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Live attenuated simian immunodeficiency virus prevents superinfection by cloned SIVmac251 in cynomolgus monkeys

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The ability of a live attenuated simian immunodeficiency virus (SIV) to protect against challenge with cloned SIVmac251/BK28 was evaluated in four cynomolgus macaques. The intravenous infection of the C8 variant of the SIVmac251/32H virus, carrying an in-frame 12 bp deletion in the *nef* gene, did not affect the CD4 and CD8 cell counts, and a persistent infection associated with an extremely low virus burden in peripheral blood mononuclear cells (PBMCs) was established. After 40 weeks, these monkeys were challenged intravenously with a 50 MID_{50} dose of SIVmac251/BK28 virus grown on macaque cells. Four naive monkeys were infected as controls. Monkeys were monitored for 62 weeks following challenge. Attempts to rescue virus from either PBMCs or bone marrow from the C8-vaccinated monkeys were unsuccessful, but in two cases virus was re-isolated from lymph node cells. The presence of the SIV provirus with the C8 variant genotype maintaining its original *nef* deletion was shown by differential PCR in PBMCs, lymph nodes and bone marrow. Furthermore, in contrast to the control monkeys, the vaccinated monkeys showed normal levels for CD4 and CD8 cells, minimal lymphoid hyperplasia and no clinical signs of infection. Our results confirm that vaccination with live attenuated virus can confer protection. This appears to be dependent on the ability of the C8 variant to establish a persistent but attenuated infection which is necessary for inducing an immune response, as suggested by the persistence of a strong immune B cell memory and by the over-expression of interleukin (IL)-2, interferon-γ and IL-15 mRNAs in PBMCs of C8-vaccinated monkeys but not in those of control monkeys.

Introduction

Infection of macaques with simian immunodeficiency virus (SIV) leads to an immunodeficiency disease which closely resembles that observed in human patients infected with human immunodeficiency virus (HIV) (Letvin *et al.*, 1985). Therefore, this animal model has been widely used to test the safety and efficacy of vaccine strategies against HIV infection in humans.

Whole inactivated (Murphey-Corb *et al.*, 1989; Desrosiers *et al.*, 1989; Dormont *et al.*, 1995) or subunit SIV vaccines (Hu *et al.*, 1992; Ahmad *et al.*, 1994; Israel *et al.*, 1994) have been shown to be effective in preventing infection or in reducing virus burden in monkeys challenged with low doses of cell-free or cell-associated homologous viruses. Nevertheless, the whole inactivated virus vaccine approach has been unsuccessful in establishing long-lasting immunity, and no protection was achieved when vaccinated monkeys were challenged with homologous or heterologous viruses that were not grown in human cell lines (Titti *et al.*, 1993; Stahl-Hennig *et al.*, 1993; Letvin, 1993). Furthermore, even formalin-fixed uninfected cells can provide protection under the same experimental conditions (Stott, 1991), suggesting that an immune response to some human cellular antigen(s) associated with SIV in vaccine preparations could be critical for induction of protective status. A common characteristic of all these vaccine trials in macaques was the ability to induce humoral and

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cellular immune responses. However, these were not always sufficient to provide complete protection or significant reduction in virus load (Giavedoni *et al*., 1993; Mills *et al*., 1993; Hulskotte *et al*., 1995).

At present, the most successful vaccines tested in the SIV–macaque model are based on live attenuated viruses. It has been shown that a functional *nef* gene is essential for the full pathogenic potential of SIV *in vivo* (Kestler *et al*., 1991). In fact, *nef*-deleted mutants replicate poorly *in vivo* and do not induce an AIDS-like disease in infected animals. Furthermore, it has been demonstrated that infection of rhesus monkeys with an attenuated SIV strain bearing a 182 bp deletion in the *nef* gene can protect the animals against challenge with pathogenic SIV (Daniel *et al*., 1992). The breadth of this protection and the type of immune responses involved have not yet been identified. A new naturally occurring variant of SIVmac251/32H virus with an in-frame 12 bp deletion in the *nef* gene, known as SIVmac251/32H/C8 (C8) has been described (Rud *et al*., 1994). It was shown to be non-pathogenic for rhesus and cynomolgus macaques, although it was able to induce low-level viraemia in these species (Rud *et al*., 1992).

Our studies were part of a European Community Concerted Action project, involving eight different institutions that examined the protection elicited by this attenuated C8 vaccine. For this cynomolgus macaques were infected with the C8 variant to: (i) evaluate its ability to establish a protective status against challenge with an infectious homologous molecular clone of SIV; (ii) verify the genotypic stability of the C8 variant before and after challenge; and (iii) study some possible factors behind this protection (humoral response, evolution of B cell memory and cytokine expression).

Methods

■ **Animals.** Adult cynomolgus monkeys (*Macaca fascicularis*) used for this study were housed in single cages within level three biosafety facilities according to the European guidelines for non-human primate care (EEC, Directive no. 86-609, November 24, 1986). Animals anaesthetized with ketamine hydrochloride (10 mg per kg) were clinically examined and their weight and rectal temperature were recorded. Blood samples, obtained the morning before food administration, were used for haematological analysis and immunological and virological assays.

■ **Lymphocyte subset determination.** Lymphocyte subsets were evaluated by direct immunofluorescence using R-phycoerythrin or fluorescein-labelled monoclonal antibodies (MAbs) anti-human CD2, CD20, CD4 and CD8 cell-surface markers (Dakopatts). Briefly, 100 µl citrated whole blood was incubated for 30 min at 4 °C with MAb (10 µl each), washed twice with PBS complemented with 2·5% foetal calf serum (FCS), resuspended and fixed in PBS pH 7·4 containing 1% (w/v) paraformaldehyde. Ten thousand lymphocytes from each sample, gated from leukocyte types based on forward and 90° light scatter, were analysed by cytofluorometry (FACScan; Becton-Dickinson).

■ **Viruses and virus isolation.** The cell-free C8 variant, with a 12 bp deletion within the *nef* gene, grown on the human C8166 cell line (obtained from M. Cranage through MRC AIDS Reagent Project, Potters Bar, UK), was used to intravenously infect (5×10^4 $TCID_{50}$) four adult male cynomolgus monkeys that tested negative for SIV and simian T-lymphotropic virus.

Forty weeks post-infection (p.i.) with the C8 variant, the four infected monkeys, along with four naive monkeys, were intravenously challenged with 50 MID_{50} of the molecular clone SIVmac251/BK28 (BK28) grown on monkey peripheral blood mononuclear cells (PBMCs; provided by A. M. Aubertine, Institut National de la Santè et de la Recherche Medical, Strasbourg, France). In this study, the four cynomolgus monkeys inoculated with the SIVmac251/32H/C8 variant are referred to as C8-infected or C8-vaccinated monkeys, whereas the four naive monkeys inoculated with SIVmac251/BK28 virus are referred to as controls.

Ficoll–Paque (Pharmacia)-purified monkey PBMCs (4×10^6) were cocultured with human CEMX174 cells (1×10^6; obtained from AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH, Mont., USA; Salter *et al*., 1985) for 30–40 days in RPMI 1640 medium containing 10% FCS and antibiotics. Cultures were scored for syncytia formation, and supernatants were monitored for the presence of the p27 antigen (SIV p27 core antigen; Coulter) and for reverse transcriptase (RT) activity. Samples that were found to be reactive in these assays at least twice were scored as positive.

■ **Detection of anti-SIV antibodies and p27 antigen in plasma.** Since there is a high sequence similarity between HIV-2 and SIV, antibody titres to SIV were determined by end-point dilution using an HIV-2 ELISA assay (Elavia Ac-Ab-Ak II kit, Diagnostic Pasteur). The measurement of SIV p27 Gag protein was done in plasma, after acidic antigen–antibody complex dissociation using an antigen-capture ELISA test (SIV p27 core antigen) with a limit of detection of 50 pg per ml as described previously (Titti *et al*., 1996).

■ **Detection of proviral *nef* sequences, differential PCR and quantification of SIV proviral copies.** DNA was extracted from Ficoll–Paque-purified PBMCs, bone marrow or lymph node cells using the phenol–chloroform method, followed by precipitation with 3 M sodium acetate and cold ethanol. Primers PCO3 and PCO4, which are specific for a 110 bp sequence of the human β-globin gene, were used to check the quality of DNA to be amplified (Saiki *et al*., 1985).

To amplify SIV-specific sequences from the *nef* region, a single-round PCR procedure was used (Thermal Cycler 9600, Perkin-Elmer-Cetus). For each reaction, 1 µg DNA was added to a PCR mixture containing primers (LS1-*nef* and LS2-*nef*) that amplify a 356 bp region of the *nef* gene. The PCR mixture (total volume 100 µl) contained 10 mM Tris–HCl pH 8·3, 1·5 mM $MgCl_2$, 0·001% (w/v) gelatin, 200 µM of each dNTPs and 400 ng of each primer. The samples, after an initial denaturation step for 4 min at 95 °C, were subjected to 30 cycles each of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. When appropriate, a nested PCR analysis was done on monkey DNA using primers LS4-*nef* and LS6-*nef* (first round). Amplification was started with an initial denaturation step of 4 min at 95 °C, followed by 35 cycles each of 1·5 min at 94 °C, 1·5 min at 55 °C, 2 min at 72 °C and finally 10 min at 72 °C. Product (10 µl) from the first-round PCR was then added to a fresh mixture containing primers amplifying an inner region of the *nef* gene (LS1-*nef* and LS2-*nef*). The same amplification procedure for the single-round PCR was then used.

To distinguish between C8 and BK28 proviruses, the nested PCR protocol described above was used. The final product was digested with *Rsa*I according to Rose *et al*. (1995), resolved by electrophoresis on a 1·5% agarose gel, and transferred to a filter. Hybridization was achieved at 42 °C with a ^{32}P-labelled oligonucleotide probe (LS3), and after washing, the filters were exposed to X-ray for 12–18 h. This analysis allowed us to distinguish between the undigested product (356 bp) of the C8 variant and the digested products (172 and 184 bp) of the BK28 virus.

Table 1. Primers and probes used to detect *gag* and *nef* regions of the SIV genome by DNA PCR and simian cytokine message

Primer name	Primer location	Sequence (5′–3′)	Product size (bp)
LS4-*nef**	8988–9010	AGATCCTCCAACCAATACTCCAG	986
LS6-*nef**	9968–9940	GCGAAATGCAGTGATATTTAATACATCAAG	
LS1-*nef**	9335–9355	GGATGATGTAGATGAGGAAGA	356
LS2-*nef**	9691–9671	GGGTCATCCCACTTGGAAGTT	
LS3-*nef* (probe)*	9587–9606	TGGCTGGCTATGGAAATTAG	
SG 1096 N*gag*†	1109–1133	TTAGGCTACGACCCGGCGGAAAGA	496
SG 1592 C*gag*†	1605–1582	ATAGGGGGTGCAGCCTTCTGACAG	
SG5 *gag*† (probe)	1470–1502	AATAGGTGGTAACTATGTCCACCTGCCATTAAG	
β-ACTIN forward	NA	GTGGGGCGCCCCAGGCACCA	548
β-ACTIN reverse		CTCCTTAATGTCACGCACGATTTC	
IL-2 forward	NA	ATGTACAGGATGCAACTCCTGTCTT	458
IL-2 reverse		GTTAGTGTTGAGATGATGCTTTGAC	
IFN-γ forward	NA	CAGCTCTGCATTGTTTTGGGT	441
IFN-γ reverse		CATCTGACTCCTTTTTCGCTT	
IL-4 forward	NA	GTCCACGGACACAAGTGCGAT	369
IL-4 reverse		CATGATCGTCTTTAGCTTTTC	
IL-10 forward	NA	ATGCCCCAAGCTGACAACCAAGACCCA	357
IL-10 reverse		GTTTCTCAAGGGGCTGGGTCAGCTATCCCA	
IL-15 forward‡	113–133	GTTTCAGTGCAGGGLTCCCTA	301
IL-15 reverse	413–393	TCCTCACATTCTTTGCATCCA	

* Primer and probe locations are based on sequences derived from GenBank, access code OD1065. Sequences were selected by computer analysis using the PRIMER program version 0.5 (Whitehead Institute for Biomedical Research).
† Primers obtained from P. Kitchin through programme EVA of the EC programme on AIDS research.
‡ Primer location based on sequences derived from GenBank, access code CAu0399.
NA, Not available.

To determine the relative number of SIV proviruses present in the experimental samples, a semi-quantitative DNA PCR was performed according to the procedure previously described (Titti *et al.*, 1996). Briefly, 1 μg DNA was used to amplify a 496 bp region of the *gag* gene of SIVmac251 (SG1096N*gag*–SG1592C*gag*) in 100 μl of reaction mixture for a total of 40 cycles. To quantify the virus copy number, a standard reference curve was prepared for each PCR experiment using pFLB10 containing the FLB10 molecular clone of SIVmac251 (Prasad *et al.*, 1990). The amplified products were quantified by densitometric analysis (Ultrascan LX Enhancer Laser Densitometer, LKB). The relationship between optical density (OD) values and the number of FLB10 molecules was detected by linear regression analysis (Statgraphics, Manugistics, Cambridge, Mass., USA). In this set of experiments, OD values were linearly related up to 1000 SIV molecules (correlation coefficient = $0{\cdot}99 \pm 0{\cdot}13$). The number of proviruses per μg DNA was obtained by interpolating OD values of monkey samples with the reference curve. The lower limit of detection was 1 SIV proviral copy per μg DNA, but samples containing 10 SIV proviral copies were invariably positive.

■ **Sequencing.** DNA extracted from PBMCs or lymph nodes was amplified using primers specific for the *nef* region (LS1 and LS2). The amplified products were separated on a 2% agarose gel and eluted using the Quick-spin gel extraction kit (Qiagen). Purified fragments were either directly sequenced with Sequenase version 2.0 (USB) or cloned into the pCMRII vector using the TA cloning system (Invitrogen). Plasmid DNA from single colonies, extracted using the Quick-spin mini plasmid preparation kit (Qiagen), were denatured and sequenced (Sequenase version 2.0, USB).

■ ***In vitro* anti-SIV antibody production.** The *in vitro* anti-SIV antibody production was done as described by Zamarchi *et al.* (1993) on purified PBMCs in the absence and presence of pokeweed mitogen (PWM, Gibco). Supernatants were collected 16 days later, and anti-SIV antibody production determined by solid-phase radioimmunoassay against whole inactivated SIVmac251 (ABI). The results were expressed as arbitrary units (AU) per ml, compared to a standard serum from an SIV-infected monkey.

■ **Detection of cytokine mRNA by RT–PCR.** Total cellular RNA was extracted, using the acid–guanidium thiocyanate–phenol–chloroform method (Chomczynsky & Sacchi, 1987), from freshly isolated PBMCs (1×10^6) obtained from four vaccinated and four control monkeys at 28, 42 and 62 weeks post-challenge (p.c.). The RT–PCR amplifications were carried out according to the manufacturer's protocol (Perkin-Elmer-Cetus). Briefly, the first strand of cDNA was generated by mixing total RNA with 100 U M-MLV RT, 2 mM of each dNTP and 2·5 mM oligo-d(T) in a total volume of 20 μl. The mixture was then incubated at 42 °C for 45 min, followed by a 5 min denaturation step at 99 °C. PCR master mix (78 μl) containing 2·5 U *Taq* polymerase (Perkin-Elmer-Cetus) was then added to each sample. The resulting cDNA product was amplified using specific primers (Table 1) and PCR conditions as described by Di Fabio *et al.* (1994).

Lymph node and bone marrow sample collection. Axillary or inguinal lymph nodes were removed surgically from vaccinated and control monkeys at 28 weeks p.c. under ketamine hydrochloride anaesthesia. Tissues were fixed in 10% (v/v) buffered formalin and paraffin-embedded. Lymph nodes were then sectioned at 4 μm intervals, treated with xylol, dehydrated in descending concentrations of alcohol (100, 95 and 70%), rehydrated in water and counter-stained with haematoxylin–eosin. Samples from bone marrow were obtained by an aspiration biopsy from the dorsal iliac crest using a 16-gauge, 4-inch needle syringe.

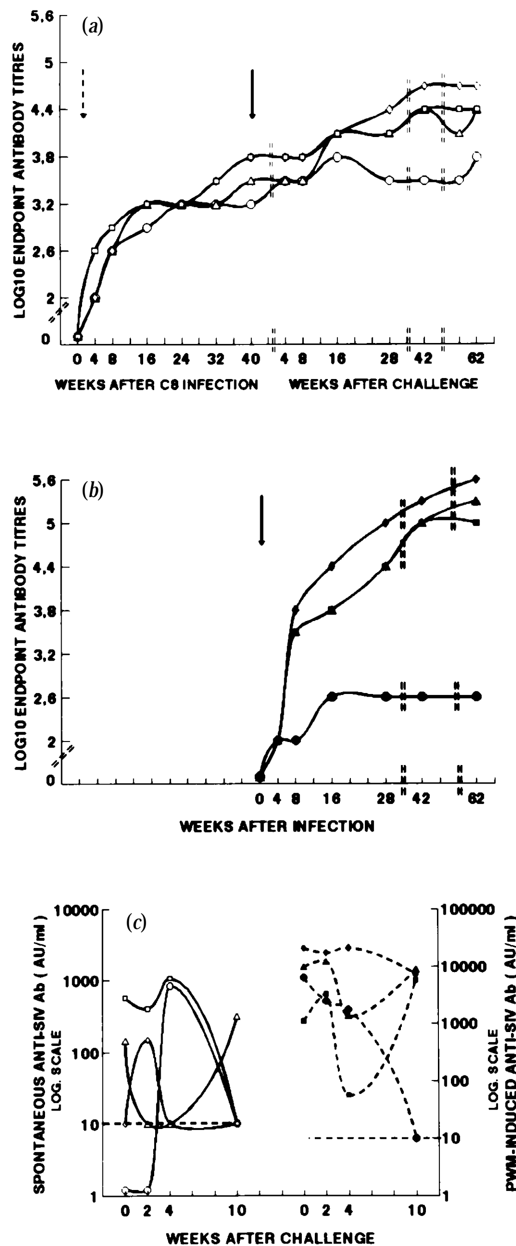

Fig. 1. (*a*) Anti-SIV ELISA antibody titres in plasma of C8-immunized monkeys before (dashed arrow) and after (solid arrow) challenge. Symbols: □, monkey 7; ◇, monkey 09; △, monkey 33; ○, monkey 46133. (*b*) Anti-SIV ELISA antibody titres in plasma of naive infected monkeys. Symbols: ■, monkey 22; ◆, monkey 28; ▲, monkey 91; ●, monkey 48126. (c) Spontaneous (continuous lines) and PWM-induced (dashed lines) anti-SIV antibody synthesis (log scale) following challenge of C8-vaccinated monkeys. The horizontal dashed lines indicate the threshold limit of the *in vitro* antibody production assay.

Results

Infection with *nef*-deleted C8 variant

Each of the four infected monkeys were seroconverted, as detected by ELISA, by the fourth week p.i. and all had progressively increasing anti-SIV antibody titres (Fig. 1*a*).

The infected monkeys were continuously monitored over a period of 40 weeks (time of challenge), and neither changes in haematological parameters (red blood cell counts, haemoglobin, haematocrit and platelet counts) nor clinical symptoms of SIV infection were observed (data not shown).

The number of $CD4^+$ cells in vaccinated monkeys fluctuated within the normal range (1200 ± 600 per mm^3) observed in adult (> 5-year-old) uninfected monkeys (Fig. 2*a*). The $CD4^+$ cell population increased in monkey 09 and, to a lesser extent, in monkey 33. It remained stable in monkey 46133 and decreased in monkey 7. When the number of $CD8^+$ cells was analysed, a moderate increase was observed in monkey 09, whereas it remained stable in monkeys 7 and 46133. At 8 weeks p.i., a decrease in the $CD8^+$ cell number below the normal range of uninfected animals (1700 ± 1100 per mm^3) was temporarily observed in monkey 33 but it returned to normal levels after that time (Fig. 2*d*).

It was possible to re-isolate the C8 variant from PBMCs of all monkeys 2 weeks p.i. (Table 2). Two monkeys (7 and 33) remained virus isolation-positive for up to 4 weeks p.i., one monkey (46133) remained positive for up to 16 weeks p.i. The remaining monkey (09) remained persistently virus isolation-positive for up to 40 weeks p.i. However, the days of cocultivation necessary for re-isolating the virus increased progressively, which probably reflected a reduction in the number of infected cells. In parallel, the SIV genome (*nef* sequences) could be detected by PCR in PBMCs of all monkeys up to 32 weeks p.i. (Table 2). PCR signals were more difficult to detect at 16 weeks p.i., when the virus could no longer be isolated from monkeys 7, 33 and 46133 or from PBMCs of monkey 09, even if it remained virus isolation-positive for up to 40 weeks p.i. Thus, from 16 weeks p.i., the presence of the SIV genome was only detected by nested PCR using primers specific for the *nef* region. At the day of challenge (40 weeks p.i.) SIV provirus was detected by nested PCR in PBMCs of only two monkeys (09 and 46133).

To verify whether the C8 variant maintained its original genotype *in vivo*, PCR-amplified products of the *nef* region (192 bp including the 12 bp deletion), obtained from PBMCs of all monkeys at 32 weeks p.i. with the C8 variant, were directly sequenced. The results indicated the maintenance of the original deletion (data not shown).

We also evaluated the cell-associated virus load by using a semi-quantitative DNA PCR that amplified the *gag* region. The

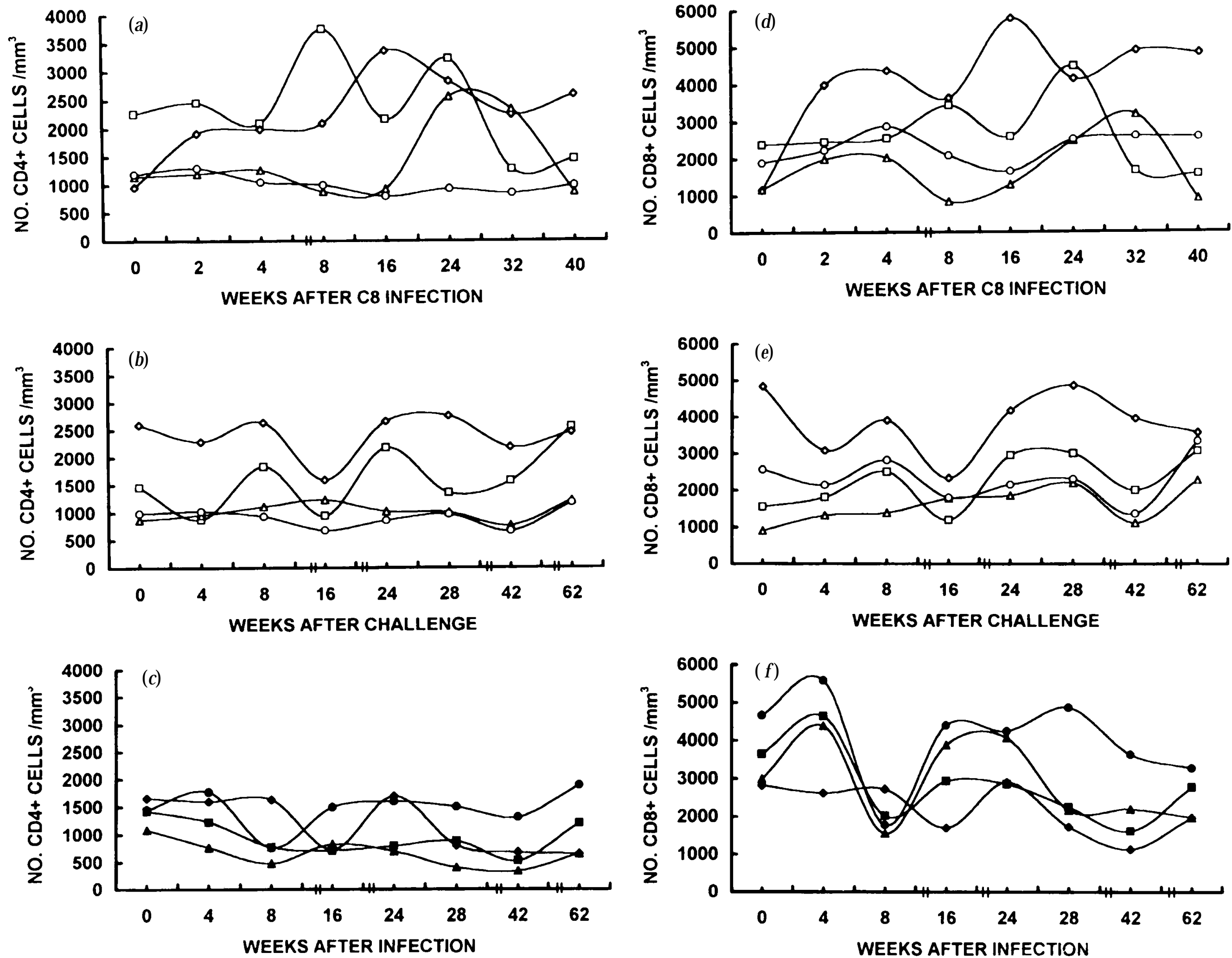

Fig. 2. Absolute counts of $CD4^+$ and $CD8^+$ cells in the blood of monkeys after infection with the C8 variant (*a* and *d*, respectively) or after challenge with BK28 virus (*b* and *e*, respectively). Symbols: □, monkey 7; ◇, monkey 09; △, monkey 33; ○, monkey 46133. The $CD4^+$ (*c*) and $CD8^+$ (*f*) cell counts in the blood of naive monkeys infected with BK28 virus are represented. Symbols: ■, monkey 22; ◆, monkey 28; ▲, monkey 91; ●, monkey 48126.

number of SIV proviral copies was constantly low, except for an initial peak at weeks 2 and 4 p.i. in monkeys 09 and 46133 (Table 2). The level of plasma antigenemia was at no time high enough to allow detection using the commercial p27 antigen-capture assay (data not shown).

Response to challenge with the infectious SIV molecular clone

At 40 weeks p.i. with the *nef*-deleted C8 variant, the four vaccinated and four naive monkeys were intravenously inoculated with the infectious molecular clone BK28 grown in monkey PBMCs.

Challenge with BK28 virus did not induce a clear serological booster response in the four C8-vaccinated monkeys. The anti-SIV antibody titres (Fig. 1*a*) appeared to be stable from 4–8 weeks p.c. in all monkeys, and then slowly increased in three of them (7, 09 and 33). In general, the antibody titres remained lower than those observed in control monkeys (Fig. 1*b*). One control monkey (48126) had an unusually low level of anti-SIV response.

Among the four C8-vaccinated monkeys, the number of $CD4^+$ cells remained stable until at least 62 weeks p.c. (Fig. 2*b*). Similarly, during the same period the $CD8^+$ cell count did not vary, although some fluctuations were observed in three of the vaccinated monkeys (7, 09 and 46133) (Fig. 2*e*). On the contrary, among the four control monkeys, three (22, 28 and 91) displayed a slight, although not statistically significant, decline in $CD4^+$ (Fig. 2*c*) and $CD8^+$ (Fig. 2*f*) cell counts.

The four C8-vaccinated monkeys remained virus isolation-negative during 62 weeks p.c., with the exception of one time-point (4 weeks p.c.) for monkey 46133 (Table 3). Virus was

Table 2. Outcome of infection with SIVmac251/32H/C8 virus in four monkeys (7, 09, 33 and 46133)

Minus (−) signs indicate that the samples were either virus isolation- or DNA PCR-negative, and plus (+) signs that the samples were positive.

Time (weeks)	Monkey 7			Monkey 09			Monkey 33			Monkey 46133		
	VC*	Virus load†	PCR‡ (*nef*)	VC*	Virus load†	PCR‡ (*nef*)	VC*	Virus load†	PCR‡ (*nef*)	VC*	Virus load†	PCR‡ (*nef*)
−2		−	−		−	−		−	−		−	−
0	−	−	−	−	−	−	−	−	−	−	−	−
+2	+(12)	< 1	+	+(9)	18·4	+	+(12)	1	+	+(9)	18·4	+
+4	+(12)	3	+	+(6)	24·9	+	+(12)	< 1	+	+(6)	61·7	+
+8	−	< 1	+	+(12)	2	+	−	< 1	+	+(12)	ND	+
+16	−	< 1	+	+(15)	< 1	+	−	< 1	+	+(18)	ND	+
+24	−	< 1	+	+(10)	< 1	+	−	ND	+	−	ND	+
+32	−	ND	+	+(18)	ND	+	−	ND	+	−	ND	+
+40	−	ND	−	+(24)	ND	+	−	ND	−	−	ND	+

* Virus isolation (VC) from monkey PBMCs cocultured with CEMX174 human cell line cells. Numbers in parentheses indicate the time (days) at which the first positive RT or p27 antigen reaction was detected in the supernatant of the cocultures.
† Cell-associated virus load (number of SIV proviral copies per μg DNA) as determined by single-round DNA PCR using primers amplifying the *gag* region (496 bp) of the SIV genome.
‡ To detect SIV-specific sequences in the *nef* region, DNA samples were analysed by single-round PCR using LS1-*nef* and LS2-*nef* primers (356 bp). From 16 weeks p.i. onwards, a nested PCR using LS4-*nef* and LS6-*nef* primers (first round, 896 bp) and LS1-*nef* and LS2-*nef* primers (second round, 356 bp) was performed.
ND, Not detectable.

frequently isolated from PBMCs of three control monkeys (22, 28 and 91), whereas it was only occasionally found in samples from monkey 48126, which showed a low antibody response to infection. Since virus was no longer re-isolated from PBMCs of C8-vaccinated monkeys, lymph nodes and bone marrow were analysed for the presence of virus at 28 weeks p.c. (Table 4). Virus was isolated from the lymph node cells of two out of three monkeys (09 and 33; lymph node of monkey 7 was missing), but not from bone marrow samples. In contrast, for control monkeys virus was easily re-isolated from the lymph node cells of three (22, 28, 91) and from the bone marrow samples of two (28 and 91), but not from either sample of monkey 48126.

Differential PCR analysis and stability of the C8 variant genotype

Although attempts to re-isolate virus from PBMCs of the vaccinated monkeys after challenge were negative (with the exception of virus isolation at 4 weeks p.c. from monkey 46133), the presence of the SIV genome was frequently detected by nested PCR (Table 3). Since the 12 bp deletion in the *nef* region eliminates one of two *Rsa*I recognition sites in the *nef* gene, a PCR-based diagnostic assay was used to distinguish between the C8 and the full-length *nef* BK28 proviruses (data not shown). Using the diagnostic PCR, no sequences that corresponded to the full-length *nef* DNA genome of the challenge virus were detected in the PBMCs (at 4, 16, 28 and 42 weeks p.c.) or lymph nodes and bone marrow (at 28 weeks p.c.) of vaccinated monkeys. Accordingly, full-length *nef* DNA could be detected in samples of control monkeys (Tables 3 and 4).

PCR-amplified products of the *nef* region present in PBMCs (monkey 7) or lymph nodes (monkeys 09, 33 and 46133) of vaccinated monkeys at 28 weeks p.c. were sequenced after cloning. The SIV genome maintained the original deletion (amino acids 144–147) in all monkeys (data not shown). Some point mutations or amino acid substitutions were observed. However, these appeared to be irrelevant for maintenance of the attenuated phenotype. In fact, no clinical signs of infection (decrease in the levels of $CD4^+$ cells and/or architectural changes in the lymph node) were observed.

In vitro spontaneous and mitogen-induced production of SIV-specific antibodies

Spontaneous *in vitro* synthesis of virus-specific antibody is a hallmark of both HIV-infected patients (Amadori *et al*., 1989, 1991) and SIV-infected macaques (Zamarchi *et al*., 1993). In fact, following C8 infection, a wave of spontaneous *in vitro* anti-SIV antibody production was seen in one monkey (09) as early as 4 weeks p.i., and in two monkeys at 25 and 40 weeks

Table 3. Virus isolation (VC) and genotype discrimination by diagnostic PCR tested on PBMCs of C8-infected (7, 09, 33 and 46133) and naive monkeys (22, 28, 91 and 48126) after challenge with SIVmac251/BK28 virus

Minus (−) signs indicate that the samples were either virus isolation- or DNA PCR-negative, and plus (+) signs that the samples were positive. Numbers in parentheses indicate the time (days) at which the first positive RT or p27 antigen reaction was detected in the supernatant of the cocultures.

	C8-Vaccinated monkeys								Control monkeys							
	7		09		33		46133		22		28		91		48126	
Time (weeks)	VC	PCR*	VC	PCR*	VC	PCR*	VC	PCR*	VC	PCR*	VC	PCR*	VC	PCR*	VC	PCR*
Day of challenge	−	−	+(24)	+	−	−	−	+(C8)	−	−	−	−	−	−	−	−
+4	−	+(C8)	−	−	−	−	+(13)	+(C8)	+(13)	+(BK28)	+(16)	+(BK28)	+(6)	+(BK28)	+(10)	+(BK28)
+8	−	+(C8)	−	+(C8)	−	+(C8)	−	+(C8)	+(6)	+(BK28)	+(20)	+(BK28)	+(6)	+(BK28)	−†	+(BK28)
+16	−	−	−	+(C8)	−	+(C8)	−	+(C8)	+(16)	+(BK28)	+(20)	+(BK28)	+(7)	+(BK28)	−	+(BK28)
+28	−	+(C8)	−	+(C8)	−	+(C8)	−	+(C8)	−	+(BK28)	+(10)	+(BK28)	+(14)	+(BK28)	−	+(BK28)
+42	−	+(C8)	−	−	−	−	−	+(C8)	−	+(BK28)	+(4)	+(BK28)	+(8)	+(BK28)	−	+(BK28)
+62	−	NT	−	NT	−	NT	−	NT	+(7)	NT	+(7)	NT	+(8)	NT	+(8)	NT

* Diagnostic PCR analysis done on DNA extracted from PBMCs of monkeys by using *Rsa*I as described in Methods.
† High cytotoxic reactivity was observed during the first days of coculture.
NT, Not tested.

p.i. (46133 and 7, respectively). In the remaining monkey (33), circulating SIV-specific B cells could not be demonstrated at any follow-up time. On the other hand, memory against SIV antigens, as judged by *in vitro* PWM-induced anti-SIV antibody synthesis, could be demonstrated in all C8-infected monkeys at 25 and 40 weeks p.i. (data not shown). Following challenge, a spontaneous *in vitro* anti-SIV antibody production was occasionally detected in all C8-vaccinated monkeys between 2 and 10 weeks p.c., whereas PWM-induced synthesis was consistently demonstrable (Fig. 1*c*). In control monkeys infected with BK28 virus, spontaneous anti-SIV antibody production was observed in only one monkey 6 weeks p.i., whereas PWM-induced anti-SIV synthesis could be detected in all monkeys 10 weeks p.i. (data not shown).

Pattern of cytokine mRNA expression in PBMCs after challenge

A comparison of the expression of several cytokine genes in freshly isolated PBMCs of vaccinated and control monkeys was done at 28, 42 and 62 weeks p.c. (data not shown). Although a quantitative RT–PCR assay was not used, the levels of interleukin(IL)-2 and interferon γ (IFN-γ) mRNAs were detected to a greater extent in vaccinated than in control monkeys, whereas IL-4 and IL-10 mRNAs were undetectable in all samples. Furthermore, a clear difference in the presence of IL-15 mRNA was observed between the two groups. In fact, IL-15 mRNA appeared to be overexpressed in PBMCs of vaccinated but not of control monkeys.

Histopathology

Histopathological analysis of the lymph nodes of vaccinated animals at 28 weeks p.c. revealed a preserved lymph nodal architecture showing a mixed lymphadenopathy (Fig. 3). In contrast, along with a significant difference in weight, the lymph nodes of three control monkeys (22, 28 and 91) revealed microhaemorrhagic areas and infiltration of lymphocytes to the germinal centre (comparable to the PGL, progressive generalized lymphadenopathy observed in HIV-positive patients).

Discussion

Infection of *Macaca fascicularis* with the C8 *nef*-deleted variant induced a specific humoral response to SIV proteins without any change in the immunological ($CD4^+$ or $CD8^+$ lymphocyte cell subsets) or haematological parameters throughout the 40 week observation period. Even if the C8 variant was only occasionally isolated from PBMCs of infected monkeys, the SIV genome was frequently detected in PBMCs, indicating that the C8 variant was able to establish a persistent but attenuated infection and, as judged by direct sequencing of the PCR products, to retain its original deletion.

Monkeys vaccinated with the C8 variant were protected from subsequent challenge with a homologous cell-free cloned

Table 4. Virus isolation (VC) and genotype discrimination by diagnostic PCR between C8 provirus in samples from PBMCs, lymph node and bone marrow cells of C8-vaccinated (7, 09, 33 and 46133) and naive infected monkeys (22, 28, 91 and 48126) at 28 weeks p.c.

Minus (−) signs indicate that the samples were either virus isolation- or DNA PCR-negative, and plus (+) signs that the samples were positive. Numbers in parentheses indicate the time (days) at which the first positive RT or antigen reaction was detected in the supernatant of the coculture.

		PBMCs		Lymph node			Bone marrow	
	Monkey code no.	VC	PCR*	Weight (mg)	VC	PCR*	VC	PCR*
C8-vaccinated monkeys	7	−	+(C8)	ND	ND	ND	−	+(C8)
	09	−	+(C8)	50	+(30)	+(C8)	−	ND
	33	−	+(C8)	50	+(30)	+(C8)	−	ND
	46133	−	+(C8)	50	−	+(C8)	−	+(C8)
Control monkeys	22	−	+(BK28)	210	+(14)	+(BK28)	−	+(BK28)
	28	+	+(BK28)	270	+(17)	+(BK28)	+(14)	+(BK28)
	91	+	+(BK28)	290	+(14)	+(BK28)	+(18)	+(BK28)
	48126	−	+(BK28)	120	−	+(BK28)	−	+(BK28)

* Diagnostic PCR analysis done on DNA extracted from monkey cells by using *Rsa*I as described in Methods.
ND, Not done.

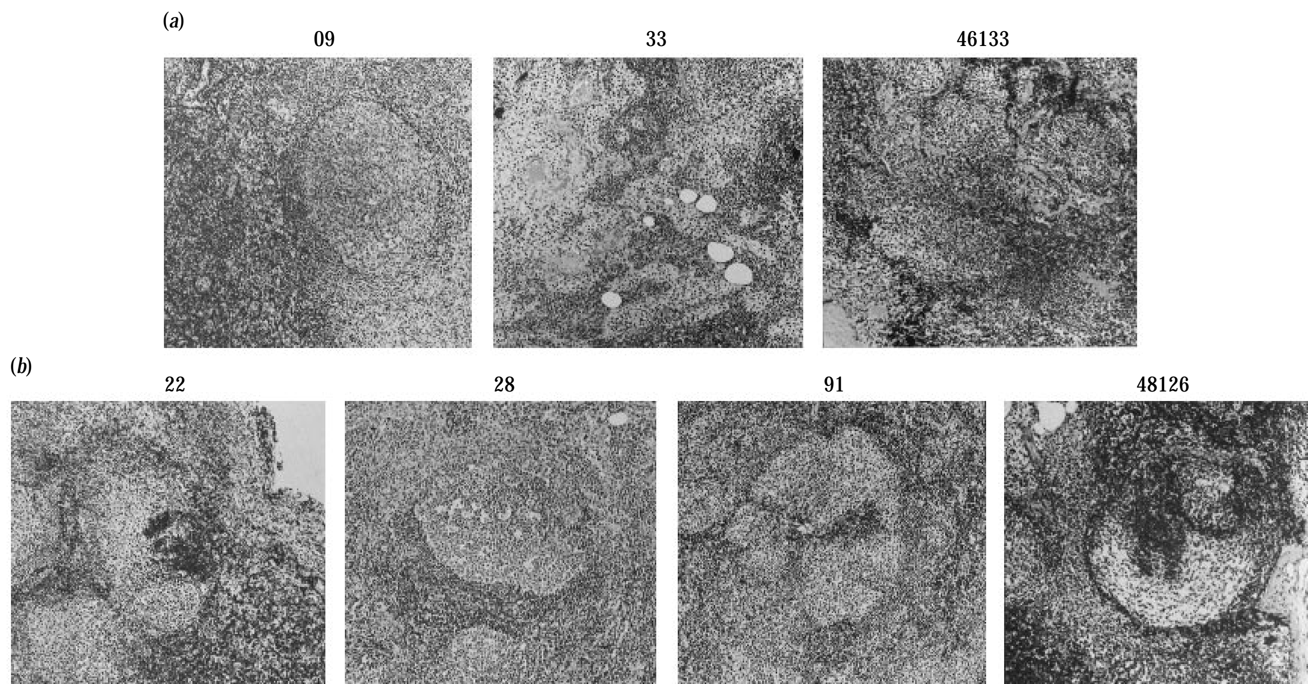

Fig. 3. Haematoxylin- and eosin-stained sections of lymph nodes from (*a*) three C8-vaccinated monkeys (09, 33 and 46133) and (*b*) four naive infected monkeys (22, 28, 91 and 48126). Magnification ×10.

SIV. In fact, the four vaccinated monkeys showed no evidence of challenge virus replication; they remained re-isolation-negative from PBMCs (except for one time-point for one monkey) and yielded a C8 variant pattern only in diagnostic PCR. Despite the fact that virus was no longer re-isolated from PBMCs of vaccinated monkeys, even after the challenge, the

persistence of infection by the vaccine virus was demonstrated by its isolation or detection by PCR from lymph nodes and bone marrow.

Our results extend previous evaluations from other groups demonstrating that in the SIV–macaque model, live attenuated virus vaccines confer protection against SIV challenge (Daniel *et al*., 1992; Clements *et al*., 1995; Almond *et al*., 1995; Norley *et al*., 1996). The strain of attenuated SIV that has been used as a vaccine in our experiment is a natural clone able to replicate in macaque PBMCs containing a small in-frame deletion of 12 bp in the *nef* gene (Rud *et al*., 1994). Furthermore, an infectious SIV molecular clone was used to super-infect monkeys instead of uncloned cell-free or cell-associated SIV. The BK28 virus is a molecular clone of SIVmac251 which is reported to have lost some pathogenic potential when compared to the parental uncloned virus (Naidu *et al*., 1988). The BK28 virus has been used as an attenuated vaccine in rhesus monkeys, resulting in successful protection against homologous and, in some animals, against heterologous challenge (Heeney *et al*., 1994). In our experimental conditions, the four naive monkeys infected with BK28 became virus isolation-positive, showing a persistent antibody response, a decreasing level of circulating $CD4^+$ and $CD8^+$ cells over a period of 62 weeks p.i. in three of the animals, and changes in the architectural structure of the lymph nodes.

By using the same live attenuated virus (SIVmac251/32H/C8) at the same dose in rhesus macaques, other groups have reported that the truncated *nef* open reading frame reverted to a wild-type genotype by restoring or duplicating the *nef*-deleted sequence. As a consequence, an AIDS-like disease similar to that present in monkeys inoculated with pathogenic SIV was observed (Whatmore *et al*., 1995; Dittmer *et al*., 1995). In the four cynomolgus monkeys infected in this study, the C8 variant maintained its original genotype during the observation period both after infection and following challenge. It remains to be elucidated whether the use of different species of monkeys (rhesus versus cynomolgus) or challenge viruses with a pathogenic potential higher than that reported for BK28 virus may have some influence on the reversion to pathogenic phenotype.

Although vaccination with a live attenuated SIV represents the most successful result in vaccine approaches, the mechanism(s) underlying the protective effect of vaccination remain unclear. As already described for murine retroviruses (Mitchell & Risser, 1992) and an *in vitro* system (Hart & Cloyd, 1990), a non-immune mechanism such as virus interference to super-infection may play an important role in establishing protection. This hypothesis is not fully sustained by the recent report that monkeys vaccinated with live attenuated HIV-2 become super-infected when challenged with heterologous uncloned SIVsm but they were still protected from the clinical signs of disease for a considerable period of time (Putkonen *et al*., 1995).

It has been suggested that vaccination of macaques with attenuated viruses induces a virus-specific immune response which appears to correlate with resistance to super-infection with pathogenic SIV (Dittmer *et al*., 1995; Lohman *et al*., 1994). Studies done with SIV molecular clones, differing in their highly or partially attenuated phenotype (Kieny *et al*., 1993; Marthas *et al*., 1990; Denesvre *et al*., 1995), seem to suggest that the establishment of protective status is inversely related to the level of attenuation and that a minimum duration of replication of the attenuated virus in the host is required to achieve good protection. It was found that, both before and after challenge, PBMCs of vaccinated monkeys retained their ability to spontaneously produce specific anti-SIV antibodies and a strong specific B cell memory, thus confirming that C8 variant replication probably occurs over the follow-up period. In HIV-infected patients, the spontaneous *in vitro* anti-HIV antibody production is a hallmark of chronic infection (Amadori *et al*., 1989, 1991), whereas B cell activation against non-persisting antigens is very rapidly lost (Kehrl & Fauci, 1983). While these findings could suggest that evolution of protective immunity following vaccination with live attenuated virus is a lengthy process, requiring a complex maturation of humoral responses in order to inhibit the uptake or to control the initial replication of the challenge virus, the role of cellular mechanism(s) in maintaining protection still needs to be fully elucidated.

In this study, it is reported that, following challenge, vaccinated monkeys have different levels of IL-2, IFN-γ and IL-15 mRNAs from control monkeys. It has already been reported that infection with attenuated rather than pathogenic SIV results in an early preferential Th1 versus Th2 cytokine profile (Benveniste *et al*., 1996; Zou *et al*., 1997). Moreover, IL-4 and IL-10 mRNAs were detected in PBMCs of monkeys during the acute phase of infection with *nef*-deleted virus (Benveniste *et al*., 1996) whereas, under our experimental conditions, these cytokines were undetectable. Furthermore, Zou *et al*. (1997) reported that the level of IFN-γ gene expression in lymph nodes of monkeys infected with pathogenic SIVmac239 was higher than that found in lymph nodes of monkeys infected with SIVmac239Δ*nef*. These differences to our results are probably due to the different stage at which the samples were examined (i.e. short time after infection versus months after challenge) or due to the different tissue origin (i.e. lymph node versus blood).

Over-expression of IL-15 mRNA is clearly found in PBMCs of vaccinated monkeys when compared to expression in control monkeys. IL-15 is mainly produced by macrophages and epithelial, muscle and placental cells, and shares many important biological activities with IL-2 such as increased proliferation of activated $CD4^+$ cell blasts, promotion of cytokine production by T helper cells, proliferation and differentiation of activated B cells, and T lymphocyte migration (Grabstein *et al*., 1994; Armitage *et al*., 1995; Seder, 1996; Mori *et al*., 1996). The identification of a macrophage-derived cytokine is very important in view of the role that macrophages play in the generation of the immune response. Thus, our

observation that the presence of IL-2, IFN-γ and IL-15 mRNAs distinguishes between monkeys infected with *nef*-deleted virus from those infected with non-attenuated virus is of importance, as it would suggest that the C8-attenuated variant had the ability to establish a chronic infection with a Th1 profile. In this respect, the involvement of IL-15 is suggestive although this still needs further investigation.

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