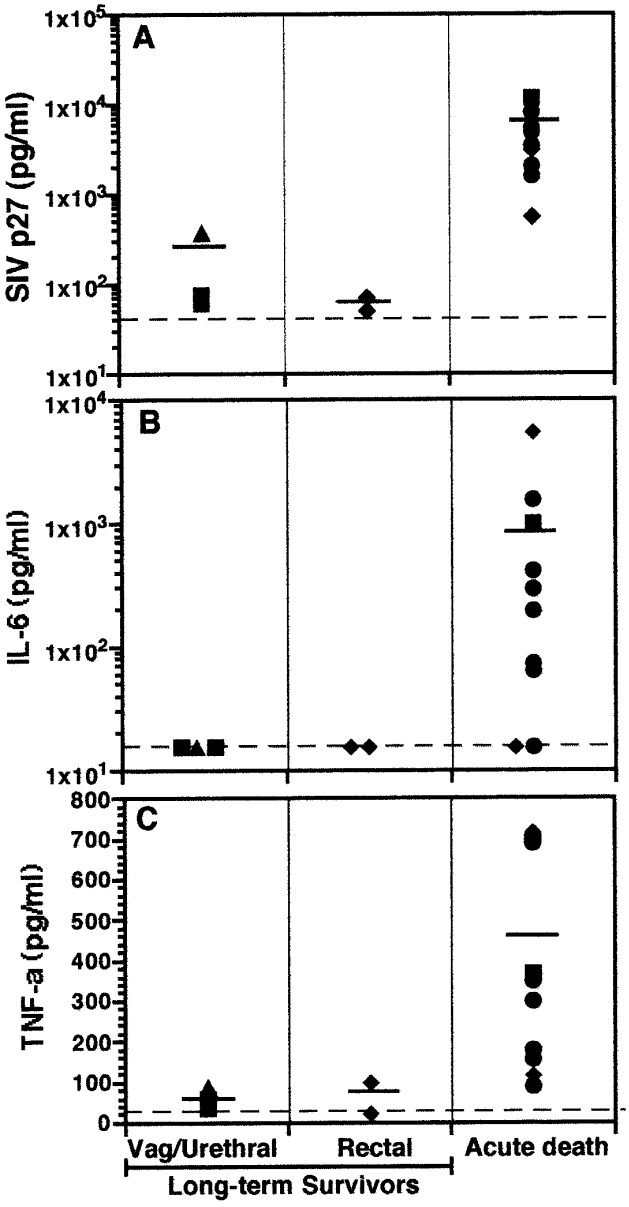

FIG. 1. Peak plasma levels of SIV p27^{gag} (A) and inflammatory cytokines IL-6 (B) and TNF-α (C) in pig-tailed macaques infected with SIV-PBj14-bcl3 by mucosal routes; all measurements were done on plasma obtained between 7 and 16 days after inoculation. Symbols represent values for individual animals and indicate route of inoculation as vaginal (■), urethral (▲), or rectal (♦). Values obtained at time of acute death for macaques (n = 8) inoculated intravenously (●) are shown for comparison and were published previously (Schwiebert and Fultz, 1994). Horizontal lines indicate arithmetic means.

and later) exhibited the phenotype of SIVsmm9, the parent virus of SIV-PBj14.

In vivo analysis of SIV-PBj14-bcl3 reisolated from a long-term survivor

As definitive evidence for loss of the acutely lethal phenotype by SIV-PBj14-bcl3 during long-term infection, we determined whether these viruses could induce the acute disease syndrome *in vivo.* The phenotype of the quasispecies obtained from macaque 3033 at death 5 months after infection resembled that of SIVsmm9, indicating that it should not induce the acute disease syndrome and death in macaques. To verify this prediction, macaques 100-103 and 9216 were inoculated intravenously with a high dose of this virus. This route was used because 100% of pig-tailed macaques inoculated by this, rather than by a mucosal, route died within 12 days (Fultz *et al.,* 1989; Fultz and Zack, 1994; Lewis *et al.,* 1992; Schwiebert and Fultz, 1994). Neither animal showed signs of acute disease following inoculation, but persistent infections accompanied by seroconversion were established. *In vitro* assays demonstrated that the virus reisolated from 100-103 on Day 6 after infection replicated poorly in resting macaque PBMC; however, virus from 9216 replicated as efficiently as SIV-PBj14 (not shown). Likewise, the percentage of cells expressing CD25 and proliferation of PBMC in cultures of virus from 100-103 were similar to those in the uninfected and SIVsmm9-infected cultures (Fig. 5), whereas virus from 9216 had properties like SIV-PBj14 (Fig. 6). That virus isolated from macaque 9216 on Day 6 resembled SIV-PBj14 phenotypically suggests that viruses with these

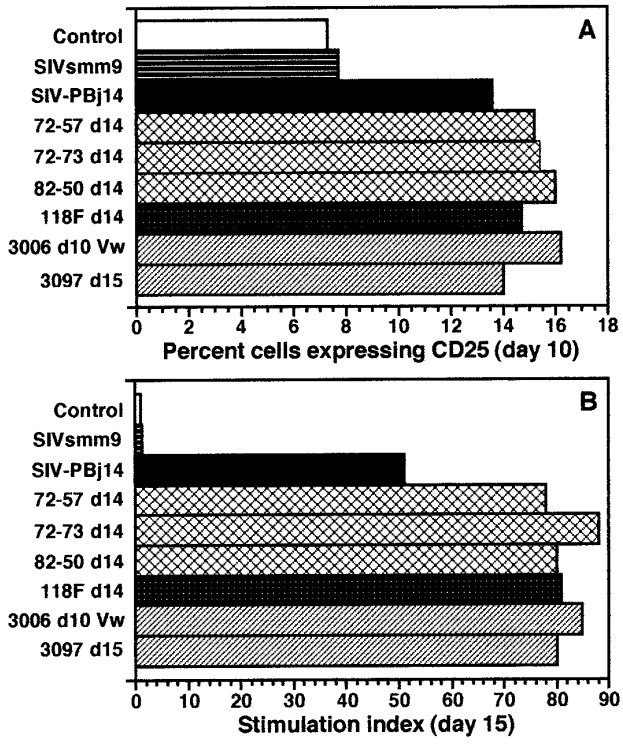

FIG. 2. Activation (A) and proliferation (B) of resting macaque lymphocytes by viruses isolated within 15 days after inoculation of macaques with SIV-PBj14-bcl3 by mucosal routes. All viruses were recovered from PBMC except as indicated by Vw, vaginal wash. Animals inoculated by the same route are grouped and indicated by bars with identical fills. The cells were evaluated on the days, indicated in parentheses, after infection of resting PBMC. Control, uninfected PBMC. Results from a representative experiment are shown.

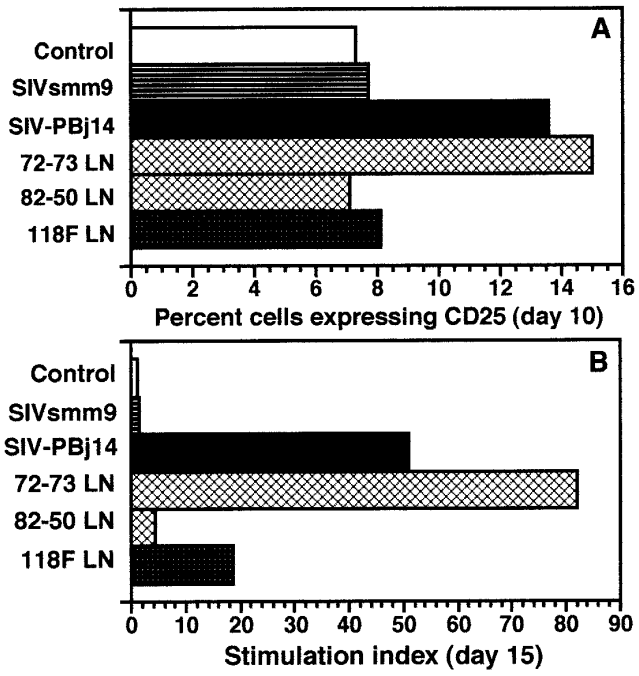

FIG. 3. Activation (A) and proliferation (B) of resting macaque PBMC induced by viruses isolated from lymph node biopsies 6 months after inoculation of macaques with SIV-PBj14-bcl3 by mucosal routes. See legend to Fig. 2 for explanations.

properties were present in low numbers in the inoculum and were amplified early after infection of 9216.

Genetic analysis of Nef

Because the presence of Y17 in the Nef protein appeared to correlate with the acutely lethal phenotype and unique *in vitro* biologic properties of SIV-PBj14 and SIVmac239YEnef, the first 55 amino acids of the *nef* genes of viruses reisolated from mucosally infected macaques at various times were sequenced. Again, regardless of the survival outcome of the animal (Table 1), viruses recovered between Days 9 and 15 after infection encoded Y17 (Fig. 7). These results correlated with the observation that viruses obtained during this period, including those from animals that survived the acute disease syndrome, exhibited biological properties (Figs. 1 and 2) associated with Y17 in Nef of SIV-PBj14 and SIVmac239YEnef (Du *et al.,* 1996, 1995).

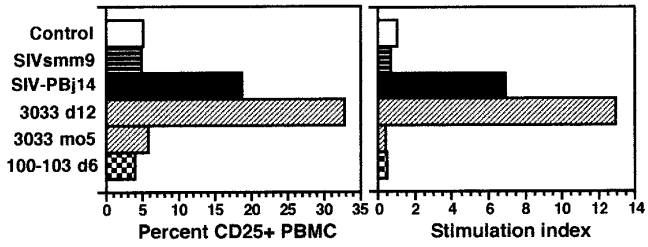

FIG. 5. Loss of the ability to activate and induce proliferation of resting PBMC by SIV-PBj14-bcl3 progeny viruses from a persistently infected and a recipient macaque. Virus isolated from macaque 3033 at time of death (5 months after vaginal inoculation) was used to infect macaque 100-103 intravenously. All viruses were isolated from PBMC at the indicated times after infection. Results from a representative experiment are shown.

With the exception of proviruses from macaque 118F, which included one variant encoding histidine at position 17 (H17), all sequenced proviruses in PBMC obtained within the first 6 weeks after mucosal infection retained Y17 in the Nef protein (Fig. 7). Between 3 and 6 months after infection, however, amino acids encoded at position 17 in *nef* of viruses in various tissues from different animals included tyrosine, histidine, or arginine (R17). Although the 6-month quasispecies found in the lymph node of macaque 72-73 phenotypically resembled SIV-PBj14, only 1 of 11 clones retained the Y17. The remaining 10 clones and all of the clones sequenced from 82-50, whose quasispecies had a phenotype similar to that of SIVsmm9, had histidine residues at position 17. After 1 year of infection of 72-73, Y17 in Nef was replaced completely by either histidine or arginine, the latter of which is the amino acid found at this position in SIVsmm9 and other SIV strains, including SIVmac239. A combination of these two amino acids also was present in proviruses from macaque 118F at 6 months (time of death). That the biologic phenotypes of the viral quasispecies from macaques 72-73 and 82-50 were different, but H17

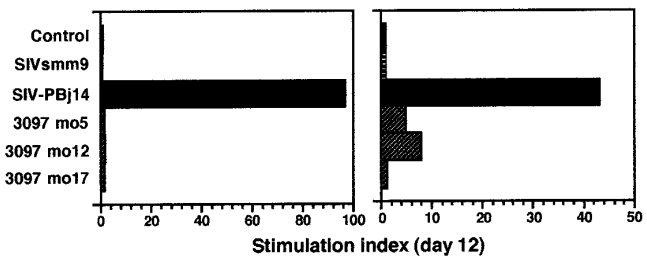

FIG. 4. Loss of the ability to induce proliferation of resting PBMC by viruses isolated from macaque 3097 at the times indicated after vaginal inoculation of SIV-PBj14-bcl3. Results from two independent experiments with PBMC from different normal macaques are shown.

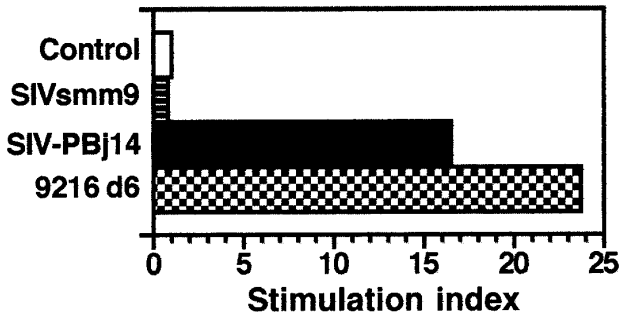

FIG. 6. Induction of proliferation of resting macaque PBMC by virus isolated from PBMC of macaque 9216 after intravenous inoculation of virus recovered from macaque 3033 at time of death. Values are means of two experiments with PBMC from two normal macaques.

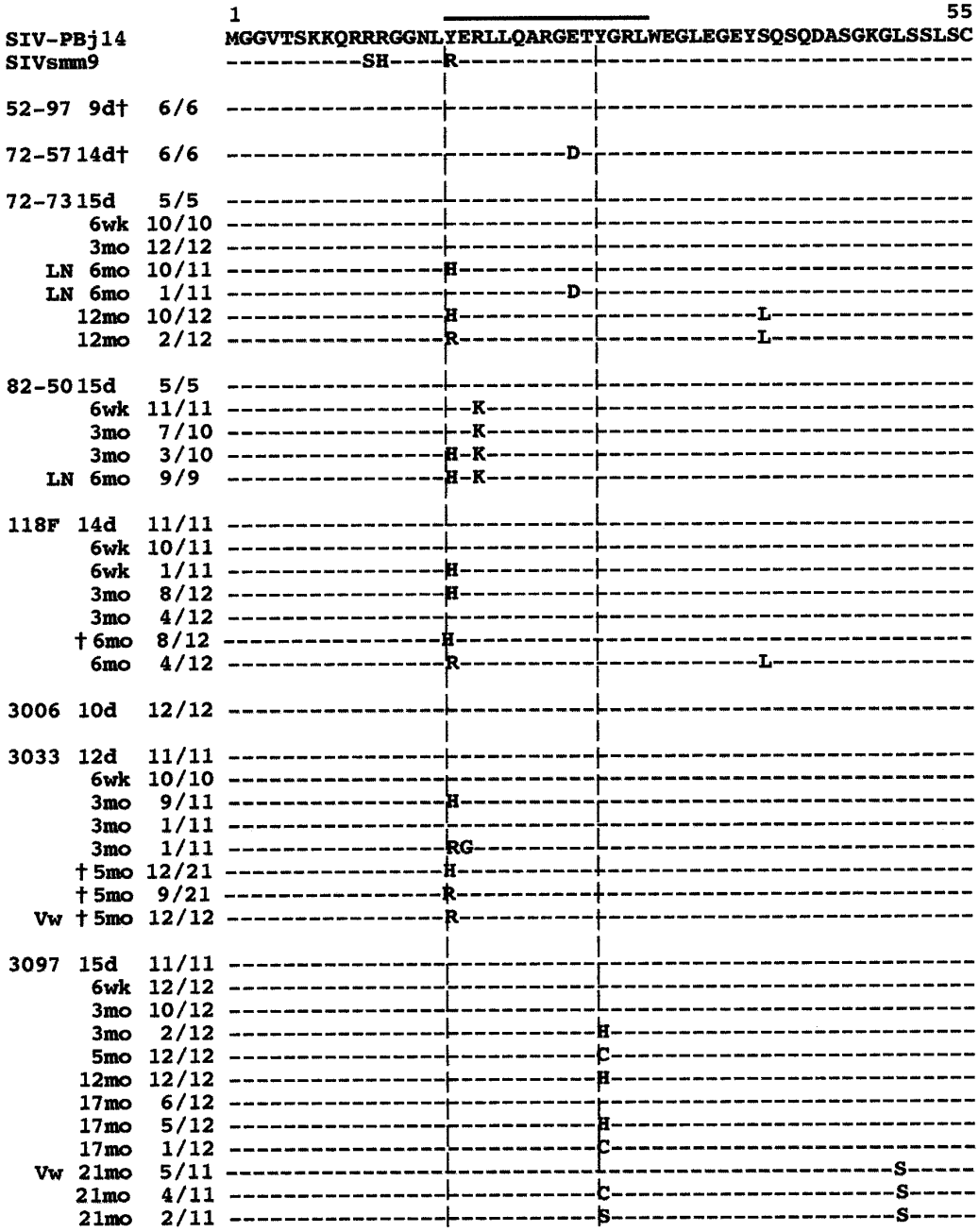

FIG. 7. Sequences of the first 55 amino acids in Nef of viruses isolated from macaques infected with SIV-PBj14-bcl3 by mucosal routes. Sequences from each animal are grouped and are relative to that of SIV-PBj14 and SIVsmm9. All of the sequences were derived from proviral DNA in cultures of PBMC, except where indicated, at the given times after inoculation. Some proviral DNA was obtained from lymph node biopsy tissues (LN) or from cultures infected by vaginal wash fluids (vw). For the sequences from macaques 52-97, 72-73, 82-50, and 72-57, RT-PCR was used to amplify virion RNA from plasma collected on Days 9 to 15 after infection. Sequences derived from samples obtained at time of death are indicated by †. The numbers to the left of the sequences reflect the number of clones with the given amino acid relative to Y17 only. The other amino acids are consensus sequences for all clones from the given animal and time, and therefore, may not be present in the same relative proportions as amino acids at residue 17. The dotted lines are for reference relative to Y17 and Y28, and the horizontal bar identifies the ITAM.

predominated in both, suggests that the SIV-PBj14 phenotype might require other regions of the genome as well as Nef Y17.

Of the two macaques that survived long-term after vaginal infection, the viruses recovered from the vaginal wash of 3033 at 5 months (when the animal died of AIDS) encoded only arginine (Y17R), whereas proviruses in PBMC contained a mixture of H17 and R17 (Fig. 7). However, when the supernatant from 3033's 5-month PBMC culture (used to inoculate 100-103 and 9216) was passaged *in vitro* in normal pigtailed macaque PBMC, 3 of 10 clones had retained the Y17 (Fig. 8), indicating prefer-

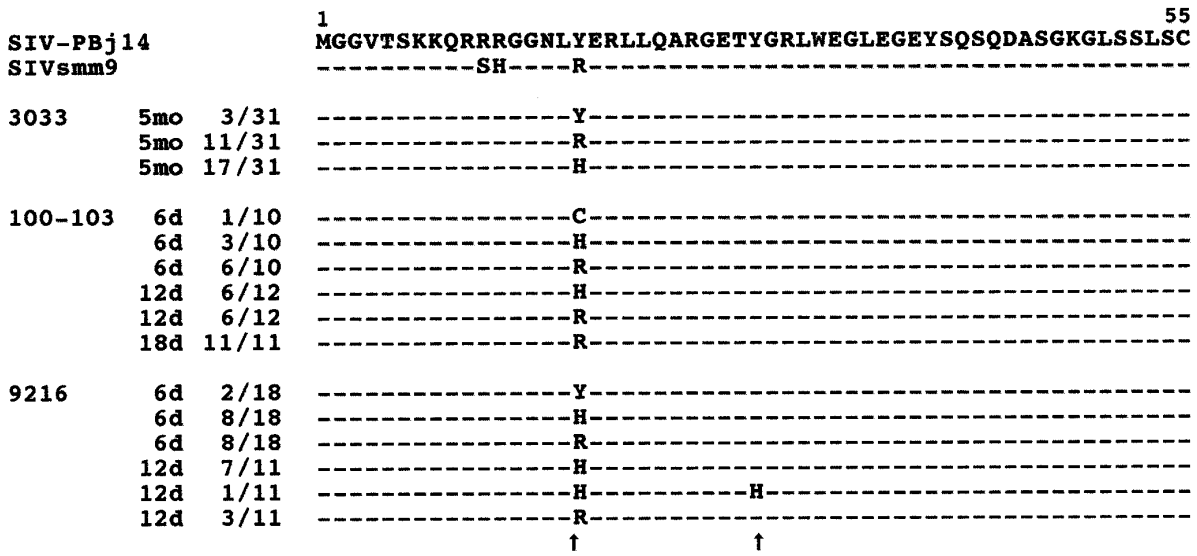

FIG. 8. Sequences of the first 55 amino acids in Nef of viruses isolated from macaques 100-103 and 9216 infected intravenously with virus recovered from macaque 3033 at death. The data for macaque 3033 include the 21 clones from PBMC (see Fig. 7) plus sequences from 10 additional clones generated from PCR products of *in vitro* passage of 3033's recovered virus. Sequences from each animal are grouped and are relative to that of SIV-PBj14 and SIVsmm9. See Fig. 7 legend for additional information. Arrows identify the positions of Y17 and Y28 in SIV-PBj14.

ential amplification of viruses with SIV-PBj14's genotype. This result and the finding of 2 of 18 clones with Y17 in virus recovered from macaque 9216 at 6 days explains this latter virus' phenotype *in vitro* (Fig. 6). Finally, of particular interest was the observation that, irrespective of time after infection, all of the proviruses from macaque 3097 that were evaluated retained Y17. However, beginning at 3 months, Y28 in some proviruses had been replaced by either a cysteine or histidine and in two cases of virus isolated from the vaginal wash at 21 months, there was a Y28S mutation. Mutations that change either of these tyrosines disrupt the activation motif encoded in SIV-PBj14 Nef amino acids 17 through 31, suggesting that mutation and loss of Y28 may be sufficient to compensate for retention of Y17.

DISCUSSION

While intravenous infection of pig-tailed macaques with SIV-PBj14-bcl3 uniformly results in death in 7 to 10 days, infection across a mucosal surface can lead either to rapid death or to persistent infections that ultimately progress to AIDS. Compared to animals that died acutely, animals that survived the primary disease syndrome had no detectable IL-6 in serum and lower virus loads and TNF-α concentrations. These results extend previous ones (Schwiebert and Fultz, 1994) by demonstrating that the correlation between replication of SIV-PBj14-bcl3, cytokine production and acute death does not extend to the acute disease syndrome (diarrhea, anorexia, depression, lymphopenia). That is, despite having low levels of inflammatory cytokines and plasma viremia, surviving macaques still developed severe acute disease. The decreased virus loads in the survivors may reflect more limited replication of SIV-PBj14 in cell types, such as macrophages and Langerhan's cells, that populate mucosal tissues, rather than in lymphocytes which would be encountered and infected preferentially after parenteral inoculation. A recent study that examined cervicovaginal mucosa within the first few days after vaginal inoculation of macaques with SIVmac251 demonstrated SIV proviral DNA in cells resembling both lymphocytes and dendritic cells (Spira *et al.,* 1996). If replication of the virus in mucosal tissues is restricted in any way, this delay may give the immune system time to respond and downregulate the primary burst of virus replication. These results demonstrate, however, that an extremely virulent lentivirus can be transmitted efficiently by mucosal routes and retain its virulence during the acute stage of infection (Table 1, macaque 3006).

Our results clearly show that progression to an AIDS-like disease did not require that biologic properties associated with the acutely lethal phenotype be retained as the major population in the quasispecies; however, it is possible that these viruses may have had an impact on pathogenesis during the acute stage of disease. In fact, most of the viruses isolated after resolution of the acute disease syndrome exhibited phenotypes more characteristic of prototypic SIV strains. The reversion to the parental SIVsmm9 phenotype was not limited to the inability to replicate in quiescent macaque PBMC and to activate and induce proliferation of lymphocytes, but also extended to *in vivo* infections.

Loss of the SIV-PBj14 phenotype was associated with step-wise mutations in Nef from Y17 to H17 to R17, the latter of which is encoded in SIVsmm9 and other SIV

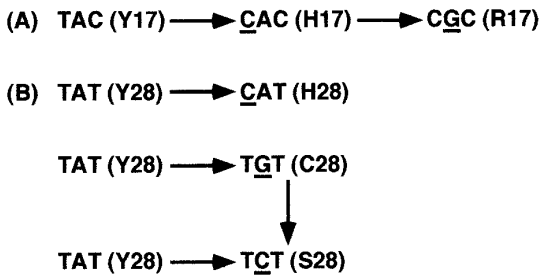

FIG. 9. Evolution of SIV-PBj14-bcl3 *nef* gene during long-term persistent infections. Nucleotide changes that occurred in codons for tyrosine residues 17 (A) and 28 (B) in Nef are underlined. SIVsmm9 encodes a tyrosine at residue 28 with the same TAT codon.

strains (Fig. 9A). Both of these changes require a single nucleotide substitution. In the case where no change in Y17 was observed during 1 year of infection (Fig. 7, 3097), it is interesting that mutations disrupted the second YXXL motif of the ITAM, resulting in replacement of Y28 with cysteine, histidine, or serine (Fig. 9B). Because the TAT codon for Y28 is essentially invariant in other HIV-2 and SIV strains, it appears that the Y28C, Y28H, and Y28S mutations compensate for retention of Y17. This possibility is supported by the loss of the SIV-PBj14 phenotype in viruses isolated from macaque 3097 at late times and suggests that the entire ITAM, and not the first YXXL SH2-binding domain alone, is required for manifestation of the SIV-PBj14 phenotype. Furthermore, Du *et al.* (1996) recently demonstrated that replacement of Y28 with phenylalanine (Y28F) in the SIVmac239/YEnef strain resulted in loss of the ability to replicate in resting PBMC. It is possible that a very small percentage of virions in the SIV-PBj14-bcl3 inoculum already encoded H17 and R17 and were amplified *in vivo.* However, this is unlikely because the rates of change for Y17 and Y28 were the same, suggesting similar selection pressures at both sites.

The other exception to the association between loss of Y17 and the SIV-PBj14 phenotype was the viral quasispecies isolated from the lymph node of macaque 72-73 at 6 months after infection. Although 10 of 11 clones had the Y17H mutation in Nef and only one clone retained Y17, the *in vitro* phenotype of the quasispecies was that of SIV-PBj14. Consistent with these results, analysis of the quasispecies isolated from macaque 9216 on Day 6 after intravenous inoculation of macaque 3033's 5-month virus revealed that only 2 of 18 clones encoded Y17, whereas the remainder were H17 and R17; however, 9216's virus had phenotypic properties like SIV-PBj14 (see Results). Previously, using biologically cloned viruses with defined phenotypes, we showed in *in vitro* mixing experiments that if only 10% of the viral population was phenotypically SIV-PBj14, then full expression of the biological properties of this virus was observed (Tao and Fultz, 1995). Thus, it is likely that a sufficient percentage of the viruses in macaques 72-73 and 9216 retained the dominant properties of SIV-PBj14. Whether this proportion is also valid for Y28 and its mutant forms remains to be tested, but the phenotypes of viruses with Y28 mutations that were isolated from macaque 3097 suggests that this may not be the case.

The importance of the *nef* gene to disease progression in HIV-1-infected individuals has not been defined precisely, but recent studies showed no correlation in the genotypic and phenotypic characteristics of *nef* when viruses from progressors and long-term nonprogressors were compared (Huang *et al.,* 1995a, 1995b; Michael *et al.,* 1995). In this regard, loss of the ITAM by mutation of the *nef* allele in SIV-PBj14-bcl3-infected macaques did not retard the development of an AIDS-like illness. However, our results imply that viral phenotypes, such as that of SIV-PBj14, that interfere with establishment of persistent infections are not advantageous for the virus. In addition, because Nef-specific antibodies and cytotoxic T lymphocyte responses are often elicited early after SIV infections (Bourgault *et al.,* 1992; Kirchhoff *et al.,* 1991; von Herrath *et al.,* 1995; Yasutomi *et al.,* 1993), their potential impact on different Nef alleles should be considered.

While it is clear that Nef is required for efficient replication and disease induction during SIV-PBj14 infection of macaques (Novembre *et al.,* 1996), as well as that of other SIV strains (Kestler *et al.,* 1991; Whatmore *et al.,* 1995), additional regions of the genome appear to contribute to the acutely lethal effects of the virus (Novembre *et al.,* 1993, 1994). Using chimeric viruses in which different regions of the genomes of SIV-PBj14 and SIVsmm9 were exchanged, Novembre *et al.* (1993) showed that the envelope of SIV-PBj14 was necessary but not sufficient for expression of this atypical phenotype. For this reason, and multiple demonstrations that *env* encodes determinants for specific biologic properties (Groenink *et al.,* 1993; Zhang *et al.,* 1993), the possibility that additional mutations occurred in *env,* other regions of the genome or elsewhere in *nef* in viruses that lost the SIV-PBj14 phenotype must be explored.

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