

with Attenuated Simian Immunodeficiency Virus

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A comparative study was undertaken to characterize the very early events that distinguish attenuated and pathogenic simian immunodeficiency virus (SIV) infections. Three rhesus macaques were inoculated with the attenuated SIVmac 251 Δ nef virus, and three others with a virus of intermediate phenotype, SIVmac 239 nef stop. They were compared to four macaques inoculated with the pathogenic SIVmac 251 isolate. Lymph nodes (LN) taken between 7 days and 2 months postinoculation were analyzed for SIV expression by *in situ* hybridization. During acute infection, SIV 251 Δ nef infected 1 to 1.5 log₁₀ fewer cells in LN tissue than the pathogenic SIV 251 isolate. The reduction was more marked in the blood, as SIV 251 Δ nef infected 2 to 3 log₁₀ fewer PBMC than the isolate and did not yield detectable antigenemia. Morphometric measurements showed that the development of germinal centers (GC) was more rapid in the Δ nef infection, which led to a more efficient trapping of viral particles, and could account for antigenemia clearance. The SIV 239 nef stop clone reverted to a nef⁺ genotype at Week 2, but induced a lower viral burden than a directly pathogenic virus. The kinetics of GC development was rapid, indicating that SIV 239 nef stop induced an immune response similar to that seen in attenuated infection. This study provides evidence that attenuated SIV elicits a more rapid immune response than pathogenic SIV and suggests that an early immunosuppressive episode may facilitate the dissemination of pathogenic SIV. © 1995 Academic Press, Inc.

INTRODUCTION

Primary infection with HIV is characterized by a transient but intense stage of viral replication and dissemination (Clark *et al.*, 1991; Daar *et al.*, 1991; Piatak *et al.*, 1993a,b; Wall *et al.*, 1987). Clinical, virological, and immunological studies now provide a body of evidence that the course of primary HIV infection conditions the progression to AIDS. Individuals who manifest prolonged symptoms such as fever and skin rash during primary HIV infection tend to have a more rapid disease course (Clark and Shaw, 1993; Niu *et al.*, 1993; Sinicco *et al.*, 1993; Tindall and Cooper, 1991). The presence of detectable p24 antigenemia prior to seroconversion is associated with rapid progression (Keet *et al.*, 1993). Immunological markers, such as low titers of anti-p24 antibodies and elevated neopterin plasmatic levels, are early predictors of clinical progression (Gaines *et al.*, 1990; Keet *et al.*, 1993; Sheppard *et al.*, 1991). Anecdotal evidence suggests that a delayed cellular cytotoxic response may be associated with a bad prognosis (Koup *et al.*, 1994) and that a more restricted V β gene usage is observed early in CTLs of patients that progress rapidly to AIDS

(Pantaleo *et al.*, 1994). Taken together, these studies indicate that pathologic events occur early and stress the need to investigate how the virus spreads and interferes with the immune response during primary infection.

Animal models of AIDS allow an in-depth analysis of the primary stage of infection, a task which is difficult in humans. SIVmac infection is the model of choice, as SIVmac is closely related to HIV in its genetic structure (Chakrabarti *et al.*, 1987; Daniel *et al.*, 1985) and causes a disease in macaques remarkably similar to AIDS in man (Desrosiers, 1990; Letvin and King, 1990). Lymphoid organs, which constitute the major reservoir and preferential target of HIV (Embretson *et al.*, 1993; Gerdes and Flad, 1992; Pantaleo *et al.*, 1993), are similarly affected by SIV (Chalifoux *et al.*, 1987; Ringler *et al.*, 1989; Rosenberg *et al.*, 1992; Wyand *et al.*, 1989). Studies of primary SIV infection in rhesus macaques have demonstrated that intense virus replication occurs in the thymus, lymph nodes (LN), and spleen (Baskin *et al.*, 1991; Chakrabarti *et al.*, 1994b; Lackner *et al.*, 1994) and that the development of the antiviral immune response in LN parallels the containment of virus replication (Reimann *et al.*, 1994). The immunological impairments seen in symptomatic primary HIV infection, such as abnormal immune activation and delayed antibody response, have been reported in SIV-infected macaques (Popov *et al.*, 1992;

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Reimann *et al.*, 1991; Zhang *et al.*, 1988). Thus, SIV infection appears a valid model to study the early immune dysregulation that precedes progression to AIDS.

We have previously shown that rhesus macaques that fail to contain virus replication, as indicated by a persistently high viral burden in LN following primary infection, progress more rapidly toward disease (Chakrabarti *et al.*, 1994a). In the present study, we compared the course of pathogenic SIV infection with that caused by SIV molecular clones of attenuated virulence. Our objective was to characterize the very early events that distinguish between pathogenic and attenuated SIV infections, in order to identify the immunopathogenic mechanisms at work in the lymphoid organs. For attenuated viruses, we used SIVmac infectious clones with mutations in the *nef* gene, because *nef* expression is known to be essential to SIV-induced pathogenesis in adult macaques (Kestler *et al.*, 1991). Rhesus macaques inoculated with attenuated SIV were compared to the previously studied animals, which had been inoculated with the pathogenic SIVmac 251 isolate. The study demonstrates that the viral burden is reduced in attenuated infection, but proportionally more in the blood than in LN. Germinal centers (GC) develop more rapidly in LN infected with attenuated SIV, allowing the efficient trapping of circulating viral particles. These results provide evidence of a more rapid development of the immune response in attenuated SIV infection and suggest that an early immunosuppression occurs in pathogenic SIV infection.

MATERIALS AND METHODS

Animals and viruses

Six adult Rhesus monkeys (*Macaca mulatta*) were infected with SIVs derived from infectious molecular clones. Prior to inoculation, the monkeys were demonstrated to be seronegative for STLV-1, SRV-1 (type D retrovirus), herpes B virus, and SIVmac. All procedures with animals were performed under anesthesia with ketamine (Imalgène, Mérieux). Three animals were inoculated with the attenuated SIVmac 251 Δ *nef* virus provided by M. P. Kieny (Transgène, Strasbourg). The SIV 251 Δ *nef* clone was derived from the BK28 clone (Kornfeld *et al.*, 1987) by deletion of nucleotides 9226 to 9400 in the *nef* gene, mutation of the *nef* initiation codon ATG to ACG at 9059, and mutation of the *env* in-frame stop codon TAG to CAG at 8785. The parental clone BK28 is partially attenuated, as it induces AIDS after a long incubation period and only in a fraction of the inoculated macaques (Israel *et al.*, 1994; Rivière and Aubertin, unpublished observations). *Nef* mutations were introduced in the BK28 clone to ensure attenuation.

The other three animals were inoculated with the SIVmac 239 *nef* stop virus provided by R. Desrosiers (NERPRC, Harvard Medical School). The SIV 239 *nef* stop

clone has a spontaneous punctual mutation in *nef* at nucleotide 9353 (Regier and Desrosiers, 1990). In both groups, the animals were intravenously inoculated with a high dose of viral supernatant corresponding to 100,000 cpm in a reverse transcriptase assay. The reference group consisted of four rhesus monkeys that were intravenously inoculated with 300,000 cpm of the pathogenic SIVmac 251 isolate, and for which the viral burden in LN and blood has been previously reported (Chakrabarti *et al.*, 1994a). A comparison could be made between the three groups as we and others have shown that, in the high range, variations in the inoculum dose have no effect on the course of infection (Chakrabarti *et al.*, 1994a; Cranage *et al.*, 1990; Lewis *et al.*, 1994).

Lymph node and PBMC collection

For each animal, four axillary or inguinal LN were collected during the early stages of infection, at Days 7, 15, 30, and 63 postinoculation (p.i.). LN were taken surgically and frozen in isopentane cooled in liquid nitrogen. After embedding in O.C.T. compound (Tissue-tek, Miles), LN were cryostat sectioned at 4- μ m intervals and the sections were stored at -20° until use.

Heparinized blood was collected from the macaques, diluted two times in RPMI 1640, and separated on a J-prep (J. Bio) density gradient (1077 g/ml). PBMC were washed two times in RPMI 1640 and resuspended at 10^7 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum. A fraction of PBMC was spotted onto slides coated with 3-aminopropyltriethoxysilane (Sigma), at a density of 2×10^5 cells per spot. The slides were air-dried for a minimum of 2 hr, fixed 10 min in acetone, and kept frozen before processing by *in situ* hybridization.

Serologic assays

Antigenemia was detected by measurement of SIV p27 gag antigen in monkey sera, using a SIVmac antigen capture ELISA test (Coulter). Antibody response to SIV was monitored with an HIV-2 ELISA test (Elavia-II, Sanofi-Pasteur). The coated antigens were derived from HIV-2-infected cells and cross-reacted with anti-SIV-specific antibodies. Serum samples were tested at serial 10-fold dilutions from 1:10 to 1:100,000.

Determination of T4 and T8 lymphocyte subsets

Lymphocyte subsets were monitored by flow cytometry, after labeling with anti-CD4 (OKT4, Ortho-mune) and anti-CD8 (Leu2a, Becton-Dickinson) monoclonal antibodies conjugated to phycoerythrin and fluorescein, respectively. EDTA-treated blood was incubated for 15 min with monoclonal antibodies added at a 1/20 dilution. Red cells were lysed with the Lyse & Fix reagents (Immunotech). The samples were washed three times in phos-

phate-buffered saline and analyzed on a Facscan flow cytometer (Becton–Dickinson).

PCR and nucleotide sequencing

LN DNA was amplified by nested PCR with SIV 239-specific primers surrounding the in-frame *nef* stop codon at 9353. PCR conditions were as previously described (Chakrabarti *et al.*, 1994b). The first set of primers, *SacI* (5'-GGG CTT GAG CTC ACT CTC TTG TGA G-3') and A2 (5'-TGG CTG GCT ATG GAA ATT AGT CC-3'), amplified a fragment of 395 base pairs (bp). Two microliters of the reaction product was further amplified with primers S1 (5'-GAA TAC TCC ATG GAG AAA CCC AG-3') and *Mla*3 (5'-AAT GAG ACA TGT CTA TTG CCA ATT TG-3'). The resulting 166-bp product was cloned in a Bluescript vector (Stratagene), and recombinant clones were sequenced in both orientations by the dideoxy-chain termination method of Sanger *et al.* (1977).

Preparation of ³⁵S-labeled RNA probes

RNA probes were derived from the transcription vector Bluescript, into which a fragment of the SIVmac 142 plasmid clone spanning the end of *env* and the beginning of *nef* (nucleotides 8234 to 8721) was inserted (Chakrabarti *et al.*, 1987). This SIV-specific riboprobe was chosen because all types of SIV RNAs, genomic as well as singly or doubly spliced, hybridize to this region of the genome. The probe did not overlap with the deletion in SIV 251 Δ *nef* and had a sequence highly homologous to that of SIVmac 251 and of SIVmac 239 (Kornfeld *et al.*, 1987; Regier and Desrosiers, 1990), so that the hybridization efficiency was equivalent for the three viruses used. The antisense riboprobe was generated from the T7 promoter by *in vitro* transcription of 1 μ g of plasmid template with 50 units of T7 RNA polymerase in the presence of 50 μ Ci of ³⁵S-UTP and ³⁵S-ATP. Two labeled nucleotides were included in the transcription reaction so as to increase the specific activity of the probe. After incubation for 1 hr at 37°, the DNA template was digested with 10 units DNase I for 15 min at 37°. To enhance the penetration of the probe into tissue sections, the ³⁵S-labeled RNA was subjected to mild alkaline hydrolysis in 80 mM NaHCO₃ and 120 mM Na₂CO₃ at 60°. The hydrolysis time was optimized to obtain a majority of fragments in the 150- to 200-nucleotide range. After neutralization with 600 mM Na acetate and 167 mM acetic acid, the probe was purified by phenol chloroform extractions and ethanol precipitation. Specific activity ranged between 1 \times 10⁸ and 2 \times 10⁸ dpm/ μ g of input DNA.

In situ hybridization

Hybridization techniques were based upon published procedures (Singer *et al.*, 1987). Hybridization was carried out in tissue sections freshly cut on the cryostat, to

minimize RNA degradation during storage. Sections were fixed in 4% paraformaldehyde and acetylated in acetic anhydride/triethanolamine (pH 8) to minimize background. Sections were denatured in 70% formamide at 70° for 2 min to enhance the accessibility of nucleic acids. The hybridization mix contained the ³⁵S riboprobe at 50,000 dpm/ μ l, 50% formamide, 10% w/v dextran sulfate, 0.3 M NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 10 mM NaH₂PO₄, 1 \times Denhardt's, 0.5 mg/ml yeast tRNA, and 100 mM dithiothreitol (DTT). The mix was heated at 80° for 2 min and applied to slides. Coverslips were mounted and sealed with rubber cement, and hybridization was carried out at 45° overnight in a humid chamber. Slides were rinsed successively in 2 \times SSC/10 mM DTT for 1 hr at room temperature (r.t.), in 50% formamide 2 \times SSC/10 mM DTT for 30 min at 60°, in 2 \times SSC for 30 min at r.t., in 0.1 \times SSC for 1 hr at r.t., and then dehydrated in ethanol with 0.3 M ammonium acetate. Slides were coated with NTB2 nuclear track emulsion (Kodak) diluted 1:1 with 0.6 M ammonium acetate and autoradiographed for 20 days at 4°. The long exposure time ensured that weak hybridization signals such as those present in germinal centers were detected. After exposure, slides were treated with Kodak D-19 developer, fixed, and stained with hematoxylin–eosin. Controls included hybridization of SIV-infected and uninfected cultured lymphocytes, hybridization of lymph node tissue from uninfected monkeys, and hybridization with a control HTLV-1 RNA probe unrelated to SIV.

Image analysis

The diffuse hybridization in GC was quantified using a computerized image analysis system. Images were acquired through a 3-CCD video camera attached to a Nikon-FXA microscope. The digitized images were analyzed with the Optilab software version 2.1 (Graftek, Mirmande). To count the number of silver grains in GC, LN sections were viewed at a final magnification of 1000. A threshold was set so as to distinguish the silver grains from the lymphoid tissues. The number of silver grains was automatically counted on a 1000- μ m² region, and the evaluated background for each section was subtracted. The mean count attained for 3 to 5 measurements in distinct GC defined the density of silver grains, reported in Fig. 3C. Productively infected cells in LN were counted manually on a 2-mm² grid. The mean count obtained for three sections is indicated in Fig. 3A. The intensity of productive infection was quantified in individual cells, by automatically counting the number of silver grains per hybridization spot. The mean count obtained for 10 measurements is reported in Fig. 3B.

The development of GC was evaluated by morphometry, using the same image analyzing system as above. To quantify the percentage of the LN surface occupied

by GC, tissue sections were viewed at a final magnification of 50. The surface of each GC was measured, and the sum was divided by the surface of the entire LN section (Fig. 5A). When GC were too numerous to be individually measured, the mean surface obtained for 10 GC times the total number of GC was used in the computation.

RESULTS

Design of the study

The objective of this work was to compare the primary infections caused by attenuated and pathogenic SIV in LN. For attenuated virus, we used a SIVmac 251 Δ nef molecular clone which has a 174-bp deletion in the *nef* gene and an additional mutation of the *nef* initiation codon (Kieny *et al.*, 1993). The second clone used, SIVmac 239 nef stop, is similar to the fully pathogenic SIVmac 239 clone except that it carries an in-frame stop codon in *nef* at nucleotide 9353 (Regier and Desrosiers, 1990). Inoculation of Rhesus macaques with the SIVmac 239 nef stop clone was shown to induce a pathogenic infection, due to the rapid reversion of the in-frame stop codon (Kestler *et al.*, 1991). We reasoned, however, that this clone would display an attenuated phenotype prior to the reversion and that it could be used to evaluate the consequences of an initial attenuation on the subsequent course of a pathogenic infection.

Three rhesus macaques were inoculated with SIV 251 Δ nef, and three others with SIV 239 nef stop. For each animal, four axillary or inguinal LN were sequentially collected between 7 and 63 days p.i. SIV infection in LN was analyzed by *in situ* hybridization, and histologic changes in LN were evaluated by morphometry. Blood samples were also regularly obtained and evaluated for viral burden and T cell subsets. The results were compared to those previously obtained in four animals inoculated with the pathogenic SIVmac 251 isolate (Chakrabarti *et al.*, 1994a).

Viral burden in the blood

Measurement of antigenemia showed that the two SIV clones induced a very low viral burden in the blood. Using an antigen capture ELISA test (Coulter) with a limit of detection of 50 pg/ml, the p27 antigen was undetectable in the serum of animals infected with SIV 251 Δ nef and with SIV 239 nef stop (Fig. 1A). In contrast, p27 was detected at high levels in animals infected with the pathogenic 251 isolate, with peak values of 200 to 3000 pg/ml.

Each virus induced a different cell-associated viremia. The number of PBMC expressing viral RNA that was detected by *in situ* hybridization reached 4% with the pathogenic isolate, while it ranged between 0.003 and

0.02% with SIV 251 Δ nef (Fig. 1B). This 2 to 3 \log_{10} difference indicated that attenuated SIV induced a particularly low viral burden in the blood. The number of PBMC expressing viral RNA in the 239 nef stop infection was intermediate, with peak values 1 to 2 \log_{10} lower than those seen in the infection with the 251 isolate. Additional experiments based on a coculture assay with CemX174 cells (Moog *et al.*, 1994) were performed to evaluate the low viral burden found in attenuated infections. The number of infected PBMC in coculture was found to be in the same range as that obtained by *in situ* hybridization, with peak values of 0.02% in the 251 Δ nef infection and of 0.1% in the 239 nef stop infection (data not shown).

The virus derived *in vivo* from the SIVmac 239 nef stop clone was genetically characterized to determine the point at which the premature nef stop codon might have reverted. For each of the three animals infected with SIV 239 nef stop, DNA was extracted from lymph node cells obtained at early time points. A 166-bp fragment of the SIV *nef* gene encompassing the stop codon was amplified by nested PCR, cloned, and sequenced. The reversion of the stop codon was found to occur early, during the second week postinoculation (Table 1). The stop codon was still present in all PCR clones at Day 7, but only in 40% of the clones at Day 15, and it was reverted in all cases at Day 30. As the peak of productive infection was observed between Days 7 and 15, it can be assumed that a significant portion of the viruses produced in primary infection harbored a functional *nef* gene and thus were potentially pathogenic. It should be noted, however, that the viral burden in PBMC remained lower than that observed for a directly pathogenic virus.

Course of attenuated SIV infection in LN

The kinetics of SIV dissemination in lymphoid organs was determined by *in situ* hybridization. The course of SIV 251 Δ nef infection in LN is represented in Fig. 2. The initial stage was characterized by the presence of numerous productively infected cells, visible as black spots on the photographs. Subsequently, the rate of productive infection decreased, while a more diffuse hybridization signal accumulated in the developing GC. The diffuse signal indicated the accumulation of viral particles trapped at the surface of follicular dendritic cells (Chakrabarti *et al.*, 1994b). The course of the Δ nef infection in LN paralleled that observed in pathogenic SIV infection, as the successive stages of productive infection and particle accumulation occurred at the same time points. The viral burden was lower in the Δ nef infection (see below). However, the number of LN cells productively infected with SIV Δ nef was not negligible at Day 7, when compared to the limited number of infected cells detected in the blood. These data showed that attenuated SIV was actively disseminated in LN during primary infection.

Individual variability was observed among animals

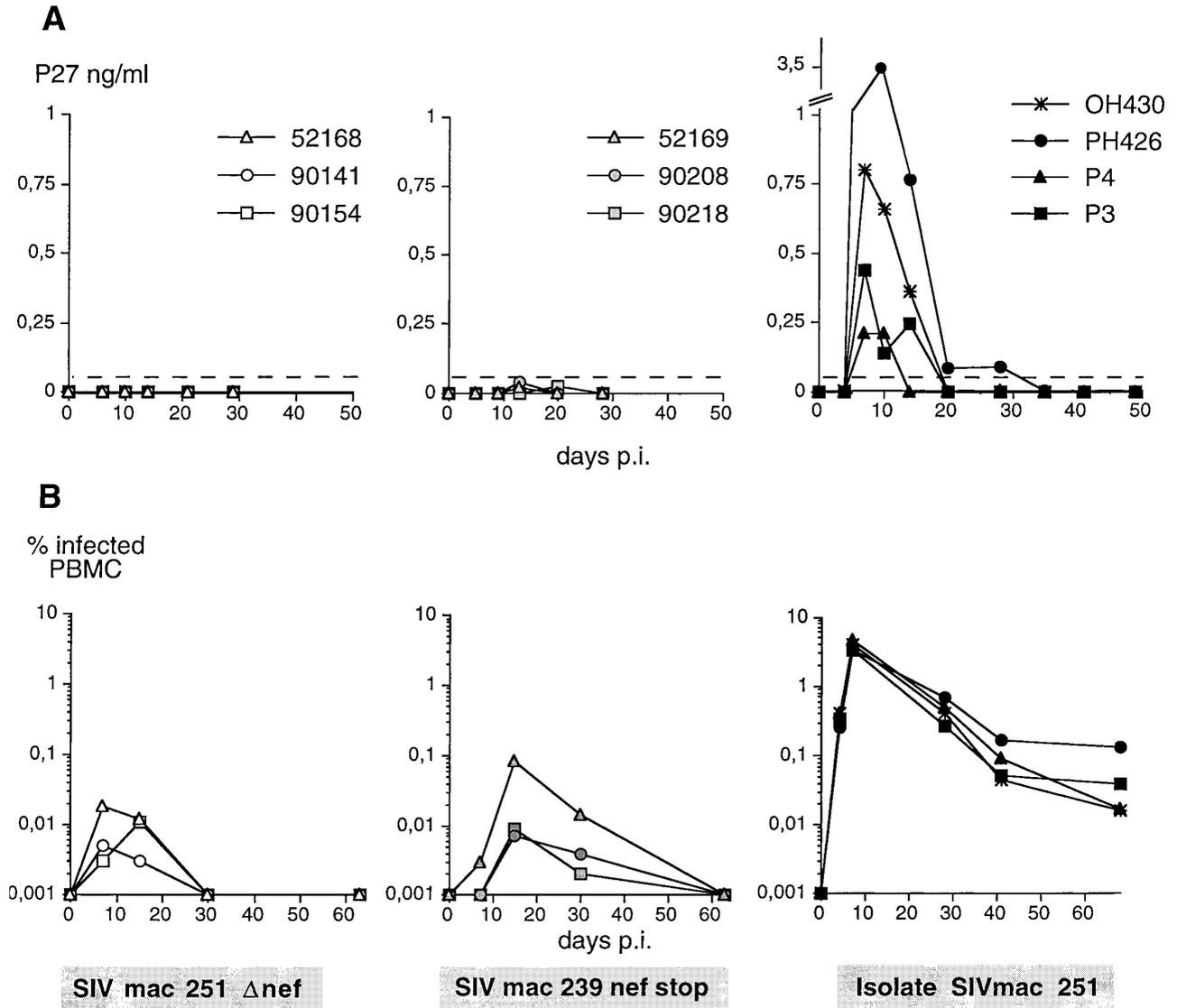


FIG. 1. Quantitation of the viral burden in blood. (A) The antigenemia was detected using an antigen capture ELISA specific for SIV Gag p27. The dashed line indicates a cutoff value of 50 pg/ml. (B) The rate of PBMC infection was determined by *in situ* hybridization of PBMC spotted onto slides with a SIV-specific riboprobe. Three slides of 10^5 PBMC were evaluated at each time point. The mean number of hybridization spots per 100 PBMC is reported.

inoculated with SIV 251 Δ nef, as shown by the different viral loads in LN of animals 90141 and 90154 (Fig. 2). The differences were visible as early as 7 days p.i., with a higher number of infected cells in 90154 LN. Differences were more marked immediately after primary infection, as viral particles accumulated in 90154 LN, while they appeared to be cleared from 90141 LN. Thus, rhesus macaques showed variable individual susceptibilities to attenuated SIV, similar to the phenomenon documented with pathogenic SIV.

Quantitation of productive infection in LN

The productive infection in LN was quantified by counting the number of hybridization spots per surface unit in

the cortical and paracortical areas (Fig. 3A). The mean number of productively infected cells detected per 2 mm^2 during the acute stage was 13 in the Δ nef infection, while it reached 150 in the pathogenic 251 infection. The viral load caused by the attenuated virus was reduced by 1 to 1.5 \log_{10} in LN, depending on the animal. These data contrasted with the 2 to 3 \log_{10} difference observed in the rate of PBMC infection between the two viruses (see above). This suggested that cells infected with the Δ nef virus preferentially localized in the lymphoid organs or that they were more efficiently cleared from the bloodstream. Intermediate levels of infection were observed in LN of animals infected with SIV 239 nef stop. At Day 7, while most of the viruses still contained a defective

TABLE 1
Reversion of the SIV 239 nef Stop Mutation *in Vivo*

| SIVmac 239 | E | R | E | K | L | A | Y | R | K | Q | N | M | D | D | I | D | E | * | D | D | D | L | V | G | V | S | V | R | P | K | V | P | L | R | T | | | | |
|---------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 days p.i. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 52169 n°02 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 52169 n°25 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 52169 n°51 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90208 n°05 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90208 n°76 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90208 n°78 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90218 n°07 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90218 n°08 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90218 n°09 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90218 n°59 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90218 n°60 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90218 n°61 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 90218 n°62 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 15 days p.i. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 52169 n°11 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Y | - | - | - | - | V | I | - | - | G | - | - | - | - | - | - | - | - | | | |
| 52169 n°12 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | |
| 52169 n°82 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90208 n°13 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90208 n°14 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90208 n°15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90208 n°85 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Q | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90218 n°16 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90218 n°33 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90218 n°34 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90218 n°88 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 90218 n°90 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 30 days p.i. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 52169 n°35 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 52169 n°37 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| 52169 n°39 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 52169 n°91 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 90208 n°41 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 90208 n°45 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 90208 n°70 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 90218 n°23 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 90218 n°48 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |



Note. The amino acid (aa) sequence of clones obtained by PCR amplification of LN DNA is presented. SIV 239-specific primers surrounding the nef stop mutation (nucleotide 9353) were used to amplify DNA from Days 7, 15, and 30 LN in the three animals studied. The aa identical to those of the original 239 nef stop clone (top line) are indicated by dashes. The arrow indicates the position of the original in-frame stop codon in *nef*.

nef gene, the mean number of infected cells was 32 per 2 mm², which represents about five times less virus than in the pathogenic 251 infection. As the difference was 1 to 2 log₁₀ in the blood, it could be concluded that SIV 239 nef stop infection was also characterized by a preferential localization of infected cells in lymphoid tissues.

To estimate the intensity of SIV replication in its target cells, we quantified the amount of viral RNA detected per productively infected cell. The mean number of silver grains per hybridization spot was automatically counted on LN sections obtained at Day 7, using a computerized image analyzing system (Fig. 3B). Slides were exposed for only 2 days in this experiment, to avoid saturation at high grain counts. The production of SIV RNA per cell was found to be equivalent in the three types of infections, with a count ranging from 150 to 300 grains/cell. Taken together, these results showed that attenuated SIV maintained the ability to replicate productively *in vivo* and that the intensity of viral gene expression could be as high as that observed for pathogenic SIV.

Attenuation was characterized by a reduced number of target cells, but not by a reduced production of virus per target cell.

Quantitation of viral particles trapped in LN

The second component of the viral burden, consisting of viral particles trapped in GC, was also quantified (Fig. 3C). The diffuse hybridization signal was measured by automatically counting the number of silver grains per surface unit in the GC area. The measurement was done in areas devoid of hybridization spots, so that only the diffuse signal associated with the FDC network was taken into account. The density of viral RNA showed a peak between Days 15 and 30 and thus appeared to follow in time the peak of productive infection. However, the levels of viral RNA deposited in the GC were not directly proportional to the peak of productive infection. Some animals infected with the 251 Δ nef and 239 nef stop viruses showed a marked accumulation of viral RNA, though they had a limited number of

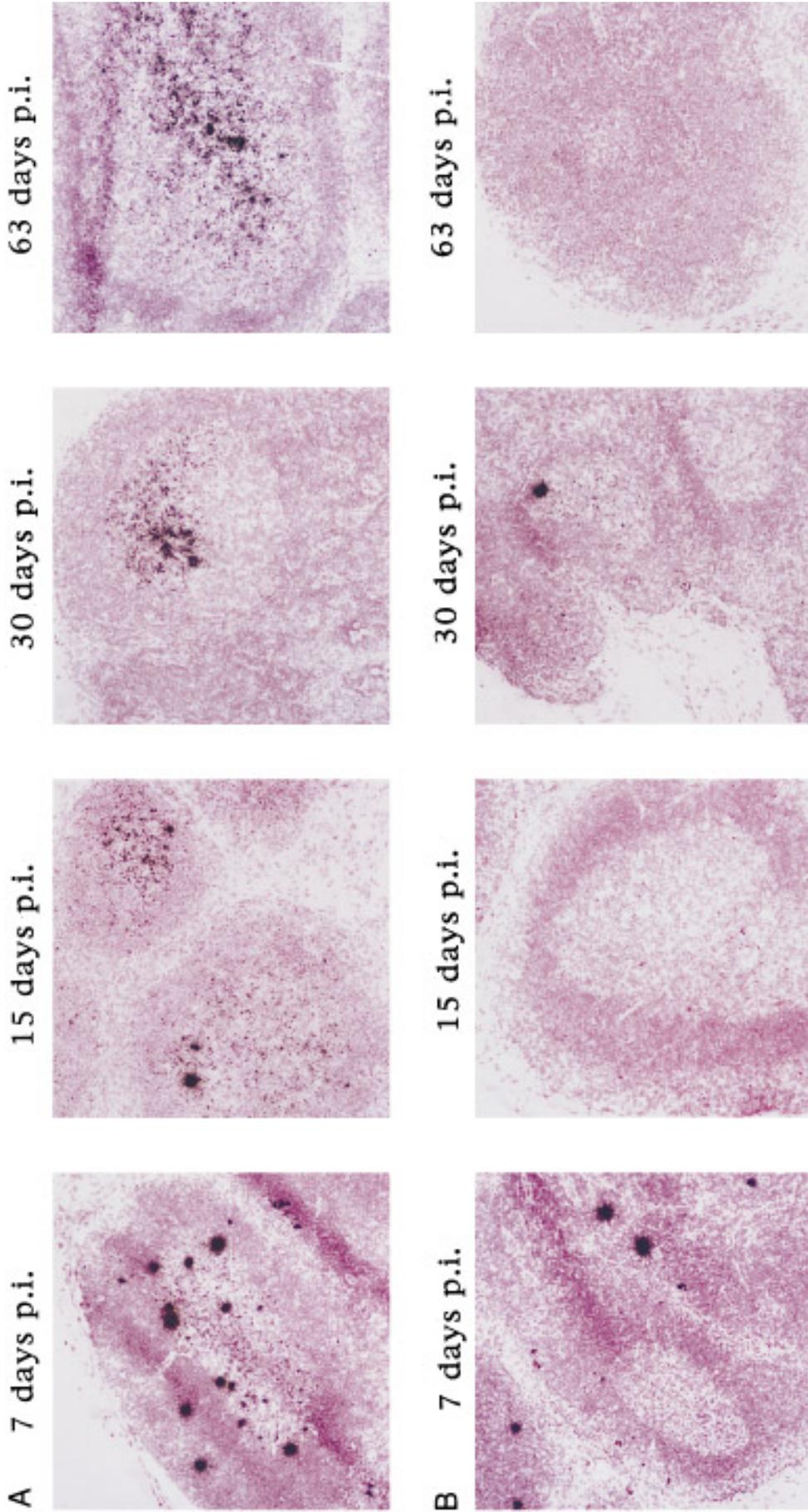


FIG. 2. Course of LN infection in macaques inoculated with the attenuated SIV 251 Δ nef clone. (A) Animal 90154, (B) animal 90141. LN obtained sequentially during primary SIV infection were analyzed by *in situ* hybridization. Viral RNA was detected with a SIV-specific riboprobe labeled with ^{35}S . Hybridization spots correspond to productively infected cells. The more diffuse hybridization signal indicates the presence of viral particles trapped in the germinal centers. Comparison of the two animals (90154 and 90141) shows that the same inoculum of SIV 251 Δ nef can induce different viral loads in LN (magnification, $\times 125$).

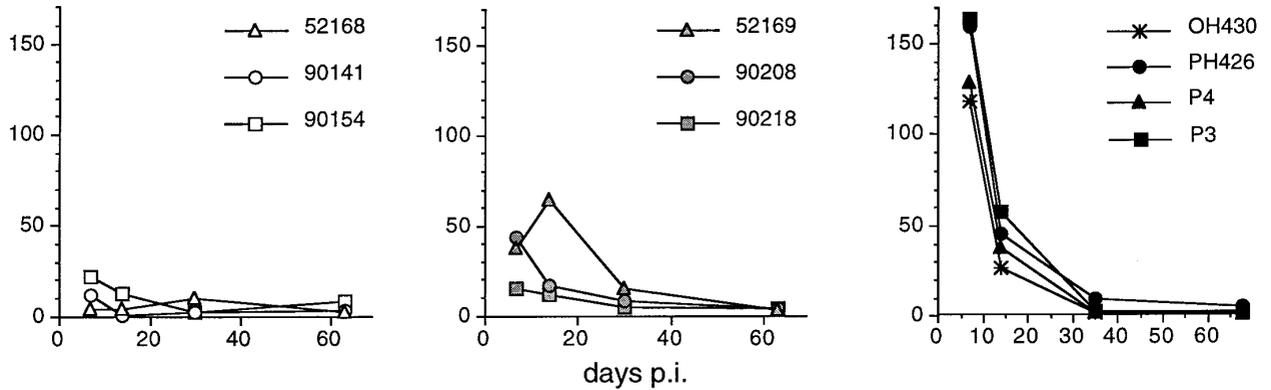
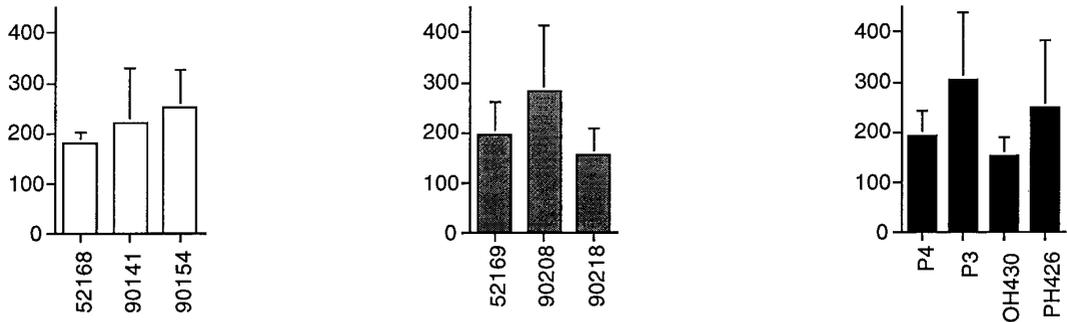
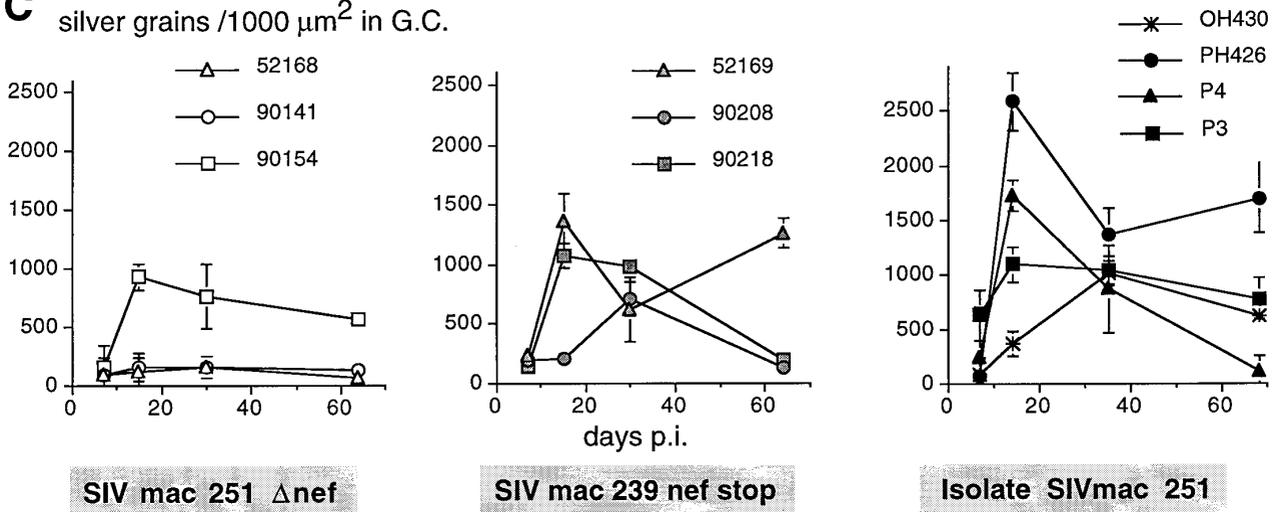
A inf.cells / 2mm² tissue**B** silver grains / infected cell**C** silver grains /1000 μ m² in G.C.

FIG. 3. Quantitation of the viral burden detected by *in situ* hybridization in LN. The two types of hybridization (spots and diffuse signal) detected in LN of SIV-infected macaques were quantified. (A) The number of productively infected cells, detected as spots, was counted in a 2-mm² area in the paracortex. The mean number obtained for three sections is reported. (B) The intensity of SIV replication in its target cells was measured by counting the number of silver grains per hybridization spot. This measurement was made by image analysis on Day 7 LN, using tissue sections exposed for only 2 days to avoid saturation. The mean and standard deviation obtained for 10 measurements are indicated. (C) The diffuse hybridization signal in GC was quantified by image analysis. The density of silver grains per 1000 μ m² represents the mean of three to five measurements made in distinct GC. Bars indicate standard deviations.

productively infected cells during the acute stage. This was apparent for animal 90154, infected with SIV 251 Δ nef. The viral RNA levels detected in GC of 90154 LN were similar to those found in certain cases of pathogenic 251 infection,

while the number of productively infected cells was 1 log₁₀ lower. These observations suggested that the proportion of viral particles that were trapped in LN could be high in attenuated SIV infection.

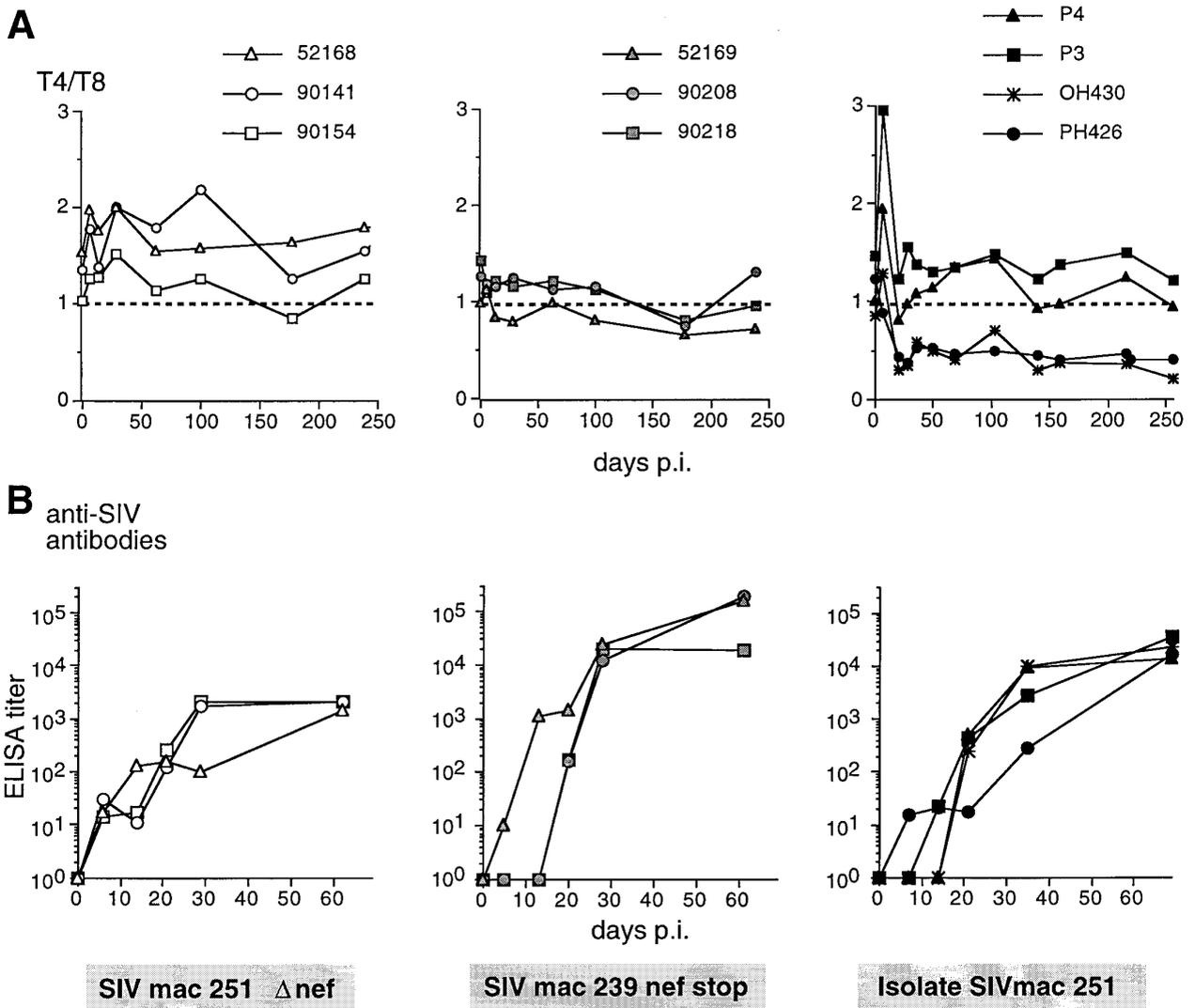


FIG. 4. Evolution of immunological parameters in the course of SIV infection. (A) Evolution of the T4/T8 lymphocyte ratio. Peripheral blood samples were labeled with OKT4 (anti-CD4) and Leu2a (anti-CD8) monoclonal antibodies conjugated to phycoerythrine and fluoresceine and analyzed by flow cytometry. The dashed line marks the level at which T4/T8 = 1. (B) Development of the antibody response against SIV. Macaque sera diluted from 1:10 to 1:100,000 were tested with an HIV-2 antibody ELISA that cross-reacts with SIV.

Clinical progression

Immunological parameters and clinical progression differed between the three types of infections. The T4/T8 ratio of circulating lymphocytes remained normal in the 251 Δ nef infections, while a ratio persistently below 1 was observed in one of the macaques infected with the 239 nef stop virus (Fig. 4A). This animal, 52169, experienced a slow decline in T4 lymphocytes, reaching a T4/T8 ratio of 0.7 at 6 months p.i. It should be noted that, during primary infection, 52169 had shown a higher viral burden in LN and PBMC than the other two macaques of its group. Among the four macaques infected with the pathogenic 251 isolate, two demonstrated a rapid decrease, with a T4/T8 ratio down to 0.4 at 6 months p.i.

One of the animals with a low ratio, PH426, developed AIDS at 8 months p.i. and was sacrificed because of intestinal necrosis. Two other animals of the group, P3 and OH430, died in the third year of infection. None of the animals infected with the Δ nef or the nef stop virus exhibited clinical symptoms over a 1-year follow-up period. In summary, SIV 251 Δ nef infection was benign, SIV 239 nef stop caused a slowly progressive infection in one animal, and SIV 251 caused a rapid disease in a subset of the infected animals.

Early humoral response

We asked whether we could detect differences in the early immune response associated with the three types

of infections. The development of the humoral response was monitored on serum samples by an ELISA test (Fig. 4B). The anti-SIV antibodies became detectable at Day 6 for the Δ nef infection, and between Days 6 and 20 in the 239 nef stop and 251 infections. Acid dissociation of immune complexes in serum did not significantly change the O.D. curves (data not shown). It could be concluded that the appearance of circulating antibodies was as rapid in attenuated infections as in pathogenic ones.

At later time points, the pathogenic SIV 251 infection induced higher antibody titers than the SIV 251 Δ nef infection, which probably reflected the higher viral burden in the former case. The 239 nef stop infections also gave rise to high titers, suggesting a progression toward a higher viral burden.

Development of germinal centers

In the course of a humoral response, affinity maturation of antibodies takes place in GC that develop in lymphoid tissues (Liu *et al.*, 1992). We investigated the kinetics of GC development as a measure of the maturation of the anti-SIV humoral response. An initial observation was that numerous GC were visible in LN infected with SIV 251 Δ nef at Day 7 (Fig. 2), which was not the case in the pathogenic 251 infection. To quantify this phenomenon, the surface occupied by GC at each time point was measured by image analysis, and the number obtained was divided by the surface of the whole LN section. This measurement indicated that the development of GC was more rapid in attenuated SIV infection (Fig. 5A). This was apparent from Day 7 to Day 15, when GC occupied 1 to 2% of LN sections in the pathogenic infection, and 3 to 8% in the Δ nef infection. These differences suggested a more efficient development of the humoral response in attenuated SIV infection or, conversely, an early impairment of the humoral response in pathogenic SIV infection.

The reaction of LN in the SIV 239 nef stop infection was similar to that observed in the 251 Δ nef infection, with GC development occurring during the first 2 weeks postinoculation (Fig. 5A, middle panel). Thus, both antibodies and GC measurements indicated that SIV 239 nef stop induced an immune response of the type seen in attenuated infection. At later time points, the trend was reversed, as the size of GC decreased in the Δ nef and nef stop infections, while they kept increasing in the pathogenic SIV 251 infection. These data could be interpreted as the normal resolution of an immune response in the first two cases, and as a progression toward follicular hyperplasia in the pathogenic 251 infection. The two animals which showed the highest percentage of LN area occupied by GC at 2 months (PH426 and OH430, Fig. 5A, right panel) were those which showed a rapid decline of the T4/T8 lymphocyte ratio (Fig. 4A). This sup-

ported the notion that ongoing development of GC into the asymptomatic phase of infection was indicative of pathologic progression.

We next addressed the question of whether rapid GC development in attenuated infection lead to an overall increase of the trapping of SIV particles at early time points. Both the density of silver grains in GC and the surface occupied by GC had to be taken into account. The density of grains was generally higher in pathogenic infections (Fig. 3C), while the percentage of LN surface occupied by GC was larger in attenuated infections (Fig. 5A). To evaluate the global amount of viral particles retained in LN, we multiplied both parameters (Fig. 5B). The resulting number, which corresponded to a density of viral particles per surface unit of LN tissue, was proportional to the total number of particles trapped in LN, given that LN sizes were approximated to be equal. This approximation could be made for early time points, before the LN infected with the 251 isolate became hyperplastic.

The quantity of viral particles retained in LN at Day 15 was found to vary with the animal (Fig. 5B). The accumulation of virus in LN could be much higher in certain cases of attenuated infection than in pathogenic infection. The two animals for which the amount of virus retained in LN was important, 90154 and 52169, were characterized by a high rate of productive infection as compared to other animals of the 251 Δ nef and 239 nef stop groups. This suggested that, in attenuated SIV infection, virus trapping could compensate for virus production. In contrast, the four animals infected with the pathogenic isolate had a limited amount of virus trapped in LN at Day 14, though the number of cells producing virus was high at this stage. It should be noted that P4, which had relatively more virus trapped in LN than the three other animals, had the lowest antigenemia in the group and progressed more slowly to disease. Taken together, these data suggested that the clearance of antigenemia depended on the capacity of GC to trap circulating viral particles.

DISCUSSION

This study indicates that attenuated and pathogenic SIVs induce different viral burdens, but also different immune responses during primary infection. An initial observation was that, in terms of viral burden, the attenuation was more marked in the blood than in LN. In peripheral blood, SIV 251 Δ nef infected 2 to 3 \log_{10} fewer cells than the SIV 251 isolate, while in LN, the number of infected cells was reduced by 1 to 1.5 \log_{10} only. A possible explanation was that SIV 251 Δ nef preferentially infected cells that homed or resided in LN, because the activation status of these cells allowed the infection with a virus defective for *nef*. Another explanation was that

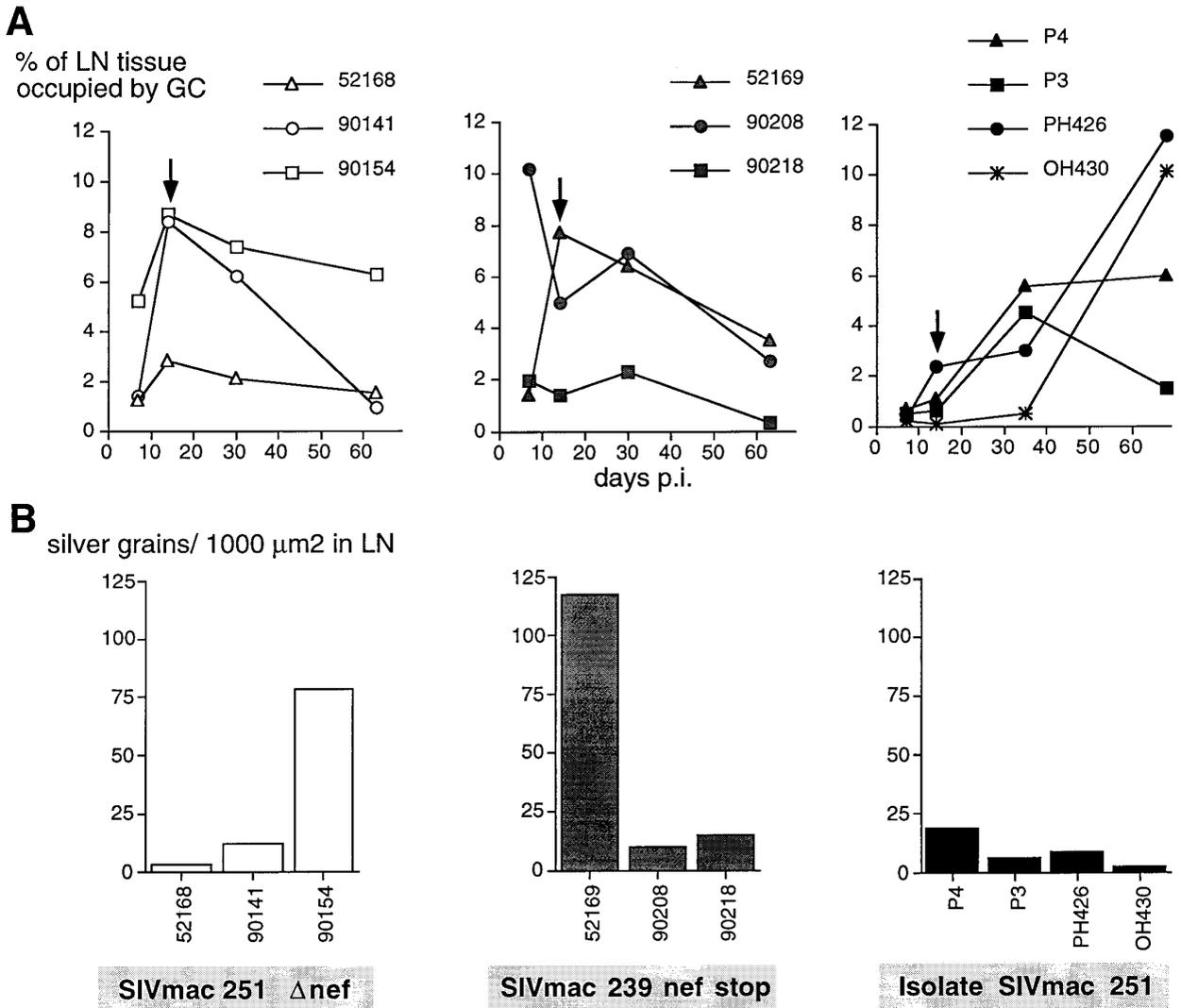


FIG. 5. Development of germinal centers during primary SIV infection. (A) The development of GC was measured by image analysis. The percentage of LN tissue occupied by GC was obtained by measuring the surface of each GC and by dividing the sum by the surface of the entire LN section. Arrows emphasize that, during the first 2 weeks, the development of GC is more advanced in SIV 251 Δnef and SIV 239 nef stop infections than in SIV 251 infection. (B) Evaluation of the quantity of viral particles retained in LN at Day 14/15. The density of silver grains in GC was multiplied by the percentage of LN tissue occupied by GC. The resulting parameter represents the quantity of viral particles trapped per surface unit of LN tissue.

the immune response was more efficient in clearing the infecting virus or the infected cells from the bloodstream. The observation that p27 antigenemia was undetectable in the sera of animals infected with SIV 251 Δnef , while an active viral replication took place in LN, supported the idea that the antiviral immunity was efficient in clearing the virus from the blood.

The seric levels of anti-SIV antibodies were monitored to evaluate whether the humoral response could account for p27 antigenemia clearance. There was no conclusive evidence for a more rapid antibody response in attenuated SIV infection. However, it should be considered that the measure of seric antibody levels only partially reflects

the total anti-SIV antibody production. Antibodies can be sequestered in LN, as FDC are known to trap and retain antigen-antibody complexes for long periods on their cell membrane (Liu *et al.*, 1992), and as HIV particle-antibody complexes are able to bind to the surface of isolated FDC in a complement-dependent manner (Joling *et al.*, 1993). The trapping of immune complexes in developing GC is likely to delay the appearance of circulating anti-SIV antibodies.

The examination of LN was needed to better evaluate the development of the anti-SIV humoral response. Morphometric measurements demonstrated that the development of GC was more rapid in attenuated SIV infection

(Fig. 5A). Other investigators have shown that cells infected with the attenuated SIVmac 1A11 localize preferentially in GC 2 weeks after inoculation, which confirms that expanded GC can be detected very early in LN infected with attenuated SIV (Lackner *et al.*, 1994). As GC development reflects the process of affinity maturation of antibodies (Liu *et al.*, 1992), our findings suggest that the synthesis of high-affinity antibodies that can efficiently complex viral particles occurs earlier in attenuated SIV infection. In addition, the development of GC means a larger FDC network into which viral particles can be trapped and thus removed from the lymph and bloodstream. The evaluation of the amount of virus trapped in LN at Day 15 confirmed that this process was more efficient in attenuated infection (Fig. 5B). The animals that showed an increased susceptibility to SIV 251 Δ nef or SIV 239 nef stop, as indicated by a higher rate of productive infection, also had a larger amount of virus trapped in LN. This could explain why these animals maintained undetectable or very low antigenemia levels. In the pathogenic 251 infection, the number of GC appeared limiting, so that despite a higher density of particles per GC, the total amount of virus trapped in LN remained low at early time points. Circulating particles could enter further rounds of replication, leading to an increased viral burden. Thus, the rapidity of GC development appeared as a key determinant of the severity of primary SIV infection.

The study of the SIV 239 nef stop infection confirmed the importance of early events in determining the clinical outcome. Animals infected with SIV 239 nef stop showed an intermediate viral burden and a slower progression to disease than those infected with a pathogenic isolate. Our interpretation is that the presence of a nef⁻ genotype during the first week of infection allowed the establishment of an efficient antiviral immune response, which, in turn, limited the spread of the nef⁺ virus. This notion is supported by the rapid development of GC seen in the nef stop infection, in a pattern similar to that seen in attenuated SIV infection. Our findings differ from those reported by Kestler *et al.*, who initially characterized the SIV 239 nef stop clone and found that it induced a high peak of viremia and a clinical progression indistinguishable from that caused by the SIV 239 clone with an open nef gene (Kestler *et al.*, 1991). The explanation may be that the Chinese rhesus macaques used in our study are less susceptible to SIV infection than the rhesus macaques of Indian origin used by Kestler *et al.*, as shown by Joag *et al.* in a comparative study (Joag *et al.*, 1994). In very susceptible animals like Indian macaques, the initial viral burden is likely to be higher, leading to a higher probability of nef reversion. Statistically, the reversion is bound to be selected earlier in susceptible animals, allowing the spread of a pathogenic form of virus prior to the initiation of the immune response.

The delayed kinetics of GC development seen in

pathogenic SIV infection suggest the occurrence of a transient immunosuppression, which could facilitate viral dissemination. This strategy is probably common to other lymphotropic viruses, such as measles and canine distemper viruses, which are known to cause an episode of immunosuppression associated with acute infection (Rouse and Horohov, 1986). The chain of events leading to a transient immunosuppression remains to be investigated. Pathogenic SIV or HIV, being initially more infectious, may rapidly induce a viral burden sufficient to partially destroy or disregulate T4 helper lymphocytes and thus may inhibit the T4-dependent steps needed to initiate specific cellular and humoral responses. The fact that T4 lymphopenia and reduced T cell proliferative responses are frequent findings in primary HIV infection (Cooper *et al.*, 1988; Pedersen *et al.*, 1990) supports the possibility of very early T4 cell dysfunction.

In conclusion, this study provides evidence that attenuated and pathogenic SIV differ not only in the viral burden, but also in the immune response they elicit during primary infection. The initial conflict between immune response development and viral dissemination appears crucial in determining the subsequent course of infection. This notion provides a rationale for attempting very early antiviral therapies. A recent study indicates that immediate AZT treatment alleviates the severity of primary SIV infection in macaque neonates and restores antibody responses in these susceptible animals (Van Rompay *et al.*, 1995). The SIV model will be valuable to test further approaches aimed at early therapeutic intervention.

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