INTERFERON-GAMMA EXPRESSION IN MACAQUE LYMPH NODES DURING PRIMARY INFECTION WITH SIMIAN IMMUNODEFICIENCY VIRUS

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Simian immunodeficiency virus (SIV) replication is rapidly downregulated in the lymph nodes (LN) of rhesus macaques after the acute stage of primary infection. The aim of this study was to evaluate a possible role of interferon-gamma (IFN-γ) in the control of SIV replication. IFN-γ expression was analysed by in situ hybridization in the LN of rhesus macaques that were inoculated either with a high dose or with a low dose of the pathogenic isolate SIVmac 251. The kinetics of IFN-γ induction in LN was found to follow that of SIV replication. However, the number of IFN-γ expressing cells was not proportional to the number of infected cells. IFN-γ expression in LN was further quantified by competitive RT-PCR. The number of IFN-γ mRNA molecules in LN was high for the animals of the high dose group. In the low dose group, the IFN-γ copy number varied over 2 log_{10} units and was particularly low for the animals that had a high and persisting antigenaemia. The analysis of a total of 10 animals inoculated with a low dose of virus showed an inverse correlation between IFN-γ expression in LN and peak antigenemia ($P < 0.01$). This study provides evidence for a marked individual variability in the IFN-γ response to primary SIV infection and supports the notion that IFN-γ production is inhibited at an early stage in animals that harbour a high viral load.

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Interferon-gamma (IFN-γ) is a cytokine that exerts both immunomodulatory and anti-viral activities.[1,2] The secretion of IFN-γ by activated T cells and natural killer cells contributes to the control of acute infections caused by a variety of viruses.[3–8] IFN-γ also plays a role during the chronic stage of some viral infections, by limiting pathologies associated with viral persistence.[9] The function of IFN-γ in human immunodeficiency virus (HIV) infection is complex and not completely understood. In vitro, IFN-γ has an anti-HIV activity that is less pronounced than that of IFN-α. Both positive and negative effects of IFN-γ on HIV replication have been reported, depending on the time of administration and on the cell culture system.[10] In vivo, HIV infection causes a chronic induction of IFN-γ expression. IFN-γ transcription is increased in peripheral blood mononuclear cells (PBMC) and lymph nodes cells of seropositive individuals, as compared to cells of uninfected individuals.[11–14] Serum IFN-γ levels peak during acute HIV infection, are usually low in asymptomatic infection, and increase again in some of the patients experiencing disease progression.[15–17] The overexpression of IFN-γ may play a role in the chronic immune activation characteristic of HIV infection.[18] On the other hand, IFN-γ production by Th_1 cells has been proposed to be beneficial in HIV infection by promoting cell-mediated immunity rather than humoral immunity.[19] The loss of cell-mediated immunity that occurs early in HIV disease is paralleled by a loss of IFN-γ and IL-2 secretion in antigen-stimulated PBMC cultures.[20] Thus, IFN-γ production may not be sufficient to fully stimulate local antigen presentation, in spite of the elevated levels of circulating IFN-γ characteristic of HIV infection.

We used the model of simian immunodeficiency virus (SIV) infected macaques to analyse IFN-γ production during the primary stage of infection, at a time critical for both viral dissemination and immune response development. SIV is closely related to HIV in its genetic structure[21,22] and causes a disease in macaques remarkably similar to AIDS in man.[23,24] This animal model allowed us to follow the time course of IFN-γ production in lymph nodes (LN),

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TABLE 1. Virological and pathological findings in SIV-infected rhesus macaques

Animals	Inoculum SIVmac 251	Peak antigenaemia p27 (ng/ml)	Survival after inoculation (wk)	Pathological signs
P4	3×10^3 $TCID_{50}$	0.70	> 191	—
P3	3×10^3 $TCID_{50}$	0.91	129	wasting, paralysis
OH430	3×10^3 $TCID_{50}$	2.10	145	splenomegaly
PH426	3×10^3 $TCID_{50}$	6.75	36	intestinal necrosis
51127	10 AID_{50}	0.48	> 146	—
51179	10 AID_{50}	0.74	> 146	—
8632	10 AID_{50}	2.17	44	lymphoma
D54	10 AID_{50}	5.64*	32	wasting, pancreatitis

*The p27 antigenaemia remained persistently positive for this animal.

which constitute a major viral reservoir in HIV and SIV infections.[25–30] We have previously shown that macaques that fail to contain virus replication, as indicated by a high antigenaemia peak and by a high viral load in LN following primary infection, progress more rapidly to disease.[31] In this study, we asked whether animals with different viral load had different IFN-γ responses. We showed that the magnitude of IFN-γ induction was variable and that, for the group of animals inoculated with a low dose of virus, levels of IFN-γ expression correlated inversely to antigenaemia.

RESULTS

Detection of IFN-γ expression in LN by in situ hybridization

In situ hybridization was performed on the LN of eight rhesus macaques that were inoculated either with a high dose or with a low dose of the pathogenic isolate SIVmac 251 (Table 1). The viral load in the LN and the blood of these animals has been described previously.[31]

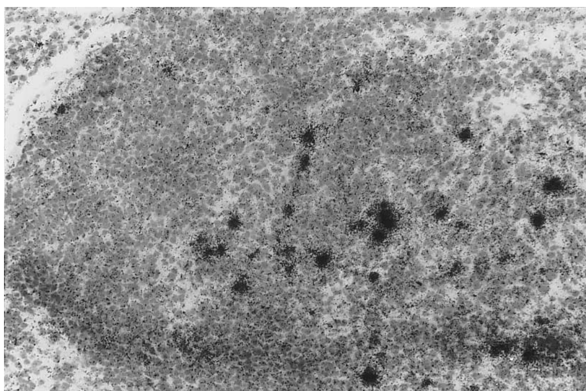

Figure 1. Detection of IFN-γ expression by in situ hybridization. Frozen lymph node sections were hybridized with a riboprobe specific for macaque IFN-γ. In this example, the lymph node taken at day 7 in animal P4 exhibits numerous IFN-γ positive spots (original magnification: ×250, reproduced here at 65%).

The IFN-γ response in LN was assayed by in situ hybridization on tissue sections. The riboprobe used was derived from the human IFN-γ cDNA and cross-hybridized with the macaque IFN-γ mRNA molecules. Primary SIV infection caused an increase of IFN-γ expression in LN (Fig. 1). The hybridization signal consisted in localized 'spots' and in a more diffuse signal, indicating that IFN-γ producing cells expressed this cytokine at different levels. Positive cells were detected throughout the tissue sections, with a preferential accumulation in the T-cell zone and in the germinal centers of the follicles.

The cells producing IFN-γ at high levels were quantified by counting the mean number of hybridization spots per surface unit of tissue section. The quantitation was performed on the five LN that were taken sequentially for each animal, which allowed to determine the time course of the IFN-γ response in lymphoid organs (Fig. 2A). The induction of IFN-γ expression was rapid for the high dose group, with the peak of expression occurring at day 7. A marked increase of IFN-γ positive cells was detected for three of the animals (30–40 cells per $2\,mm^2$), while a moderate increase was seen for the fourth animal OH430. The number of IFN-γ positive cells detected in the LN of three uninfected animals ranged between 0 and 4 per $2\,mm^2$ (data not shown). In SIV-infected animals, the induction of IFN-γ persisted until 2–4 weeks post-inoculation and declined after the acute stage of infection. In the low-dose group, the kinetics of IFN-γ induction was delayed by approximately 1 week, with the peak of expression occurring at day 14 or 19. The level of IFN-γ induction was lower than that seen in the high-dose group, except for one animal (51127). We next compared the kinetics of IFN-γ induction to that of SIV replication in LN (Fig. 2B). The time of IFN-γ induction and that of acute SIV replication coincided: they occurred both at day 7 in the high-dose group and one week later in the low dose group. Though the times of onset were similar, there was no direct correlation between the

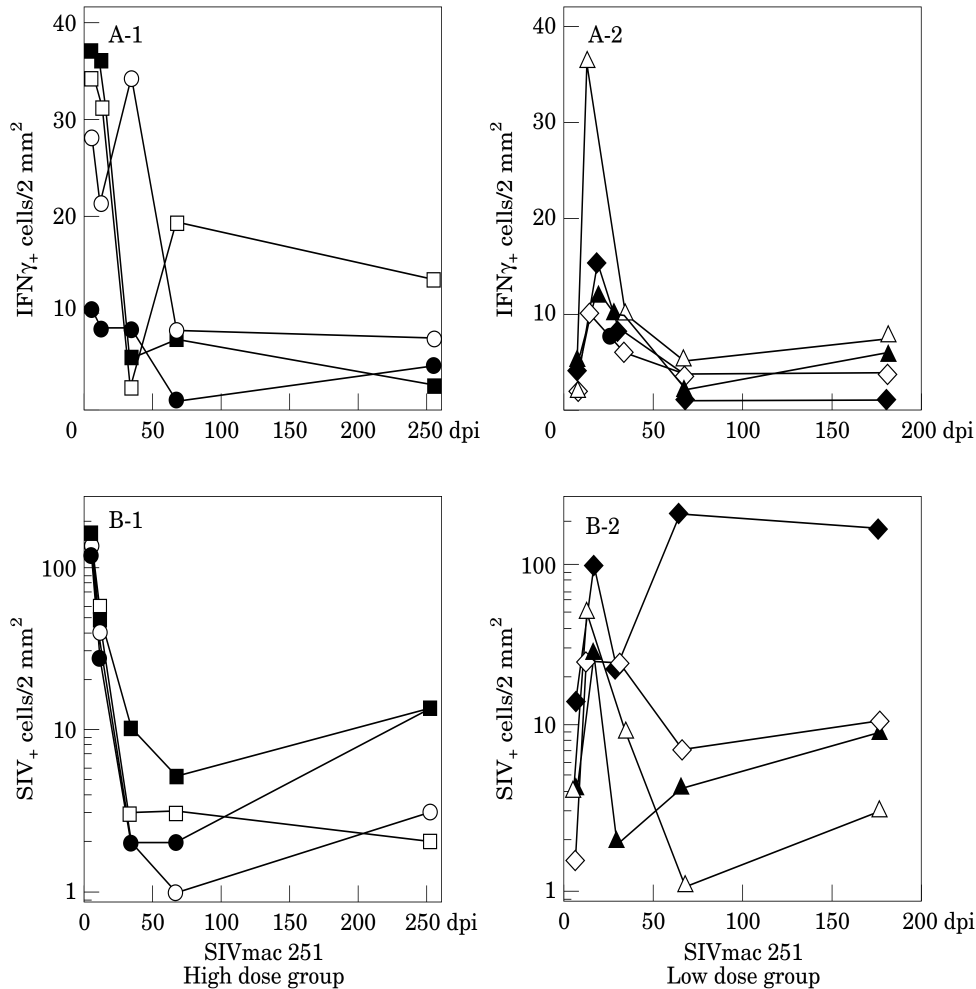

Figure 2. Comparison of the kinetics of IFN-γ expression to that of SIV replication in macaque lymph nodes.
Lymph nodes taken sequentially during the early stages of SIV infection were analysed by in situ hybridization with [A-1 and A-2] a riboprobe specific for macaque IFN-γ [B-1 and B-2] a riboprobe derived from the *nef* gene of the SIVmac 142 clone. For each lymph node, the number of hybridization spots was counted on a 2-mm^2 area of tissue section. The mean count obtained for three sections is indicated. (d.p.i.: days post-inoculation). (○), P4; (□), P3; (●), OH430; (■), PH426; (△), 51127; (◇), 51179; (▲), 8632, (◆), D54.

levels of IFN-γ expression in LN and those of virus production. On the opposite, it was interesting to note that in the high-dose group, the animal P4 which controlled SIV replication to very low levels 2 months after inoculation had a more prolonged IFN-γ response than the others. In the low-dose group, the number of IFN-γ expressing cells remained moderate except for the animal which exhibited the lowest viral load in LN at the outcome of acute infection (51127). These data suggested that the production of IFN-γ was greater in the LN of animals that efficiently controlled SIV replication.

Development of a competitive RT-PCR assay for macaque IFN-γ

A competitive RT-PCR assay was developed to quantitate the global amount of IFN-γ mRNA synthesized in LN. The advantage of this assay was that it took into account the cells that expressed both high and low levels of IFN-γ mRNA. The competitive PCR technique uses a competitor DNA construct engineered to carry a sequence similar but not identical to the target sequence.[36,37] The co-amplification of a known amount of competitor molecules with the sample allows to determine the copy number of gene-specific cDNA molecules present in the sample. The competitor DNA construct used in this study was engineered by PCR-driven mutagenesis of the macaque IFN-γ plasmid provided by F. Villinger.[35] A 58-bp deletion was introduced in the plasmid by amplification with a 42-mer primer designed to overlap the deletion, as described in Materials and Methods. The amplified fragment was cloned and sequenced to verify the presence of the deletion. The resulting mutated plasmid was used as competitor DNA.

The competitive PCR assay was calibrated by

using serial dilutions of the undeleted IFN-γ plasmid as samples. As shown in Fig. 3A, the assay gave a linear response over the range of concentrations studied. The estimated IFN-γ DNA copy number was found to be four times higher than the expected copy number. This factor was corrected in further experiments by adjusting the concentration of competitor molecules. For competitive RT-PCR, the RNA sample was converted to cDNA and co-amplified with serial dilutions of the competitor DNA. Typical products of a competitive RT-PCR assay are shown in Fig. 3B. The results, which corresponded to the estimated number of IFN-γ-specific cDNA molecules derived from the original RNA sample, were expressed as copy equivalents (CE) per 0.25 μg RNA.

Quantitation of IFN-γ expression in LN by competitive RT-PCR

For each of the macaques studied, RNA was extracted from the LN obtained at day 14 or 19, during the acute stage of infection. The competitive RT-PCR analysis showed that IFN-γ expression was increased by up to 2 $\log_{10}$ units in LN of infected animals as compared to controls (Fig. 3C). The magnitude of IFN-γ induction suggested that SIV could cause an acute activation of the immune system in some of the animals. IFN-γ expression varied depending on the animal and on the dose group. IFN-γ mRNA levels tended to be high in the high-dose group, with values superior or equal to 10^5 CE, and were widely variable in the low-dose group. No simple relationship was

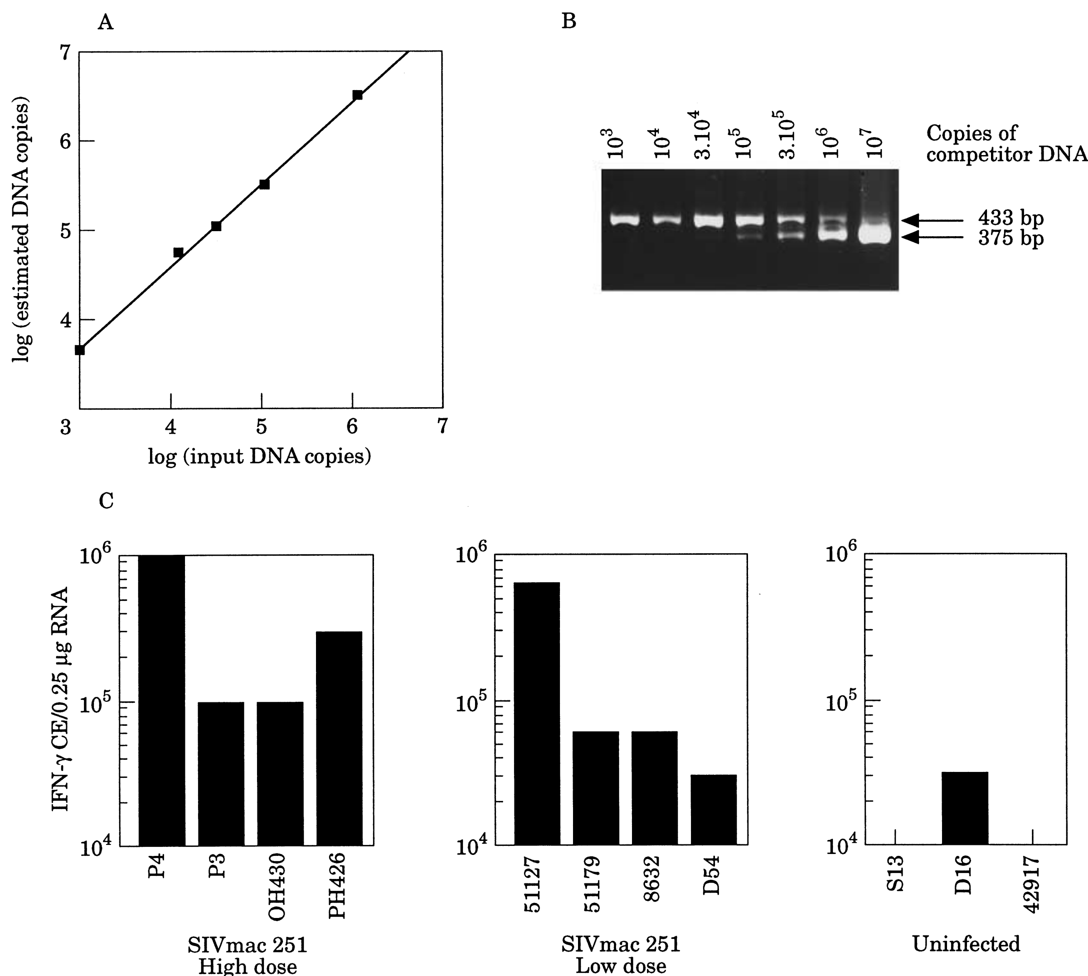

Figure 3. Detection of IFN-γ expression by competitive RT-PCR. (A) Calibration of the competitive PCR assay: test samples containing known numbers of IFN-γ plasmid molecules were assayed by competitive PCR. The number of IFN-γ DNA copies estimated in the assay was plotted as a function of the number of input DNA copies. Each plot symbol corresponds to the result of a series of amplifications in the presence of different dilutions of competitor DNA fragment. The competitive PCR assay gave a linear response over the range of concentrations studied. (B) Example of competitive RT-PCR products: a RNA sample was converted to cDNA and amplified in the presence of serial dilutions of competitor DNA. The PCR products were separated by agarose gel electrophoresis. The endogenous IFN-γ product can be distinguished from the competitor product by a size larger by 58 base pairs (bp). The number of copy equivalents, corresponding to the number of endogenous IFN-γ cDNA copies derived from the sample, was determined by the switch point at which the endogenous and competitor products gave similar densitometric measurements. (C) Quantification of IFN-γ expression in the lymph nodes of macaques: For each infected animal, RNA from the lymph node taken at day 14 or 19 was assayed by competitive RT-PCR. The RNA obtained from 3 uninfected macaques was processed similarly. The results are expressed as copy equivalents (CE) per 0.25 μg RNA.

TABLE 2. Virology findings in vaccinated animals

Animals	Type of vaccine	Inoculum SIVmac 251	Peak antigenaemia p27 (ng/ml)
8674	gp120 + liposomes	10 AID_{50}	2.32
8701	gp120 + liposomes	10 AID_{50}	7.98*
8725	gp120 + liposomes	10 AID_{50}	5.25*
8718	gp120 + syntex	10 AID_{50}	6.79
8777	gp120 + syntex	10 AID_{50}	21.66*

*Animal with persistently positive p27 antigenaemia.

found between IFN-γ mRNA levels and the number of SIV infected cells in LN at day 14 or 19. SIV replication was uniformly high in LN taken at this early stage and did not discriminate between animals with rapid or slow progression to disease. The p27 antigenaemia peak value was more informative as it was the earliest parameter associated with the risk of progression (Table 1).[31] In the high-dose group, a maximal IFN-γ expression of 10^6 CE was observed for the animal with the lowest antigenaemia peak (P4). However, the animal with high antigenaemia and rapid progression (PH426) also exhibited a relatively high IFN-γ copy number of 3×10^5 CE, Thus, in this group, IFN-γ expression in LN did not correlate either positively or negatively with the viral load. In contrast the levels of IFN-γ mRNA detected in the low-dose group appeared inversely related to the antigenaemia. It was striking that animal D54, which exhibited a persistently high antigenaemia and the most rapid progression to disease, had IFN-γ mRNA levels as low as those seen in uninfected animals (3×10^4 CE).

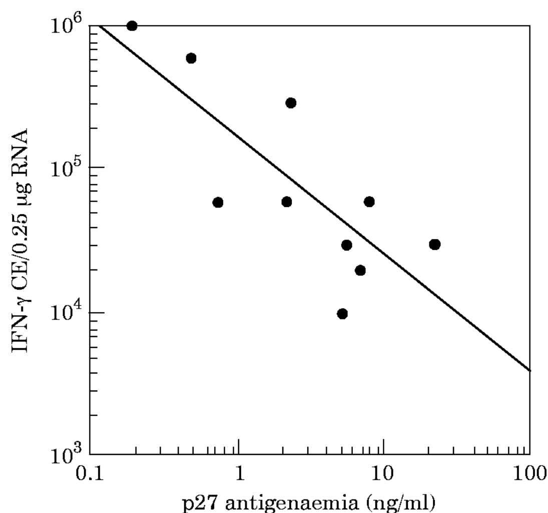

Figure 4. Inverse correlation between IFN-γ expression and antigenaemia. The number of IFN-γ copy equivalents (CE) detected by RT-PCR in lymph nodes taken at day 14 or 19 was plotted as a function of the peak antigenaemia value. This analysis was performed for ten rhesus macaques inoculated with a low dose of SIVmac 251. The significance of the correlation was estimated by the Student's *t*-test. $P < 0.01$; $r = -0.793$.

We extended the study to further explore the relationship between IFN-γ expression and antigenaemia. We analysed the LN of six additional macaques that received the same inoculum as the animals of the low-dose group. Five of these macaques were vaccinated and got infected upon challenge (Table 2). Three of them were particularly susceptible to SIV infection and remained persistently antigenaemia positive. The levels of IFN-γ expression were analysed on one LN taken at day 14 or 19, as described above. The results obtained for the total of the ten animals inoculated with a low dose of SIVmac were plotted as a function of the p27 antigenaemia peak value (Fig. 4). IFN-γ expression levels ranged between 10^4 and 10^6 CE, indicating that the IFN-γ response varied markedly between animals. The comparison with the viral load provided evidence for an inverse correlation between IFN-γ expression and p27 antigenaemia, which was statistically significant ($P < 0.01$). These data supported the notion that IFN-γ expression was inhibited in animals with a high viral load.

DISCUSSION

This study demonstrates that primary SIV infection induces the expression of IFN-γ in the LN of macaques and that the kinetics of IFN-γ induction follows that of virus replication. The magnitude of IFN-γ induction is widely variable, as indicated by the occurrence of up to 2 log differences in RT-PCR measurements. The differences in IFN-γ expression indicate that the cellular response mediated by T cells or NK cells is individually variable, which may in turn account for the variable susceptibility of rhesus macaques to SIV-induced disease. We observed an inverse correlation between IFN-γ expression and viral load in animals inoculated with a low dose of virus, which suggested that IFN-γ was essential to the control of SIV replication. However, the inverse correlation was not found for the four animals inoculated with a high dose of virus, as IFN-γ induction was high and rapid for all of them. It is possible that a high intravenous inoculum favoured the induction of a rapid innate response that lead to a massive activation of NK cells. We have observed that the expression of interferon-alpha (IFN-α) was higher in the LN of the animals of the high dose group as compared to those of the low-dose group[38]. As IFN-α is the major activating agent for NK cells during a virus infection,[39,40] it is likely that the activation of NK cells was higher in the high dose group. The production of IFN-γ by NK cells may then have masked the individual variations in the production of IFN-γ by specific T cells. Thus, one interpretation is that IFN-γ expression reflects two separate immunological

phenomena: the NK cell response, that is dependent on the inoculum dose, and the T cell response, that is more dependent on host factors.

We used two different techniques to evaluate the production of IFN-γ mRNA in LN. Both in situ hybridization and competitive RT-PCR yielded concordant results for the low dose group, in that the animal with the lowest viral burden (51127) was distinguished by a higher IFN-γ expression. The results obtained for the high dose group indicated that IFN-γ expression was high in the LN of three of the animals. One exception was OH430, for which the number of hybridization spots was low while the RT-PCR was relatively high (10^5/CE). Such discrepancy is likely to result from the lower sensitivity of the in situ hybridization technique, for which the threshold of detection is estimated to range between 10 and 20 mRNA copies.[41,42] Thus, cells producing lower levels of IFN-γ mRNA may be detectable by RT-PCR only. The comparison of the data obtained by the two techniques indicates that the IFN-γ response is mediated by a large number of LN cells that produce a relatively low amount of IFN-γ mRNA in addition to a much smaller population of cells that overexpress IFN-γ mRNA.

IFN-γ is one of several cytokines that are induced in primary HIV and SIV infections. Levels of IL-1 are increased in most of the patients experiencing the acute HIV syndrome and TNF-α secretion is increased in some of the patients.[15,43] The induction of the pro-inflammatory cytokine TNF-α is also characteristic of primary SIV infection.[44] Macrophages are activated, as indicated by the release of neopterin.[15] Signs of T8 lymphocyte activation, including the increased expression of major histocompatibility complex class II molecules and the release of soluble CD8, are detected both in patients and in monkeys.[45–48]. These data fit a picture of massive immunological activation that is consistent with the rapid development of the cellular and humoral responses detected in primary infections with HIV and SIV.[30,49–51] However, the results presented in this study suggest that the antiviral response may be impaired at a very early stage of infection. The limited expression of IFN-γ in the LN of the most susceptible animals may reflects defect in the function of either T4, T8, or NK cells. These possibilities are not mutually exclusive. Other studies have shown that T-cell proliferative responses to recall antigens are severely compromised during acute HIV and SIV infections, which suggests that the function of T4 lymphocytes is inhibited.[52–54] This would make sense as the population of T4 lymphocytes is the major target of HIV and SIV.[55,56] A dysfunction in T4-mediated help may in turn lead to defective T8 responses. As T8 lymphocytes represent the predominant population of IFN-γ secreting cells in HIV infection,[13] limited IFN-γ production may reflect a defective cytotoxic response. In this respect, it is interesting to note that in five patients for which the activity of cytotoxic T lymphocytes (CTL) was assessed during acute HIV infection, the one with the lowest CTL response had the fastest progression to AIDS.[50]

In conclusion, this study provides evidence for a marked individual variability in the IFN-γ response to primary SIV infection, and supports the notion that immunosuppression occurs at a very early stage in the most susceptible animals. Further studies are warranted to elucidate the immunogenetic factors that determine the susceptibility to immunodeficiency viruses.

MATERIALS AND METHODS

Animals and virus

Seventeen adult rhesus macaques (*Macaca mulatta*) were included in this study. Prior to inoculation, they were demonstrated to be seronegative for STLV-1, SRV-1 (type D retrovirus), Herpes B Virus and SIVmac. All procedures with animals were performed after anaesthesia with ketamine (Imalgène, Mérieux, Lyon). Three uninfected macaques were used as controls. The other macaques were infected intravenously with the pathogenic SIVmac 251 isolate provided by R. Desrosiers.[21] The kinetics of infection in LN has been described previously for eight of the animals.[31] Among these eight animals, four were inoculated with a high dose of SIVmac 251 corresponding to 3×10^3 $TCID_{50}$ (tissue culture infectious doses); the four others were inoculated with a low dose corresponding to 10 AID_{50} (animal infectious doses) of an in-vivo titrated stock of SIVmac 251 provided by Anne-Marie Aubertin (Université Louis Pasteur, Strasbourg). The remaining six rhesus macaques, which were part of unpublished vaccination studies, were inoculated with 10 AID_{50} of the titrated SIVmac 251 stock.

Antigenaemia

Serum samples were collected regularly and stored frozen at −70°C until use. The antigenaemia was detected by an antigen capture enzyme-linked immunosorbent assay (ELISA) specific for SIVmac p27 Gag protein (Coulter, Hialeah, FL). Serum samples were used at dilutions of 1:2 and 1:10, each dilution being tested with and without acid treatment prior to the ELISA. The acid treatment was performed to ensure dissociation of immune complexes that could mask the p27 antigen. The acid and neutralizing solutions were used according to the manufacturer's recommendations (Sanofi diagnostic Pasteur, Marnes-la-Coquette). The maximum value obtained with and without acid treatment is reported in Tables 1 and 2.

Lymph node collection

Axillary or inguinal LN were collected sequentially for eight of the rhesus macaques. Four LN were taken during the

early stages of infection, between days 7 and 68 post-inoculation (p.i.), and a fifth LN was taken 6 or 8 months later to estimate disease progression. For the six rhesus macaques of the vaccine group, a single LN was obtained at day 14 or 19, at the peak of primary infection. LN were removed surgically and frozen in isopentane cooled in liquid nitrogen. After embedding in O.C.T. compound (Miles, Elkhart, IN), LN were cryostat sectioned at 4-μm intervals. The sections were fixed for 10 min in acetone and stored at −20°C until use.

Preparation of ^{35}S-labelled RNA probes

RNA probes were derived from a human IFN-γ plasmid provided by Michael Tovey (CNRS UPR 274, Villejuif). The plasmid contained the transcription vector pSP65 (Promega, Madison, WI) into which a Taq1-Sau3a fragment (603 to 909) of the IFN-γ cDNA was inserted[32]. The antisense riboprobe was generated from the SP6 promoter by in vitro transcription of 1 μg of plasmid template with 50 U of SP6 RNA polymerase in the presence of 200 μCi of [^{35}S]UTP. After incubation for 1 h at 40°C, the DNA template was digested with 10 U DNase I for 15 min at 37°C. To enhance the penetration of the probe into tissue sections, the ^{35}S-labelled RNA was subjected to mild alkaline hydrolysis in 80 mM $NaHCO_3$ and 120 mM Na_2CO_3 at 60°C. The hydrolysis time was optimized to obtain a majority of fragments in the 150–200 nucleotide range. After neutralization with 600 mM sodium acetate and 167 mM acetic acid, the probe was purified by phenol/chloroform extractions and ethanol precipitation. Specific activity ranged between 3×10^8 and 5×10^8 dpm/μg of input DNA.

In situ *hybridization*

Hybridization techniques were based upon published procedures.[33] Briefly, the tissue sections were fixed in 4% paraformaldehyde and acetylated in acetic anhydride 0.25%/triethanolamine (0.1 M, pH 8). The sections were then denatured in 70% formamide at 70°C for 2 min to enhance the accessibility of nucleic acids. The hybridization mix contained the ^{35}S riboprobe at 50 000 dpm/μl, 50% formamide, 10% W/V dextran sulfate, 0.3 M NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 10 mM NaH_2PO_4, 1 × Denhardt's, 0.5 mg/ml yeast tRNA and 100 mM dithiothreitol (DTT). The mix was heated at 80°C for 2 min and applied to slides. Coverslips were mounted and sealed with rubber cement, and hybridization was carried out at 45°C overnight in a humid chamber. Slides were rinsed successively in 2 × saline sodium citrate (SSC)/10 mM DTT for 1 h at room temperature (RT), in 50% formamide 2 × SSC/10 mM DTT for 30 min at 60°C, in 2 × SSC for 30 min at RT, in 0.1 × SSC for 1 h at RT, 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°C. The long exposure time ensured that weak hybridization signals were detected. After exposure, slides were treated with Kodak D-19 developer, fixed, and stained with haematoxylin–eosin. Controls included hybridization of lymph node tissue from uninfected monkeys, and hybridization with a probe unrelated to IFN-γ.

RNA extraction and RT-PCR

Total cellular RNA was extracted from LN tissues by homogenization in RNA B solution (Bioprobe system, Montreuil) followed by chloroform extractions and isopropanol precipitation. The RNA was controlled for integrity on a 1% agarose gel and stored at −70°C. The RNA concentrations were normalized according to the amount of RT-PCR product detected using primers for human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) purchased from Clontech (Palo Alto, CA).

For the synthesis of dT_{16} primed cDNA, 0.5 μg of LN RNA was incubated in reverse transcription buffer (5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3) with 1.25 U/μl ribonuclease inhibitor (RNAguard, Pharmacia Biotech) 1 mM each deoxynucleotide, 2.5 μM oligo dT_{16}, and 50 U of Moloney murine leukaemia virus reverse transcriptase (Superscript RNAse H-, Gibco BRL) in a final volume of 20 μl. This mixture was incubated 30 min at 42°C, heated for 5 min at 99°C, and chilled for 5 min at 5°C. PCR amplification was performed with oligonucleotide primers specific for macaque IFN-γ: 5′-ATG AAA TAT ACA AGT TAT ATC TTG GCT-3′ and 5′-GCG ACA GTT CAG CCA TCA CTT G-3′. These primers, designed by Villinger *et al*.,[34] amplified a 433-bp fragment from the cDNA of macaque IFN-γ (Genebank accession number L26026). For amplification, the cDNA solution was diluted to a final volume of 100 μl in PCR buffer (2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3) with 0.15 μM of the two specific primers and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). After denaturation for 2 min at 95°C, the reaction was subjected to 35 cycles of amplification (30 s at 95°C, 1 min at 55°C, and 1 min at 72°C). A final extension step was performed for 7 min at 72°C. PCR products were analysed on agarose gels stained with ethidium bromide. The amount of DNA in each band was determined by densitometry, using a digitized image of the negative photograph of the gel. Densitometric measurements were made with the Optilab 2.5 image analysis software (Graftek, Mirmande, France). The specificity of IFN-γ PCR products was controlled by Southern hybridization with the oligonucleotide probe 5′-TGA CCA GAG CAT CCA AAA GAG TGT G-3′.[34] Amplifications without reverse transcriptase or with water instead of RNA in the reaction mixture were used as controls for possible contaminants.

Competitive RT-PCR for IFN-γ

To generate competitor DNA, a 58-bp deletion spanning nucleotides 354–411 was introduced in the amplified fragment of the macaque IFN-γ plasmid.[35] The deletion was obtained by PCR-driven mutagenesis, using the IFN-γ 5′ primer and a second primer overlapping the deletion (5′-GCG ACA GTT CAG CCA TCA CTT GAG CTT TTC AAA GTC ATC CCG-3′). The PCR product was purified on Chromaspin 400 columns (Pharmacia Biotech), cloned in a Bluescript vector (Stratagene, La Jolla, CA), and sequenced. The resulting competitor DNA was diluted in a log 3.16 series in siliconized microcentrifuge tubes starting from 10^6 to 10^2 copies/μl. Ten μl of each dilution were then co-amplified with a constant amount of sample cDNA (obtained from 0.25 μg RNA), using the PCR conditions described above. The

PCR products resulting from endogenous and competitor IFN-γ DNA were distinguished by their different size on 2% NuSieve agarose 1% standard agarose gels (FMC Bio-products, Rockland, ME). The number of copies of IFN-γ cDNA in the sample was determined by the dilution at which the endogenous and the competitor PCR products gave similar densitometric measurements. When the two products differed by more than a factor 2 at all the dilutions tested, the arithmetic mean between the dilutions flanking the switch point was determined.

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