Viral load in tissues during the early and chronic phase of non-pathogenic SIVagm infection


Abstract: African green monkeys (AGMs) persistently infected with SIVagm do not develop AIDS, although their plasma viremia levels can reach those reported for pathogenic HIV-1 and SIVmac infections. In contrast, the viral burden in lymph nodes in SIVagm-infected AGMs is generally lower in comparison with HIV/SIVmac pathogenic infections, at least during the chronic phase of SIVagm infection. We searched for the primary targets of viral replication, which might account for the high viremias in SIVagm-infected AGMs. We evaluated for the first time during primary infection SIVagm dissemination in various lymphoid and non-lymphoid tissues. Sixteen distinct organs at a time point corresponding to maximal virus production were analyzed for viral RNA and DNA load. At days 8 and 9 p.i., viral RNA could be detected in a wide range of tissues, such as jejunum, spleen, mesenteric lymph nodes, thymus and lung. Quantification of viral DNA and RNA as well as of productively infected cells revealed that viral replication during this early phase takes place mainly in secondary lymphoid organs and in the gut (5×10^3–5×10^6 RNA copies/10^6 cells). By 4 years p.i., RNA copy numbers were below detection level in thymus and lung. Secondary lymphoid organs displayed 6×10^2–2×10^6 RNA copies/10^6 cells, while some tissue fragments of ileum and jejunum still showed high viral loads (up to 10^9 copies/10^6 cells). Altogether, these results indicate a rapid dissemination of SIVagm into lymphoid tissues, including the small intestine. The latter, despite showing marked regional variations, most likely contributes significantly to the high levels of viremia observed during SIVagm infection.

Introduction
The natural history of HIV infection includes three phases. The acute phase of infection is usually characterized by high viremia (up to 10^8 copies/ml of plasma) and extensive viral replication in lymphoid tissues [57, 61]. The virus can also be detected in other tissues, such as the lung [73]. Within 4–6 weeks from the onset of symptoms, a significant decrease in viral burden then generally occurs, coincident with the emergence of HIV specific immune responses [13, 18]. These virological and immunological events characterize the beginning of a second phase of infection, a long period (6–10 years) of clinical latency [13, 18, 25]. This asymptomatic phase is characterized, however, by a continuous replication of the virus that takes place primarily in secondary and tertiary lymphoid tissues, such as lymph nodes (LN) and mucosa associated lymphoid tissues [3, 28, 51], and to a considerably lesser extent in primary lymphoid organs, such as the thymus [7, 63, 64]. Lymphoid tissues are thus considered to be the major replication sites for HIV-1 in humans during the acute and chronic phases of infection. However, compartments such as the central nervous system may play major roles as viral reservoirs [1, 23, 42]. The third stage, corresponding to the stage of infection with AIDS, is preceded by an increase in peripheral viral load [16] and the establishment of an active
viral replication in almost all organs [22]. Presence of virus in the lung is more frequently observed during this stage than during the asymptomatic phase, and may be associated with infiltrations of infected macrophages [14, 40, 55].

The critical factors involved in HIV pathogenesis still remain to be elucidated. Progression towards disease in HIV-1 infected humans is closely associated with the level of replication in blood and tissue cells, long-term non-progressors (LTNP) generally showing lower viral burden than rapid progressors [9, 52]. Moreover, plasma viral RNA levels as early as 6 months post-infection (p.i.) are highly predictive of the outcome of infection in patients [43]. Finally, viral replication can be profoundly suppressed for prolonged periods in 70–80% of individuals treated with highly active anti-retroviral therapy (HAART), and progression to AIDS is then seen to be consistently delayed [19, 70].

In addition to quantitative considerations, tropism of HIV for specific tissues may be closely associated with disease progression profiles. Thus, viruses from HAART treated patients showing discordant responses (no decrease in viremia, but increased levels of CD4+ cells), have been shown to display an impaired ability to replicate in the thymus [66]. Furthermore, studies in the major animal model for AIDS, macaques infected by SIVmac, have suggested that AIDS is principally a disease of the gut. Studies in this animal model revealed indeed the early viral replication to be particularly abundant in gut-associated lymphoid tissues and associated with a very rapid and profound depletion of CD4+ T cells [71]. A depletion of intestinal CD4+ lymphocytes has also been demonstrated early in HIV infection in humans [15, 41]. It has been suggested that the preferential depletion of intestinal CD4+ T cells is related to a preferential tropism of HIV/SIV for CD4+ T cells of the activated CCR5+ CXCR4+ phenotype that are more frequent in the gastrointestinal mucosa than in secondary lymphoid organs [56, 72].

Studies in animal models of non-pathogenic infection allow further evaluation of the relevance of tissue tropism in pathogenesis. Such non-pathogenic infections are observed in African non-human primates, the natural hosts for SIV. Among them, African green monkeys (AGM, Chlorocebus aethiops) are frequently infected by SIV [44, 53]. SIVagm infects CD4+ lymphocytes and macrophages and uses CCR5 as a main coreceptor [6, 24, 38]. Another coreceptor frequently used by SIVagm in vitro is Bonzo [20]. AGMs infected with SIVagm show up to 10^8 RNA copies/ml of plasma during the acute phase of infection [21, 32]. As in HIV-1 and SIVmac infections, the plasma viremia levels in SIVagm-infected AGMs then decrease after this initial phase [21]. During the chronic phase, virus replication is continuous [45], some AGMs displaying viremia levels similar to long term non-progressors (LTNP), whereas viremia remains at high levels in others (>10^6 copies/ml of plasma) [6, 21, 24, 32]. Similar high levels in the absence of pathogenesis have also been described in other natural SIV infections, i.e. sooty mangabeys, mandrills and chimpanzees infected by SIVsm, SIVmnd-1 and SIVcpz, respectively [11, 49, 59, 68]. The patterns of viremia and CD4 cell numbers are therefore discordant in these African monkeys and reminiscent of the profiles in discordant responders among HAART treated patients mentioned above. It is not clear if the reasons for the discordance patterns observed in some humans and in the African monkeys are the same or not. In order to better understand the mechanisms underlying the discordant profile in African monkeys, it is crucial to gain insights into the tissue targets of the virus during infections with a non-pathogenic outcome. Data on viral burdens in tissues during non-pathogenic SIV infection in natural hosts are, however, limited, since they have been mainly studied during the chronic phase. During chronic SIVagm infection, many lymphoid tissues, including secondary lymphoid organs (spleen, LNs) and intestine, are sites of viral replication [24]. The SIVagm load had been most accurately quantified in LNs. The numbers of infected cells in LNs are generally lower than those described for pathogenic HIV-1 and SIVmac infections [4, 6, 21, 50].

For the gut, semiquantitative measurement of SIVagm expressing cells in two naturally infected AGMs indicated a higher viral load in small intestine than in other parts of the gut, such as the duodenum, concordant with HIV-1/SIVmac distribution in humans and macaques [24, 29]. Another study, based on quantification of DNA copy numbers in the gut of two naturally infected AGMs did not show a higher viral load in jejunal in comparison with duodenum or colon, but indicated proviral load levels in the gut similar to that in chronically infected macaques [6].

In the experiments described here, we defined the tissue targets of SIVagm during primary infection of AGMs. We examined tissue distribution of SIVagm at a time point of maximal virus production, i.e. just before or around the peak of viremia. We searched for the potential existence and possible role of additional viral replication sites more important than the peripheral LNs in...
SIVagm-infected AGMs that could account for the high viremia in these animals. The viral replication patterns determined in lymphoid and non-lymphoid tissues during this early phase of infection were then compared with those in AGMs chronically infected for 4 years with the same SIVagm virus. Our results indicate a rapid dissemination of SIVagm into lymphoid tissues, including the small intestine. Although showing a significant intra-individual variability, the small intestine was among the tissues showing the highest levels of viral RNA (up to 10⁹ copies/10⁶ cells) in chronically infected AGMs. Our study suggests that viral replication in the intestine contributes significantly to the high levels of viremia observed during SIVagm infection. The implications on our findings for the understanding of HIV pathogenesis will be discussed.

Materials and methods

Animals

Seven juvenile AGMs (body weight approximately 2.5 kg and between 2 and 3 years old at the time of inoculation) from the C. sabaeus species were used in this study. All the animals were wild born in Senegal. They were negative for SIV and STLV, as determined by commercial Elisa assays (Genelavia mixt and Diagnostics Biotechnology, respectively). The absence of SIV infection was confirmed by polymerase chain reaction (PCR). The latter was negative on peripheral blood mononuclear cells (PBMC) and LN cells collected at day 0 before infection ([21], and data not shown).

Viruses and infections

Animals were infected intravenously with a primary SIVagm virus (SIVagm.sab92018). The inoculum was prepared as previously described [21]. Five AGMs (00008, 96011, 96023, 97026 and 97027) were inoculated with either 100 or 300 TCID₅₀ of the virus stock. These infectious doses were previously determined to give reproducible and comparable infection patterns and viral loads in blood similar to that in the naturally infected donor monkey AGM92018 [21]. Two AGMs (AGM 96028 and 00010) served as negative controls.

Sample collection

In order to evaluate as precisely as possible the time point closest to the peak of viremia for animals AGMs 97026, 97027 and 00008, blood samples were collected every day starting from day 3 p.i., and plasma samples were tested for p27 viral antigen by using an SIV antigen capture enzyme-linked immunosorbant assay (Coulter Corporation, Hialeah, FL, USA). The animals 97026, 97027 and 00008 were sacrificed at days 8, 9 and 11 p.i., respectively. Plasma antigenemia levels were 4.73, 4.56 and 3.94 ng p27/ml, respectively. The mock-infected control animals AGM 00010 and 96028 were sacrificed, respectively, at days 11 and 12 post-inoculation. Animals AGM 96011 and 96023 had already been followed for viremia during primary infection [21] and were sacrificed for tissue analysis during the chronic phase, 4 years p.i.

Blood and tissues were collected under ketamine anesthesia. PBMC were harvested on Ficoll gradients and frozen in fetal bovine serum containing 10% DMSO. Plasma was aliquoted and stored at −80°C until use. Lymphoid and non-lymphoid tissues, such as thymus, axillary LNs, inguinal LNs, cervical LNs, mesenteric LNs, spleen, tonsils, small intestine, colon, liver, lung, kidney, brain, spinal cord and/or skin were obtained from animals 97026, 97027, 96011, 96023 and 96028. They were immediately washed in saline solution, and fragments weighing between 100 and 150 mg were frozen at −80°C for RT-PCR and PCR analysis. Tissues collected from AGMs 00008 and 00010 were cryo-preserved as previously described [21] and used for in situ hybridization analysis (ISH). Animals were then sacrificed by intravenous administration of a lethal dose of ketamine.

Virus isolation and determination of infectious titers

PBMCs were stimulated for 3 days with 20 μg/ml of ConA and cultured in the presence of IL-2. Every 3 days, blasts (ConA stimulated PBMC from normal AGMs) were added to the culture. Viral production was evaluated by p27 antigen measurement (Coulter) in supernatants collected every 3 days. The positive supernatants collected at days 7, 10 and 14 were pooled, and the infectious titer was determined by limiting dilution on SupT1 cells, as previously described [21]. The infectious titer of plasma was determined by the same assay.

Nucleic acids extractions and viral load assays

Viral DNA and RNA, obtained from PBMCs and plasma, respectively, were extracted as previously described [21]. Extractions from tissue cells were performed either by using a Qiagen RNA/DNA extraction kit (Qiagen, Courtaboeuf, France) or by using a Qiagen RNeasy Extraction Kit, according
to the manufacturer’s protocol. RNA and DNA were extracted from two distinct fragments of each tissue in order to control for differences in viral distribution within a single organ. Viral load measurements were performed separately on the two extracts of each tissue. In addition, each RNA extract was submitted to two distinct RT reactions, and separate PCR reactions were performed for each cDNA or DNA. An average of four RNA and DNA measurements were performed for each tissue. Plasma RNA and PBMC DNA were submitted to two measurements each.

RNA viral loads were evaluated by a real-time PCR assay specifically developed and validated for SIVagm.sab, as previously described [21]. Briefly, in each assay, RNA amounts between 100 and 500 ng of RNA from tissues or PBMCs, or RNA corresponding to 30–100 μl of plasma, were first submitted to a reverse transcription step by using a TaqMan Gold RT-PCR kit with random hexamers (Applied Biosystems, Courtaboeuf, France). Primers and a probe specific for SIVagm.sab were designed using conserved regions of the SIVagm.sab LTR [21]. An in vitro transcribed LTR standard RNA was obtained as previously described [21] and serial dilutions from 10^5 to 1 copy were used to generate a standard curve. Quantification of 18S ribosomal RNA served to normalize the RNA input from cells. The standard curve for 18S rRNA was generated by using the ribosomal RNA control reagent kit from Perkin Elmer. 18S rRNA was amplified simultaneously in the same tube as the RNA sample. PCR conditions were as described [21]. The sensitivity of the assay was 10^3 RNA copies/ml of plasma and 200 RNA copies/10^5 cells. The coefficient of variation (71%) was within the same range as reported for a commercial HIV RNA load assay [69].

Viral DNA was also quantified by real-time PCR, using the same conditions as those described above for the RNA measurements, except that the RT step was omitted. In addition, a real-time PCR assay was developed for quantification of a simian gene in order to normalize the DNA used as input for quantification. The CCR5 gene was chosen for calibration, because it is a single copy gene (i.e., two copies per diploid cell), at least in humans, and thus reflects the input number of cells used in each assay. Primers were designed in conserved regions among the reported AGM and macaque CCR5 coding sequences [12, 20, 38, 46]. The primers were respectively CCR5fwd105 (5’-CAAGGTGACTGTCTGGCTTT-3’) and CCR5rev201 (5’-TTC TCT GGAATCTTCTTCATCC-3’), and the FAM labeled probe used was CCR5rev134 (5’-FAM-AACACAGCATGGGACGATAGCCAGG-TACC-3’-ROX). CCR5 copies were quantified in parallel with each DNA sample to be tested. The sensitivity of the CCR5 assay was 100 copies/reaction. The sensitivity of the SIVagm.sab DNA assay was 10 copies/10^5 cells.

In situ hybridization

SIVagm expression in tissues was evaluated by ISH, as previously described [10]. In order to construct a probe that recognizes specifically SIVagm.sab genomic RNA as well as RNA coding for viral structural genes, we amplified a 1150 bp env fragment from the SIVagm.sab reference virus, SIVagm.sab1 [34], by using the SIVagm.sab specific primers EnvAsab and EnvBsab [21]. The fragment was cloned into an expression vector [21], and a radioactively labeled 1.2 kb transcript was generated according to the protocols described [10] and used as an antisense probe. As a positive control, SupT1 cells productively infected with a primary SIVagm isolate (SIVagm.sabD46 [44]) were used. As a negative control, we used tissue fragments from the uninfected control animal AGM 00010.

Results

Determination of the time point of maximal virus production in acutely SIVagm.sab92018 infected AGMs

Previous studies have shown that AGMs infected with SIVagm.sab92018 display high viremia levels during primary infection (peak levels between 2 × 10^6 and 2 × 10^8 SIVagm copies/ml of plasma), similar to those described for both HIV and SIVmac infections [21, 43, 57, 67]. We wanted to identify the tissues responsible for the high virus levels detected in the blood very early after infection. To date, tissue viral load during primary infection in AGMs has been studied only for peripheral LNs [21]. Viral burdens were relatively low in SIVagm.sab92018-infected animals, the peak values detected ranging between 3 × 10^4 and 3 × 10^5 RNA copies/10^5 LN cells [21]. In order to elucidate the tissues that contribute to viremia in primary SIVagm infection, we determined the viral burdens during the days just preceding the peak of viremia, since these time-points were most likely to correspond to those of high if not highest replication in tissues. To determine as accurately as possible the moment immediately preceding the peak of viral replication, we inoculated two AGMs (97026 and 97027) with SIVagm.sab92018 and monitored p27 antigen levels each day starting from day 3 p.i. Levels above 1 ng p27/ml of plasma...
were reached at days 7 and 8 p.i. in AGMs 97026 and 97027, respectively (Fig. 1), and the animals were sacrificed the day after, at days 8 and 9 p.i., respectively, when plasma antigenemias were high (4.73 and 4.56 ng of p27/ml, Fig. 1). The kinetics of antigenemia during the first days following viral exposure in these two animals were very similar to those previously described in five other AGMs infected with the same virus, with peaks of antigenemia observed at day 10 p.i. [21]. The two animals described above were therefore sacrificed most likely at the time point just before or at the peak day of viremia. Antigenemia remained negative in the control animal (AGM 96028) until day 12, at which time it was sacrificed.

In order to confirm that the levels of p27 antigen observed in the animals corresponded to high levels of virus, we evaluated viral RNA copy numbers at day 8 p.i. in the plasma of one of the two acutely infected AGMs (97026). The RNA burden was high (4 × 10^8 SIVagm copies/ml). We further confirmed high viremia at day 8 p.i. in this animal by evaluating the level of infectious virus in plasma. The plasma was used to infect SupT1 cells, and its infectious titer was determined to be 7 × 10^3 TCID_{50}/ml (data not shown). These titers were thus significantly higher than those we obtained previously for the chronic phase in the naturally infected donor animal (AGM 92018), in which the titer in PBMCs was 10 TCID_{50}/10^6 cells, and that in plasma was below the detection limit [21]. Finally, PBMCs obtained at days 8 and 9 p.i. from AGMs 97026 and 97027, respectively, were cultured in vitro and p27 monitored in the supernatants. For both animals, viral replication was evidenced as early as 4 days after in vitro culture (Fig. 2). The viral isolate SIVagm.sab.97026 (corresponding to the pooled supernatants collected at days 7, 10 and 14 of PBMC culture) showed a high infectious titer in SupT1 cells (4 × 10^2.8 TCID_{50}/ml; data not shown). The level of infectious virus in the blood was not determined for AGM 97027, but antigenemia was similar and in vitro viral replication in PBMCs was even more rapid than for AGM 97026, suggesting that infectious titer was at least comparable with that of AGM 97027. Thus, these results demonstrate that both animals were sacrificed at time points where both cell-free and cell-associated virus levels were elevated in the blood. This high viral load in blood most likely reflects a time point of maximal virus production in tissues.

Widespread distribution of SIVagm RNA in lymphoid and non-lymphoid tissues during primary infection

We then analyzed which tissues are responsible for the high viremias we observed at days 8–9 p.i. We quantified the tissue viral load in 16 distinct anatomic compartments of the body. As a marker...
for SIVagm replication, we first measured RNA copy numbers using a real-time RT-PCR assay that allows quantification of genomic RNA as well as mono- and multi-spliced RNAs. We detected viral RNA in all lymphoid tissues analyzed (Fig. 3A). The inter-individual variability of viral load in lymphoid tissues was low, below 1 log, except for the cervical LNs, where the difference reached two logs between the two animals studied. The RNA levels in axillary and inguinal LNs (5 × 10⁴–5 × 10⁶ SIV copies/10⁶ cells) were similar to those previously determined at days 7 and 10 p.i.

Fig. 3. Viral load in tissues during the exponential phase of early viremia. Tissues were obtained from SIVagm.sab92018-infected AGMs 97026 and 97027 at days 8 and 9 p.i., respectively. Quantification of SIVagm RNA and DNA copy numbers was performed by real-time PCR. For each organ, nucleic acids were extracted from two distinct fragments, and viral loads were evaluated separately for each extract. The viral load is expressed as a mean of four measurements performed for each tissue, and the SD is indicated. The viral load in the mock infected animal was negative (data not shown). The white and black bars stand for AGMs 97026 and 97027, respectively. (A). Quantification of SIVagm RNA. (B). Quantification of SIVagm DNA.
in five other AGMs infected with the same virus [21, 27]. The mesenteric LN of both animals showed slightly higher viral load levels (6.6 × 10^6 and 1 × 10^7 SIV RNA copies/10^6 cells) than most peripheral LNs analyzed, except for the cervical LN of AGM 97027. The viral RNA loads detected in primary or tertiary lymphoid organs (thymus, lung and gut) reached similar levels (between 0.6 × 10^7 and 0.6 × 10^8 SIV RNA copies/10^6 cells) to those observed in the secondary lymphoid organs during this early time point following infection (Fig. 3A).

Two non-lymphoid organs (liver and kidney) showed slightly lower viral RNA copy numbers (10^6–10^7 copies/10^6 cells) at days 8–9 p.i., whereas viral RNA was undetectable in other non-lymphoid tissues. Thus, the viral burden was below detection level in the skin of one animal tested and in brain and spinal cord in both animals.

Secondary lymphoid organs and gut-associated tissues show the highest levels of SIVagm DNA during primary infection

In order to evaluate the numbers of infected cells in the tissues as well as to identify those tissues which could represent major virus reservoirs in AGMs, without necessarily expressing viral RNA, we studied viral DNA burdens during primary infection. We quantified viral DNA in total cells from the same tissues analyzed for viral RNA. Our method detects the total viral DNA (both integrated and non-integrated), allowing evaluation of both chronically and recently infected cells. The cell numbers were normalized by measuring CCR5 expression using a real-time PCR assay specifically developed for precise quantification of this simian gene.

We detected the highest viral DNA copy numbers in the secondary lymphoid organs and gut-associated tissues, such as inguinal LNs and cecum (Fig. 3B). DNA levels ranged between 2 × 10^5 and 6 × 10^8 copies/10^6 cells, except for tonsils where the values were lower (6 × 10^5 copies). In the chronic phase, viral load in tonsils was not significantly different from viral load in other peripheral LNs (see below). Occasional differences in the RNA/DNA ratios between the animals or among distinct tissues might be explained by distinct frequencies of latently infected cells and/or frequencies of defective particles produced.

In contrast to gut, LNs and spleen, other lymphoid organs, such as the lung and especially the thymus, exhibited low DNA levels (<10–6 × 10^5 DNA copies/10^6 cells) (Fig. 3B). Similarly, liver and kidney contained low numbers of viral DNA copies (10^5–10^7/10^6 cells), despite high RNA copy numbers. In brain and skin, viral DNA was below the detection limit, as was the case for viral RNA.

Altogether, relatively high viral DNA levels (above 10^7 copies/10^6 cells) at an early stage of infection were detected only in secondary lymphoid organs and intestine. These tissues also showed high SIV RNA levels, suggesting that they are sites of major virus production during primary infection in non-pathogenic SIVagm infection. In contrast, the lungs, kidney and liver were characterized by low viral DNA levels despite high RNA copy numbers. Finally, brain and skin showed consistently low levels of both RNA and DNA, indicating a low level or absence of virus replication in the latter tissues.

Productively infected cells in peripheral and mesenteric LNs during primary infection detected by in situ hybridization

In order to confirm the patterns of viral replication indicated by measurements of SIVagm RNA and DNA copy numbers, we analyzed the frequency and localization of productively infected cells in specific tissues during primary infection by in situ hybridization. We performed this analysis in tissues representative of the three patterns we observed, i.e. in tissues showing both high RNA and DNA levels (peripheral and mesenteric LNs), in a tissue with high RNA but low DNA copy numbers (liver) and finally in a tissue that had both low RNA and DNA loads (brain). One AGM (00008) was infected with SIVagm.sab92018 and tissues were collected again at a time point of maximum viremia (day 11 p.i.). AGM 00010 served as negative control. Both LNs harvested from the SIVagm-infected monkey (AGM 00008) showed significantly higher numbers of productively infected cells (10–12 cells/2 mm^2) than did liver (0–1 positive cells/2 mm^2) or brain (0–1 positive cells/2 mm^2) (Fig. 4). The numbers were similar for peripheral and mesenteric LNs. SIVagm infected cells were mainly localized within the T zone of the LNs, outside of the germinal centers, whereas productively infected cells within a follicle were very rare (Fig. 4C and D). No signs of viral trapping were detected in the germinal centers at day 11 p.i.

The results obtained by in situ hybridization therefore correlate with the observations based on RNA and DNA quantifications. They confirm that productively infected cells are more frequent in secondary lymphoid organs, such as peripheral and mesenteric LNs, than in brain and liver.
Major sites of SIVagm replication in tissues during chronic infection

The data presented above indicate that during acute infection of AGMs with SIVagm, the virus is present in a large number of tissues, but that viremia is mainly the result of ongoing replication in intestine and secondary lymphoid organs, such as LNs. It is of interest, therefore, that AGMs exhibit lower LN DNA viral loads than macaques during the chronic phase of infection despite equivalent plasma RNA levels [4, 6]. Hence, we considered it likely that tissues other than LNs contribute significantly to viremia during chronic, chronic...

Fig. 4. Detection of SIVagm RNA expressing cells in tissues during the exponential phase of early viremia. *In situ* hybridization was performed on tissue sections obtained at day 11 p.i. from SIVagm.sab92018-infected AGM 00008, and at day 12 from mock infected AGM 00010. Hybridization was performed with an antisense SIVagm env probe. (A) SupT1 cells infected with a primary SIVagm.sab isolate; (B) axillary LN from the mock-infected animal; (C) inguinal lymph node from the SIVagm-infected animal AGM 00008; (D) mesenteric lymph node from AGM 00008; (E) liver from AGM 00008; (F) brain from AGM 00008. The arrows indicate representative cells positive for SIVagm RNA. The highest numbers of positive cells were detected in the LNs. Positive cells were detected most frequently in the T zone and only exceptionally within follicles. Such a follicle is shown in (C). The average numbers of positive cells in inguinal and mesenteric lymph node sections were similar. 

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non-pathogenic SIVagm infection. In order to address this, we quantified DNA and RNA copy numbers in tissues of two AGMs infected 4 years previously with SIVagm.sab92018 (Fig. 5A). These two AGMs (96011 and 96023) experienced high viremia during the acute phase of infection, similar to those observed in AGMs 97026 and 97027 (Fig. 1 and [21]) and displayed $3 \times 10^3$ and $2 \times 10^5$ RNA copies/ml of plasma, respectively, during the chronic phase [21]. The proviral loads in AGMs

![Graph of viral load in tissues during chronic infection.](image)

**Fig. 5.** Quantification of viral load in tissues during chronic infection. Tissues were obtained from SIVagm.sab92018-infected AGMs 96011 and 96023 at 4 years p.i. SIVagm RNA and DNA was quantified in the same manner as described in the legend of Fig. 3. Jejunum/ileum corresponds to the median portion of the small intestine. Striped and dotted bars indicate AGMs 96023 and 96011, respectively. (A) SIVagm RNA. (B) SIVagm DNA. The standard deviation is represented when quantification was performed at least on two distinct extracts of a same tissue.
96011 and 96023 corresponded, respectively, to $10^5$ and $10^4$ DNA copies/10^6 PBMC at the peak during primary infection [21], to $10^3$ and $10^2$ DNA copies/10^6 PBMC at 3 months p.i. [21] and to $8 \times 10^1$ and $2 \times 10^0$ copies/10^6 PBMC at 4 years p.i. (data not shown).

As expected, RNA and DNA copy numbers in LNs at 4 years p.i (Fig. 5), were significantly lower than in primary infection. Thus, the RNA copy numbers were decreased by 1–4 log as compared with the levels described in LNs from the same animals during the primary infection [21]. Other secondary lymphoid organs, such as spleen, exhibited RNA levels within the same range as peripheral LNs (Fig. 5A). The levels were not higher in mesenteric than in peripheral LNs, at least in the two animals we tested.

In other lymphoid tissues, the viral RNA levels were also generally lower during the chronic phase in AGMs 96011 and 96023 than those measured during acute infection in AGMs 97026 and 97027. Thus, viral RNA copy numbers were below detectable levels in the thymus and the lung, indicating that these two organs were not sites of high viral replication in the two chronically infected animals. In contrast to LNs, thymus and lungs, the RNA levels in some gut-associated tissues were as high as those detected during acute infection in AGMs 97026 and 97027 (Fig. 5A). This was the case for the ileum and Peyer’s patches from AGM 96011 and for the median fraction of the small intestine (interface jejunum/ileum) from AGM 96023 (up to $10^9$ copies/10^6 cells). In contrast to the small intestine, no RNA could be detected in the colon of either animal. The variation of viral load in the small intestine was high and more pronounced than in other tissues. Such marked inter-regional variations in viral load levels have also been described in SIVmac-infected macaques for distinct sections from the same intestinal compartment and is characteristic for this organ [17, 29].

In the brain, where viral RNA could not be evidenced by RT-PCR during the acute phase, RNA was detectable in the chronic phase, but only in one of the two AGMs, and the level was moderate ($10^3$ RNA copies/10^6 cells).

Viral DNA could be detected in peripheral secondary lymphoid organs in five of seven cases (Fig. 5B) and ranged between $3 \times 10^3$ and $1.2 \times 10^2/10^6$ cells. In the remaining two cases (axillary LN of AGM 96011 and tonsils of AGM 96023) the levels were below the detection limit (<10 DNA copies/10^6 cells). The proviral levels in peripheral LNs correspond to those previously reported in naturally and experimentally infected AGMs, ranging between >2 and $3.3 \times 10^3$ DNA copies/10^6 cells [4, 6, 21, 24]. In contrast to peripheral secondary organs, viral DNA could be consistently detected in all gut-associated tissues analyzed (Fig. 5B). SIVagm DNA was undetectable in the brain of AGM 96011 and the thymus of AGM 96023, consistent with undetectable viral RNA (Fig. 5).

Altogether, the determination of RNA copy numbers indicates a continuous replication in secondary lymphoid organs and in the small intestine during the chronic phase of infection. Those fragments showing the highest SIVagm RNA levels were most often, albeit not always, associated with the small intestine. The quantification of viral DNA levels confirms the existence of a viral reservoir in the small intestine.

**Discussion**

In pathogenic HIV and SIVmac infections, early host–virus interactions are predictive of the outcome of the infection [43, 67]. Studying the nature of those interactions in a non-pathogenic model of infection, such as SIVagm infection of AGMs, helps in understanding lentiviral pathogenesis. SIVagm infection in AGMs is asymptomatic despite extensive viremia [6, 21, 24, 32] and it seems of fundamental importance to understand this discordant profile, which is reminiscent of patients showing discordant patterns in their virological and immunological responses to HAART [54, 66]. In order to better understand this paradoxical phenomenon, it is crucial to gain insights into the tissue targets of the virus during infections with non-pathogenic outcome. Previous studies have not addressed specific patterns of tissue replication of SIV in natural host species during primary infection. Here, we developed a study to identify the replication sites and tissue reservoirs of SIVagm in vivo by analyzing RNA and DNA levels in distinct lymphoid and non-lymphoid compartments in acutely infected AGMs. We studied viral replication in specific tissues during the logarithmic phase of viremia in primary infection. We detected a wide tissue distribution of SIVagm RNA at days 8 and 9 p.i., mainly in lymphoid tissues, but also in some non-lymphoid organs such as kidney and liver. However, the number of virus producing cells in peripheral and mesenteric LNs was 10 times higher than in the liver, as measured by in situ hybridization. Furthermore, wide distributions of virus in non-lymphoid organs have also been described for pathogenic HIV and SIVmac infections [8, 31, 39]. As for HIV/SIVmac, the consistent detection of SIVagm RNA in kidney and liver, but
not in skin or brain, is most likely a consequence of their high vascularization (liver) and their physiological functions (kidney, liver) [33, 36, 74]. The liver for instance is thus known as a major organ for virus clearance [74].

Among lymphoid tissues, only secondary lymphoid organs and gut appeared to be major sites for SIVagm replication. We also detected viral RNA and DNA during the primary infection in the lungs of both animals, but the RNA levels were below detection limit during the chronic phase. This might eventually be associated to an early migration of antigen-specific cells to the lung, as described in other models [58]. The thymus showed high viral RNA levels during primary infection, but viral DNA levels were low in both animals, and virus DNA and RNA were below the detection limit in the thymus during chronic infection in the one animal tested. It is not clear whether the high RNA/DNA ratios in thymus during primary infection represents high level of virus production. Such a discrepancy has been reported in SIV infected macaques where for one animal, the thymus was negative by PCR but positive at a low level by virus culture [2]. Our study does not exclude that the thymus is targeted during early SIVagm infection, but more animals should be studied and the tropism of SIVagm for thymocytes analyzed in order to better understand whether differences exist or not at the level of virus replication in the thymus between pathogenic and non-pathogenic infections.

Thus, high levels of both viral DNA and RNA in both acute and chronic phase of SIVagm infection were detected consistently only in secondary lymphoid organs and in gut-associated tissues. In some fragments of jejunum and ileum, the RNA viral loads were elevated (up to \(10^6\) copies/\(10^6\) cells) at 4 years p.i. However, the virus levels measured in the small intestine during chronic infection were highly variable. Such a high variability of viral load within the gut is in line with previous reports in human and macaques, describing marked inter-regional variations of viral burden as a characteristic feature of the gut [30]. This variable virus load profile might also explain the inconsistent detection of SIVagm replication in gut tissue sections from some naturally infected AGMs [24]. Further studies on viral load within the gut of chronically infected AGMs are warranted. We studied only two animals here. However, the high RNA viral load we observed in some gut-associated tissues in the chronic phase correlates with a study that reported 1–3 logs higher DNA copy levels in the gut in comparison with LNs and spleen in a naturally infected AGM [6]. Altogether, our data reveal that the gut is an early target for SIVagm and support its role as one major site of SIVagm replication during the chronic phase. Such a replication pattern could be explained by the fact that intestinal tissues can provide a particularly high number of target cells for the virus. Indeed, the frequency of activated CD4\(^+\) T cells is generally higher in the intestinal mucosa than in peripheral lymphoid organs in normal, uninfected primates [62, 72]. Furthermore, intestinal lymphocytes of non-human primates express the major coreceptors for SIVagm, i.e. CCR5 and Bonzo [20, 38]. Finally, the intestinal mucosa corresponds to the largest immunological organ of the body and contains more than 60% of the body lymphocytes [5]. Consistent viral replication in the gastrointestinal tract can therefore result in a production of high numbers of virus particles and significantly contribute to high viremia in SIVagm infection.

Although these data show that SIVagm is replicating preferentially in gut and secondary lymphoid organs, as do pathogenic HIV/SIVmac [8, 31, 39, 57, 71], there was a lack of evidence for virus trapping at day 11 p.i. in germinal centers of the LNs from AGM 00008. This contrasts with trapping of viral particles described during the second week of SIVmac infection [10]. We cannot exclude virus trapping at later stages of SIVagm infection, since we studied only the time point around the peak of viremia and only one animal was analyzed. The lack of viral trapping at day 11 p.i. correlates, however, with data reported for the chronic phase of SIVagm infection. Thus, chronic SIVagm infection is characterized by a lack of detectable signs of virus trapping in germinal centers of LNs [4, 24]. Lack of viral trapping in the chronic phase has also been reported for two other non-pathogenic SIV infections in African monkeys, i.e. SIVsm and SIVcpz infections in their respective hosts [37, 59]. A lack of capture of free virus by the follicular dendritic cells in the germinal centers could contribute, at least in part, to the high plasma viral load seen in AGMs. Together with a continuous replication in the large immune organ, which is the gut, this can explain the high viremia observed in SIVagm infected AGMs.

Altogether, our data suggest that during the acute phase of SIVagm infection, rapid systemic spreading of the virus allows high viral replication in many lymphoid organs. At that stage, viral load in the peripheral LNs is not significantly different from that in the gut. Thus, during primary infection, there seem to be enough target cells to support viral replication in both compartments. In
contrast to the acute phase, the viral load in LNs during the chronic infection was lower than in some gut-associated tissues, which is in agreement with published data on the lower viral load in LNs during chronic SIVagm infection in comparison with HIV/SIVmac infections [4, 6]. This lower viral load in LNs is probably not due to strong immune responses, since viremia remains high, and no strong CTL activity could be evidenced so far in SIVagm-infected AGMs [48]. Rather, the lower viral load in LNs in AGMs in comparison with that in HIV-1/SIVmac infections might be explained by a lower frequency of activated target cells in LNs during chronic infection. In this regard, data collected during SIV infection in natural host species indicate indeed a lack of chronic activation of lymphocytes in LNs [11, 21, 27, 49]. In contrast to LNs, the constantly elevated proportion of activated cells in the gut due to environmental antigens might offer sufficient target cells at all stages of SIVagm infection, even in the absence of virus induced activation of immune cells. Our studies thus have direct implications for the understanding of HIV immunopathogenesis. SIVagm, while targeting similar tissues as HIV and SIVmac, does not induce AIDS. These data are in support of other studies showing that high levels of CD4+ T cell tropic lentiviruses per se is not lethal to the host [35, 60, 65]. Together with data showing that SIVagm can be highly cytopathic in vitro [6, 47], our studies are strongly in favor of indirect mechanisms, in particular HIV- and SIVmac-induced chronic immune activation, as a driving force for pathogenesis [26]. Further studies are needed, however, on the consequences of SIVagm infection on host cells, in particular on CD4+ cell dynamics and activation profiles of LN and intestinal T lymphocytes in response to SIVagm infection. Such studies represent a key element for deciphering the mechanisms leading to CD4+ cell depletion in tissues, such as the intestine, in SIVmac infected macaques and HIV infected humans.

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