Dissemination of SIV after Rectal Infection Preferentially Involves Paracolic Germinal Centers

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Homosexual transmission remains a major mode of contamination in developed countries. Early virological and immunological events in lymphoid tissues are known to be important for the outcome of HIV infections. Little data are available, however, on viral dissemination during primary rectal infection. We therefore studied this aspect of rectal infection in rhesus macaques inoculated with the biological isolate SIVmac251. We show that infection is established initially in lymph nodes draining the rectum. Infected cells and virions are localized mainly in germinal centers at that stage. With increasing viral burden, infected cells are found throughout the lymph node parenchyma. In addition, the difference in viral load between lymph nodes draining the rectum and other lymph nodes is attenuated or abolished. We discuss this pattern of viral dissemination with respect to the physiology of the mucosal immune system. The pattern and kinetics of viral dissemination after rectal infection have important implications for the development of efficient mucosal vaccines. © 1999 Academic Press

INTRODUCTION

Despite the importance of mucosal transmission of HIV (Holmberg et al., 1989; Royce et al., 1997), few studies have evaluated the early viral events after mucosal infection. A knowledge of these events could orient strategies in the development of mucosal vaccines. Such knowledge also would help our understanding of the pathogenesis of HIV infection. Indeed virologic and immunological events in lymphoid tissues are known to be important for the outcome of HIV infections. Little data are available, however, on viral dissemination during primary rectal infection. We therefore studied this aspect of rectal infection in rhesus macaques inoculated with the biological isolate SIVmac251.

The sequence of viral events in lymphoid tissues after intravenous infection has been studied in animal models such as macaques infected by SIV (Chakrabarti et al., 1994a,b; Lackner et al., 1994; Baskin et al., 1995; Sasseville et al., 1996) and cats infected by FIV (Bach et al., 1994; Beebe et al., 1994). Infected cells are present in lymph nodes (LN) as early as 4 days p.i. with uncloned SIVmac251. The overall density of infected cells in LN peaks between 7 and 14 days p.i. in sequential studies of individual animals (Chakrabarti et al., 1994a; Reimann et al., 1994). Sasseville and coworkers (1996) found, on the other hand, that the density of infected cells peaks in LN 3 weeks p.i. for two molecular clones of SIV. The variations in timing of the peak of viral replication in LN likely reflect animal-to-animal as well as strain-to-strain variations.

During the first 2 weeks of infection, infected cells can be present in the whole LN parenchyma (LN) (Chakrabarti et al., 1994a,b; Lackner et al., 1994; Baskin et al., 1995), or in extrafollicular areas only (Reimann et al., 1994). In all these studies, infected cells may be detected in germinal centers (GCs) as soon as these are identifiable in LN, 1–3 weeks p.i. (Chakrabarti et al., 1994a,b; Reimann et al., 1994; Baskin et al., 1995). Chakrabarti and coworkers (1994a,b) have shown a high density of infected cells in the paracortex 7–14 days p.i. The density of infected cells in GCs was too high to be quantitated in those studies. Other studies have shown that the paracortex is infected more heavily than the follicles 1–2 weeks p.i. whether with uncloned SIV or with pathogenic molecular clones (Lackner et al., 1994; Baskin et al., 1995; Sasseville et al., 1996). In addition, extracellular trapping of virions on follicular dendritic cells (FDC) has been observed as early as 2 weeks p.i. by some authors (Chakrabarti et al., 1994a,b; Baskin et al., 1995), but not as easily by others (Lackner et al., 1994; Reimann et al., 1994).

Few studies have addressed the issue of potential differences in viral replication between LN from different anatomic sites. At the AIDS stage mesenteric LN (including ileocaecal nodes) show a higher viral load than
peripheral LN (axillary and inguinal) (Scharko et al., 1996). This is not the case during primary intravenous infection. Indeed Chakrabarti et al. and Beebe et al. did not observe differences in density of infected cells between mesenteric and peripheral LN during primary SIVmac251 (Chakrabarti et al., 1994b) or FIV (Beebe et al., 1994) infection. Veazey et al. (1998) report very similar percentages of CD4⁺ T cells in peripheral and mesenteric LN during primary intravenous SIVmac239 and SIVmac251 infection. This again suggests that there is no major difference in viral replication between peripheral and mesenteric LN during primary intravenous infection.

Dissemination of a viral infection usually involves LN draining the point of entry of the virus (Tyler and Fields, 1996). The issue of regional confinement of viral replication after mucosal retroviral infection, however, seldom has been addressed. Prolonged confinement to mesenteric LN has been described in the newborn mouse after oral infection with MMTV (Karapetian et al., 1994). Infected cells do not diseminate beyond mesenteric LN for >2 weeks in that model. The localization of infected cells in LN was not addressed in that work. The natural targets of MMTV are B lymphocytes, and this confinement could reflect the migratory behavior of mucosal B cells. In contrast, dissemination to distal LN in <4 days has been described in the rhesus macaque after vaginal infection with SIVmac251 (Spira et al., 1996) or SHIV (Joag et al., 1997). Infected cells initially reach iliac (Spira et al., 1996) or pelvic (Joag et al., 1997) LN as early as Day 2 p.i. Infected cells localize to the paracortex of LN (Spira et al., 1996; Joag et al., 1997), with no infection of germinal centers for up to 9 days p.i. (Spira et al., 1996). The natural targets of SIV and SHIV are T-cells, dendritic cells, and macrophages. Spira et al. suggested that transport of SIV to the draining LN after vaginal inoculation was accomplished by dendritic cells of the lamina propria of the female genital tract. The rapid dissemination of SIV and SHIV observed after vaginal infection therefore could reflect the migratory behavior of these cells. Only one study has been published regarding viral dissemination after rectal infection of pigtail macaques (*Macaca nemestrina*) with a clone of SIVMne (Kuller et al., 1998). Potential quantitative differences in viral load between draining and nondraining LN have not been investigated. Distribution of infected cells within the LN are also not described in this article, which focuses on the mucosal humoral response. The authors nevertheless show that most lymphoid organs are infected in two animals 1 week p.i.

Rectal transmission of HIV remains a major mode of contamination in developed countries (European Center for the Epidemiological Monitoring of AIDS, 1996). There is evidence that it is different from parenteral transmission. Studies of macaques infected atraumatically by the rectal route with SIVmac251 show that establishment of infection requires a higher inoculum than infection by the parenteral route (Dormont and Le Grand, 1993; Pauza et al., 1993; Cranage et al., 1997). Other elements suggest that establishment of infection is different after intravenous and rectal inoculation. All animals die within 2 weeks after intravenous infection with the acutely lethal SIV isolate PBj14, whereas 50% survive after intrarectal inoculation (Schwiebert et al., 1997). Attenuation of the virus occurs, but only after several months of chronic infection (Schwiebert et al., 1997). Viremia can be transient after rectal infection (Pauza et al., 1993; Trivedi et al., 1996), and selective amplification of minor viral genotypes of the inoculum has been demonstrated (Trivedi et al., 1994, 1996). In humans, minor viral variants are selected within the viruses present in sperm during homosexual transmission of HIV (Zhu et al., 1996). In the same line of thought, different founder virus populations in homosexual men vs. intravenous drug users have been proposed (Lukashov and Goudsmit, 1997). Several studies report a more rapid progression to AIDS after homosexual vs. parenteral contamination (Biggar, 1990; Jason et al., 1996; Eskild et al., 1997; Pehrson et al., 1997). Whether there is a correlation between selection of viral variants during rectal transmission and progression to disease remains to be determined.

We decided to characterize viral dissemination in rhesus macaques inoculated rectally with the biological isolate SIVmac251. We targeted our study to the first 2 weeks of the primary infection. We studied 12 animals in that time frame to assess animal-to-animal variation. We show both quantitative and qualitative differences in viral load between draining and nondraining LN. We did not find such differences in four animals infected intravenously in the same time frame.

**RESULTS**

**Autopsy findings**

All macaques infected by the rectal route had markedly enlarged paracolic LN compared with uninfected animals or animals infected with a nonpathogenic clone of SIV (data not shown). Other LN were enlarged only in macaques sacrificed 14 days p.i. In contrast no difference in LN enlargement could be observed in macaques infected intravenously, with animals sacrificed early showing poor or no enlargement, and animals sacrificed late showing marked enlargement of all LN.

**Paracolic lymph nodes are the first LN to replicate the virus after rectal infection**

Infection could be demonstrated in all rectally inoculated animals by PCR amplification of provirus from
PBMC (Table 1). When present in LN, SIV always was found in paracolic LN. In animals R7.3, R7.4, and R14.2, this was the only LN positive. Negative samples were tested repeatedly to rule out the possibility of a very low level of infection. In the other animals, provirus also could be detected in axillary LN.

Productively infected cells were detected by in situ hybridization in eight animals (Fig. 2). Paracolic LN showed the highest viral load in all animals, but more strikingly so 7 and 10 days p.i. At early time points, axillary LN were either negative by in situ hybridization (ISH; macaques R7.3, R7.4, R10.1) or infected at a very low level (macaques R7.5, R10.2). Inguinal LN were negative in R7.3 and R10.1, and slightly positive in R7.4 and R10.2. Similarly, parajejunal LN could be negative (R7.3), or show a low (R7.4) or an intermediate (R10.1, R10.2) level of infection. At later time points (R13, R14.3, R14.4), the difference in overall viral load between paracolic and other LN was much less important than at early time points. Overall the density of infected cells increased between Days 7 and 14 p.i.

Infectious virus could be recovered by coculture in two of five macaques at Day 7 p.i. (R7.3, R7.5, Table 2), and in all animals tested at further time points except R14.1. In macaques R7.3 and R14.2, infectious virus was recovered in paracolic, but not in axillary LN. Three of the animals had more than a 10-fold difference in cellular viral load between paracolic and axillary LN. In macaques R7.5 and R10.1, the difference was 15-fold. In macaque R10.2, it was 17-fold (and 23-fold with inguinal LN). The other animals (R13, R14.3, R14.4) showed little or no difference in cellular viral load between paracolic and peripheral LN.

Paracolic germinal centers are heavily infected after rectal infection

In macaques sacrificed 7 days after rectal infection, there was a very striking concentration of infected cells in GCs (Figs. 2 and 3), which were present in all paracolic LN. Densities could reach 710/mm² in macaque R7.4 and 450/mm² in macaque R7.5 (Appendix Table 4). The density of infected cells was much lower in other areas of the LN. This explains the overall low density of infected cells of these LN. In parajejunal and inguinal LN of macaque R7.4, the cells were concentrated in the few GCs that were present, with cell densities reaching 250/mm². In axillary LN of macaque R7.5 (the only animal in which these LN were positive), there was no preferential localization of infected cells in GCs.

In macaques R10.1 and R10.2, two subsets of paracolic LN could be identified, one (COL1 in Fig. 2) being more heavily infected than the other (COL2 in Fig. 2). In animal R10.1, infected cells of COL1 were concentrated in GCs, reaching densities of 305 cells/mm². The density of infected cells varied considerably from one GC to the next within a given LN (data not shown). The average density in GCs varied also considerably from one LN to the next for a given location (Appendix Table 4). In axillary and inguinal LN, there was a slight concentration of infected cells in the GCs. The paracortex and medulla were significantly infected only in the paracolic LN.

Later in infection, GCs were less prominent in paracolic LN but were nevertheless heavily infected (Figs. 1 and 2, Appendix Table 4). They could reach densities of 510 (R13) 310 (R14.3), and 340 (R14.4) infected cells/mm² (Appendix Table 4). These densities are comparable with those observed at early time points. The increase in overall viral load of these LN as compared with early

### TABLE 1

<table>
<thead>
<tr>
<th>Macaque</th>
<th>R7.1</th>
<th>R7.2</th>
<th>R7.3</th>
<th>R7.4</th>
<th>R7.5</th>
<th>R10.1</th>
<th>R10.2</th>
<th>R13</th>
<th>R14.1</th>
<th>R14.2</th>
<th>R14.3</th>
<th>R14.4</th>
<th>V7.1</th>
<th>V7.2</th>
<th>V14.1</th>
<th>V14.2</th>
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<td>Rectal</td>
<td>Rectal</td>
<td>Rectal</td>
<td>Rectal</td>
<td>Rectal</td>
<td>Rectal</td>
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<td>Rectal</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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**Note.** Provirus was detected by nested PCR. IV, intravenous infection; PBMC, peripheral blood mononuclear cells; AXL, axillary lymph nodes; COL, paracolic lymph nodes; +, provirus detected; −, provirus not detected; ND, not done.
time points is attributable to the presence of infected cells at a high density outside GCs. In particular, the extrafollicular cortex, paracortex, and medulla were heavily infected, reaching a density of 470 (R14.3) and 280 (R14.4) infected cells/mm$^2$. In axillary LN, infected cells were localized to the extrafollicular cortex and paracortex (Fig. 1) with variable infection of the medulla and GCs.

Lymph nodes of all sites are equally infected after intravenous infection

Provirus was detected in all lymphoid tissues tested of intravenously infected animals (Table 1). Overall the density of infected cells in LN was lower than in rectally infected animals. V7.1 was negative by ISH. V7.2 was very weakly positive, as 0–15 positive cells per LN section were observed (data not shown). They were found in lymphoid follicles (axillary, paracolic, and parajejunal LN) or in the extrafollicular cortex and paracortex (paracolic, parajejunal, and inguinal LN). Infected cells were found throughout the LN parenchyma of animals sacrificed 14 days p.i. (Figs. 1 and 2). The average density of infected cells was equivalent in LN from different anatomic sites, except for one parajejunal LN in V14.1 and one paracolic LN in V14.2 (Appendix, Table 3). GCs were found in some of these LN. When they were infected, the density of infected cells was higher than that in the surrounding parenchyma (Figs. 1 and 2). Infectious virus was not recovered from animal V7.1, but was recovered in all lymphoid tissues analyzed from V7.2, V14.1, and V14.2. In V14.2, paracolic LN were more infected than axillary LN, but the difference was under sixfold. Cellular viral load was not available for axillary LN in V7.2, but the difference between inguinal and paracolic LN was only threefold in that animal. No significant difference in cellular viral load was otherwise observed between mesenteric and peripheral LN (Table 2).

**Cellular targets of SIV in LNs**

The majority of infected cells were identified as T cells by combining immunohistochemistry and ISH on the same LN section. A small number of infected macrophages could be identified by performing immunohistochemistry and ISH on serial LN sections. Figure 4 shows the results of such an analysis for a paracolic LN of R10.2. Similar results were obtained in all LN and at all time points regardless of the route of infection (data not shown). Immunohistochemistry with the anti-p55 antibody (to identify dendritic cells) was unfortunately not possible in conjunction with ISH.

FDCs were seen to carry trapped virions (Figs. 1 and 3) in some, but not all, animals even when GCs were present (Table 3). This was observable as a reticulate pattern at low magnification. At high magnification, the label was seen to be associated with the surface of cells with a highly convoluted morphology. A similar pattern was observed when labeling frozen sections of LN with the DRC1 antibody, specific for FDCs (Fig. 5).

### TABLE 2

<table>
<thead>
<tr>
<th>Macaque</th>
<th>R7.1</th>
<th>R7.2</th>
<th>R7.3</th>
<th>R7.4</th>
<th>R7.5</th>
<th>R10.1</th>
<th>R10.2</th>
<th>R13</th>
<th>R14.1</th>
<th>R14.2</th>
<th>R14.3</th>
<th>R14.4</th>
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<th>V7.2</th>
<th>V14.1</th>
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**Note.** Cellular viral load is given in TCID$_{50}$/10$^6$ cells. ING, inguinal lymph nodes; JIE, parajejunal lymph nodes; na, not available.

### TABLE 3

<table>
<thead>
<tr>
<th>Macaque</th>
<th>R7.3</th>
<th>R7.4</th>
<th>R7.5</th>
<th>R10.1</th>
<th>R10.2</th>
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<th>R14.3</th>
<th>R14.4</th>
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<tr>
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<td>ND</td>
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</table>

**Note.** Virions on follicular dendritic cells were recognized as a positive reticulate network by in situ hybridization on GCs. Lymph nodes from different anatomic sites were scored as follows: +, reticulate network in GCs; −, no reticulate network observed; ND, not done. Note that all LN of a given site displayed GCs and that all GCs were not necessarily positive in a given anatomic site.
Unfortunately combining ISH and immunohistochemistry with the DRC1 antibody was not possible. In rectally infected animals, trapped virions were seen over FDCs as early as Day 7 p.i. in paracolic LN in macaques R7.3 and R7.4 (Figs. 3A and 3B) but not in macaque R7.5 (Figs. 3C and 3D). FDCs were positive for trapped virions in paracolic LN but not axillary LN in animals R10.2, R13, R14.3, and R14.4 (Figs. 1 and 3, Table 3). In macaques infected by the intravenous route, virions trapped on FDCs could be observed in both paracolic and axillary LN 14 days p.i. (Table 3, Figs. 1E and 1F).

**DISCUSSION**

Dissemination of SIV after rectal infection likely involves primarily paracolic LN but could occasionally involve inguinal LN as well. In humans, the upper two-thirds of the rectum are drained by pararectal LN and by the paracolic LN closest to the rectum (Rouvière, 1932). There is anastomosis of lymphatic vessels between these LN that varies from individual to individual (Rouvière, 1932). In macaques, the upper part of the rectum appears to be drained by paracolic LN (F. Rigoud, personal communication). We occasionally observed pararectal LN as well (e.g., animal R13). The lower part of the rectum most often drains by inguinal LN in humans (Rouvière, 1932) and probably in macaques as well.

To investigate dissemination of SIV, we harvested and studied four types of LN. Paracolic LN were considered to be directly draining the inoculation site, as inoculation was performed in the upper half of the rectum. Axillary LN were considered representative of peripheral LN. We expected inguinal LN to behave as peripheral LN if the inoculum stayed strictly in the upper half of the rectum and as draining LN if the inoculum redistributed to the lower part of the rectum. We also harvested parajejunal LN. These LN do not drain directly the rectum. They could be reached by SIV at the approximately same time as peripheral LN if dissemination occurred via cells with peripheral homing properties. However, they could be reached by SIV earlier than peripheral LN if dissemination initially occurred via cells of the entero-enteric cycle, with homing to the gut followed secondarily by draining to mesenteric LN.

**Regional confinement in LN after rectal infection**

Our results indicate that paracolic LN (the essential draining LN) are the first hit by SIV infection in rectally infected macaques. Virus appears to infect secondarily axillary LN (the typical nondraining LN). These may remain negative for ≤2 weeks. Infection of inguinal and parajejunal LN is more variable, as discussed below. We believe the dissemination process to be a continuous one, progressing at a varying speed from animal to animal. Five stages of LN infection can nevertheless be individualized (Fig. 6). Stage 1 is the detection of provirus in paracolic LN only. At stage 2 infected cells can be identified by ISH in paracolic LN. They localize highly preferentially to GCs. At further stages, infected cells are found by ISH throughout the parenchyma of paracolic LN. Preferential localization in GCs is not always obvious. Infection also reaches axillary LN. Stage 3 corresponds to detection of infected cells only with no other sign of infection in these LN. Stage 4 corresponds to detection of infected cells by ISH in the extrafollicular cortex and paracortex but not in other areas of these LN. Stage 5 corresponds to general dissemination of the infection. Four animals (R7.1, R7.2, R14.1, R14.2) had a very low viral load. Slow viral replication after rectal infection has been previously reported (Pauza et al., 1993). Animal R14.2 was positive by PCR in paracolic LN as well as in PBMC. Infectious virus could also be recovered by coculture from the paracolic LN but not from the PBMC. This animal was in stage 1. In R7.1, R7.2, and R14.1, infection could be evidenced only by PCR in PBMC. We suspect viral replication to have been initiated in minute LN draining the rectum. We have occasionally observed such LN in rhesus macaques. In addition to being difficult to sample, these LN are too small to allow analysis.

We therefore believe that these animals were also in stage 1. We, however, cannot exclude that PBMC are the only site of infection in these animals. These infected LN were considered representative of peripheral LN. We occasionally observed pararectal LN as well (e.g., animal R13). The lower part of the rectum most often drains by inguinal LN in humans (Rouvière, 1932) and probably in macaques as well.

**FIG. 1.** Localization of SIV in paracolic and axillary LN two weeks after inoculation. SIV RNA was detected by ISH with a nef probe. (A) Prominent germinal center in paracolic lymph node of macaque R14.3; magnification, ×260. (B) Higher magnification shows virions trapped on FDC and an infected cell; magnification, ×625. (C) Low paracolic node in macaque R13. Note numerous infected cells and infected germinal centers; magnification, ×130. (D) Axillary node in the same animal. The density of infected cells is lower; magnification, ×130. (E) Paracolic node in macaque V14.2. Note infected germinal centers and infected cells in the parenchyma; magnification, ×130. (F) Axillary node in the same animal. The distribution of SIV is very similar; magnification, ×130. Arrowheads point to infected cells and arrows to virions trapped on the surface of FDC in all figures. GC, germinal center; FM, follicular mantle; F, follicle. (A) and (B) are counterstained with eosin.

**FIG. 3.** Localization of SIV in paracolic LN 7 days after rectal inoculation. SIV RNA was detected by ISH with a nef probe. (A) Both infected cells and virions trapped in the FDC network are observed in macaque R7.4. Occasional cells are visible in the parenchyma; magnification, ×260. (B) At high magnification, the reticular pattern on the surface of FDCs is clearly visible, as well as infected cells; magnification, ×625. (C and D) Cortical area of two paracolic nodes from macaque R7.5. Note high density of infected cells in GC, lack of virions trapped on FDC. Several infected cells are observed in the follicular mantle in (C) but few in (D); magnification, ×260. Arrowheads point to infected cells and arrows to virions trapped on the surface of FDC in all figures. GC, germinal center; FM, follicular mantle. (A) and (B) are not counterstained. (C) and (D) are counterstained with eosin.
cells could be memory mucosal cells. Such cells are likely to patrol the organism continuously and therefore be present in the peripheral blood. In any case, infected cells in the PBMC of these animals have very poor replicative capacity.

Stage 2 was observed only 7 days p.i. In animal R7.3,
paracolic LN was the only site infected. This is probably due to the fact that the virus is inoculated in the upper half of the rectum. In animal R7.4, inguinal and parajejunal LN were also positive, albeit at a lower level. Infection of inguinal LN could be due to redistribution of the inoculum to the lower part of the rectum during experimental infection. One explanation of the infection of parajejunal LN at that stage is the entero-enteric cycle. Infected cells escaping from paracolic LN would home to the small intestine. They would then either migrate to parajejunal LN or transmit infection to cells migrating to these LN.

Stage 3 was observed only in animal R10.1. In that animal, infected cells were observed in parajejunal LN but not in inguinal LN. This is likely due to an inoculation strictly limited to the upper half of the rectum. Infection of parajejunal LN is likely due to recirculation of infected mucosal cells by the entero-enteric cycle as described above. Two subsets could be identified in paracolic LN. Subset 1 was heavily infected and could correspond to LNs directly draining the rectum in that animal. The level of infection of subset 2 was similar to that of parajejunal LN. This could be due either to anastomosis of subset 2 with subset 1 or to infection by the entero-enteric cycle as described for parajejunal LN. The detection of provirus in axillary LN at that stage suggests that infection has now progressed to cells with peripheral homing properties.

Stage 4 was observed at 7 (R7.5) and 10 (R10.2) days p.i. The level of infection of axillary LN (and inguinal LN in R10.2) remains much lower than that of paracolic LN. The pattern of infection showed by R10.2 is very reminiscent of that of R10.1 (e.g., two subsets of paracolic LN), suggesting similar mode of dissemination of the virus in these two animals. Overall stages 3 and 4 differ only by the level of infection of axillary LN (and of inguinal LN, which behave in these animals as peripheral LN).

Stage 5 was observed only in animals sacrificed 2 weeks p.i. A higher viral burden in paracolic vs. axillary LN is still observed to variable degrees. This is most pronounced in animal R14.4. In this animal, the lack of difference in viral load between axillary, inguinal and parajejunal LN suggests direct progression from a situation similar to that of R7.3.

Our results in two animals in stages 1 and 2 show initial confinement of SIV in LN directly draining the rectum. This confinement can still be observed in some cases 14 days p.i. Even when there was no strict confinement, there is a strong gradient in viral load between paracolic and axillary LN, indicating initial confinement. The data presented by Kuller et al. (1998) suggest that their animals are likely at stage 5 1 week p.i., even if no quantitative data are available in their study. The differences in kinetics could reflect species or strain differences. It would be very interesting to compare data at earlier time points in the model of Kuller et al. (1998), with our own results.

The regional confinement of infected cells that we observe is more reminiscent of the data of Karapetian et al. (1994) than of those of Spira et al. (1996) and Joag et al. (1997). SIV could be transported by dendritic cells from the rectum to the paracolic LN. Dendritic cells have been described in the digestive tract. Two subsets of dendritic cells have been described in mouse Peyer's patches (Ruedel and Hubele, 1997). In the colon, lamina propria (Liu and MacPherson, 1995; Pavli et al., 1996) as well as intraepithelial (Maric et al., 1996) dendritic cells have been described. The differences in kinetics of dissemination of SIVmac251 after vaginal infection and rectal infection suggest that if SIV is indeed transported by rectal DC, their migratory behavior is different from that of dendritic cells of the female genital tract. Transport of SIV from the rectum to paracolic LN could also be accomplished by infected T lymphocytes, which we could identify in the LN. The migratory behavior of T cells is likely to be close to that of B lymphocytes. This would explain the similarity between our results and those of Karapetian et al. (1994). Last SIV could also gain access to the draining LN as free virions. These transport routes are discussed below.

No preferential site of viral replication after intravenous infection

Our animals infected intravenously showed homogeneous patterns of dissemination at each time point. One week p.i., the level of viral replication was very low, with <10 infected cells per LN section. Two weeks p.i., viral replication was more active. The overall level of infection did not reach that of rectally infected animals. Overall the results suggest direct progression from the Day 7 situation to the Day 14 situation. We did not observe preferential viral replication in LN from any site at any time point. This is in agreement with what Chakrabarti and coworkers reported during primary intravenous SIVmac251 infection (Chakrabarti et al., 1994b). Preferential viral replication in mesenteric LN has only been reported at the AIDS stage by Scharko and coworkers (Scharko et al., 1996). Opportunistic infections of the digestive tract are frequent in end-stage disease, and Scharko and coworkers report that mesenteric LN are the most activated at that stage. This likely explains the increased susceptibility to viral replication of mesenteric LN at the AIDS stage. Our data and that of Chakrabarti and coworkers in primary intravenous infection indicate that intrinsic increased susceptibility of mesenteric LN to viral replication does not account for our observation in rectally infected animals during primary infection.
A striking feature of draining LN after rectal infection was the very high density of infected cells in GCs. In animal R7.3, which we believe to be at a very early stage of viral dissemination, infected cells were detected by ISH in only one LN. The density of infected cells in GCs of that LN was not very high but higher than that of the rest of the LN parenchyma. In animals R7.4 and R7.5, the density of infected cells in GCs was, respectively, 28- and 145-fold higher than in the rest of the LN. GCs were therefore the only area of the LN with a significant level of infection. We did not find a similar situation after intravenous infection. The density of infected cells was enriched only seven- and fivefold in GCs of V14.1 and V14.2, respectively. Chakrabarti and coworkers (Chakrabarti et al., 1994b) show that infected cells can be found in GCs as early as 7 days post intravenous infection. In that case, however, infected cells can also be found throughout the LN parenchyma. In contrast, GCs are not infected during primary vaginal infection (Spira et al., 1996; Joag et al., 1997). Starting 10 days post rectal infection, the density of infected cells in other parts of the LN becomes significant (paracolic LN of R10.1 and R10.2 and all LN of R13, R14.3, and R14.4).

Overall the data suggest that, after rectal infection, infected cells accumulate preferentially in GCs early in infection. Then infected cells redistribute to the whole LN parenchyma with the duration of infection. This chronology of events appears different from that in intravenously infected animals. All kinetic studies show that infected cells are first present throughout the LN parenchyma, then appear in GCs (Chakrabarti et al., 1994b; Lackner et al., 1994; Reimann et al., 1994; Sasseville et al., 1996). Our own intravenously infected animals show similar kinetics, albeit with a lower overall level of infection in LN.

Preferential localization of infected cells to GCs has been reported for animals infected with nonpathogenic viruses (Lackner et al., 1994; Chakrabarti et al., 1995). Attenuated variants of the uncloned pathogenic isolate SIVmac251 we used for infection could have been selected by the rectal mucosa. This could explain preferential localization of infected cells to GCs in draining LN of our rectally infected animals. However, the density of infected cells in GCs in our animals is too high to be due to replication of an attenuated variant. The density of infected cells in GCs is indeed very low in animals infected with nonpathogenic viruses (1–5 positive cells per section for 1A11 (Lackner et al., 1994)).

Preferential transport of SIV to lymphoid follicles could account for the preferential infection of GCs after rectal inoculation. SIV could be transported from the rectal mucosa to the draining LN as free virions, as cell-associated virions, or as infected cells. Free viral particles could be drained by the lymphatics directly from the rectal mucosa. FDCs can extend processes in the subcapsular sinuses in some circumstances (Tenner-Racz et al., 1988) and could therefore directly capture the free virions. LN colonization by virions would not be observed after IV infection, as free virions cannot exit from blood capillaries. Chakrabarti and coworkers have indeed concluded that the colonization of LN occurred by infected

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**FIG. 6.** Stages of SIV dissemination to LN. Five stages in SIV dissemination to LN can be individualized (see text for details). The inner oval symbolizes the medulla, the outer stripe, the extrafollicular cortex, and paracortex. Lymphoid follicles are indicated as large circles within the outer stripe. Infected cells are represented by two concentric circles. PCR indicates LN positive only by PCR. Localization of infected cells in such LN is unknown. Animals belonging to each stage are indicated on the figure.

**FIG. 4.** Infected cells in LN are mostly T cells. Infected cells in GC of a paracolic LN of macaque R10.2 are identified as T cells (arrows) by yellow ring in combined ISH and CD3 immunohistochemistry (A), and as a macrophage (arrowhead) by ISH (B) and CD68 immunohistochemistry (C) performed on serial sections. Red fluorescence (A and B) is due to the HNPP/fast red precipitate obtained after ISH for nef. Green fluorescence (A and C) is due to fluorescein-coupled secondary antibodies. A–C are serial sections. Final magnification ×260.

**FIG. 5.** Follicular dendritic cell network. Follicular dendritic cells were labeled with the DRC1 antibody (A). Sections were counterstained with Hoescht's stain (B). Note the heavy density of follicular dendritic cells in the light zone of the germinal center (arrowheads), and the looser network of the dark zone (arrows). Final magnification, ×260.
cells incoming via afferent lymphatics. Transport of virions from the rectal mucosa could of course be accomplished by dendritic cells. This is the currently accepted hypothesis for vaginal transmission. This, however, would mean that rectal dendritic cells migrate to lymphoid follicles in draining LN rather than to T areas as would dendritic cells of the female genital tract. Dendritic cells can indeed be found in human GCs (Grouard et al., 1996). Last, infected rectal T lymphocytes could have homing potential for lymphoid follicles in draining LN.

Preferential replication in GCs could also account for preferential infection of GCs after rectal inoculation. A strong mucosal B-cell response would lead to the rapid formation of GCs in mesenteric LN. GC CD4+ T cells are known to be preferential targets for HIV replication in vivo when compared with CD4- T cells from other areas of LN (Hufert et al., 1997). Optimal conditions would therefore be present for preferential replication of virus within these GCs (Heath et al., 1995; Burton et al., 1997; Rosenberg et al., 1997). The propagation of the infection to other LN areas would be associated with generalized activation of the LN. Kuller et al. have observed in pig tail macaques low levels of SIV-specific IgA in rectal secretions as early as 1 week p.i. (Kuller et al., 1998). This agrees well with a rapid B-cell response after rectal inoculation. However, the level of rectal SIV-specific IgA becomes significant only 12 weeks p.i. The initial magnitude of the B-cell response in draining LN remains therefore to be determined. It should be noted that in our hands the relative surface area occupied by GCs in follicles was not very different between paracolic and axillary LN in rectally infected animals (data not shown). Activation of GCs per se does not therefore seem to be sufficient to induce accumulation of infected cells in GCs.

FDC trapping of virions

Last we observed virions trapped on FDCs in most animals infected rectally, but not all (R7.5, was negative for example). This trapping was observed in paracolic but not axillary LN, consistent with observations on the density of infected cells at these sites. In intravenously infected animals, FDC trapping of virions, when present, was observe at all sites. The association of HIV with FDCs has been recognized as a hallmark of infection very early in the epidemics (Armstrong and Horne, 1984; Biberfield et al., 1986; Tenner-Racz et al., 1989) and is most prevalent in the asymptomatic phase of infection (for review, see Embretson et al., 1993; Pantaleo et al., 1993, 1994; Haase et al., 1996). Virions trapped on FDCs are a prominent feature in macaques in the asymptomatic phase (Ringler et al., 1989; Wyand et al., 1989), and can be observed as early as 2 weeks p.i. (Chakrabarti et al., 1994b; Baskin et al., 1995). Trapping is usually thought to occur through opsonization of HIV by antibodies, followed by complement (Pantaleo et al., 1994). In our experiments, trapped virions 14 days p.i. are likely due to the presence of antibodies at an early stage of the immune response. Trapped virions 7 days p.i. could indicate direct interaction of SIV with complement or complement receptors on FDCs, which has been reported for HIV (for review, see Stoiber et al., 1997). However, Kuller et al. detect SIV-specific antibodies in rectally infected pig tail macaques as early as 7 days p.i. (Kuller et al., 1998). Their animals show more rapid kinetics in viral dissemination and could also have more rapid kinetics in the development of immune responses than rhesus macaques. It is nevertheless possible that FDC trapping of virions in our animals indicates early induction of an antibody response in LN draining the rectum. This would agree with the early presence of GCs in these LN. Further studies will be needed to address this point.

Perspectives

The compartmentalization that we observe between LN and within draining LN after rectal infection has important implications for pathogenesis. The delayed delivery of infected cells to peripheral LN may for example delay the induction of systemic immunity, even if only by a very short time. Within LN, GC CD4+ T cells are an important site for HIV replication (Hufert et al., 1997). In addition, virions trapped on FDCs remain infectious for very long periods of time (Heath et al., 1995; Burton et al., 1997). These phenomena (and others) could be factors in the more rapid evolution of homosexually vs. parenterally acquired HIV infection (Biggar, 1990; Jason et al., 1996; Eskild et al., 1997; Pehrson et al., 1997). Our results also provide a clue to the efficiency of locally targeted LN immunization for protection from rectal infection (Lehner et al., 1996). They underscore the necessity to take in account the route of entry for vaccine development.

MATERIALS AND METHODS

Animals

Rhesus macaques (Macaca mulatta, Chinese subspecies) were housed at the P3 macaque facility of the Pasteur Institute in accordance with European Economic Community guidelines. All manipulations of animals were performed under ketamine anesthesia.

Sixteen macaques were infected experimentally with SIVmac251. The viral stock, originally obtained from R. Desrosiers, was a generous gift of A.-M. Aubertin (Strasbourg, France). This stock established by a single passage on macaque PBMC has been titrated in vivo either by the intravenous (IV) or by the rectal route. One AID50 (i.e., the dose infecting 50% of animals) has been deter-
mined to be equivalent to 1 TCID50 by the IV route (A.-M. Aubertin and R. Le Grand, personal communication) and 200 times higher by the rectal route (Dormont and Le Grand, 1993). Infection was performed with 10 AID50, either intravenously (10 TCID50, macaques V7.1, V7.2, V14.1 and V14.2) or rectally (200 TCID50, macaques R7.4, R7.5, R10.2, R13, R14.3, and R14.2).

Animals were sacrificed 7 days (macaques V7.1, V7.2, R7.4, and R7.5), 10 days (macaque R10.2), 13 days (macaque R13), or 14 days (macaques V14.1, V14.2, R14.3, and R14.2) p.i. by a lethal dose of pentothal. Peripheral blood was collected on heparin prior to sacrifice. Axillary LN and LN draining the sigmoid colon (referred to as paracolic LN) were collected separately at autopsy in all animals and either processed for paraffin embedding or kept under sterile conditions in Hanks’ balanced salt solution (Gibco, Cergy-Pontoise, France). In some animals, inguinal LN and LN draining the jejunum (referred to as parajejunal LN) were collected and processed as above.

**Lymphocyte preparations**

PBMC were obtained by centrifugation of heparinized blood on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) gradient. For each type of LN, several LN were pooled and lymphocytes were obtained by mechanical disruption on sterile nylon mesh and centrifugation on Ficoll–Hypaque.

**Titration of cell-associated virus**

Indicator cells (CEMx174) were cocultured in triplicate with serial 1:3 dilutions of macaque PBMC (starting from 300,000 per well). The presence of p27 gag antigen in supernatants was evaluated after 14 days by an antigen-capture assay (Coulter, Margency, France). Viral titers were then calculated according to the method of Reed and Muench (1938).

**Polymerase chain reaction (PCR)**

DNA was prepared from 5.10⁶ cells of each sample. Extraction was performed with phenol/chloroform after proteinase K treatment. The amount of DNA in each sample was normalized with respect to actin using sense (5’ GGG TCA GAA GGA TTC CTA TG 3’) and antisense (5’ GGT CTC AAA CAT GAT CTG GG 3’) actin primers (Genset, Paris, France).

Integrated SIV was detected by nested PCR performed on duplicates samples of 1 μg of DNA (corresponding roughly to 150,000 cells). The first SIV gag-specific PCR reaction was performed to amplify a 744-bp fragment with the primers EVA8003.1/1368N (5’ GAA ACT ATG CCA AAA ACA AGT 3’) and EVA8003.2/2129C (5’ TAA TCT AGC CTT CTG TCC TGG 3’) as previously described (Le Grand et al., 1992). The nested PCR was then performed on 1/50 of the first PCR products with the primers EVA8003.2/2129C and SPRG1731N (5’ CCG TCA GGA TCA GAT ATT GCA 3’) generating a 399-bp fragment. The final PCR products were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The SIV primers were obtained through the EVA (European Vaccine against AIDS) program. The experiment was repeated at least twice for each sample analyzed. To rule out the presence of PCR inhibitors in the sample, negative samples were reanalyzed by nested PCR over a range of 10 pg to 5 μg of DNA. The detection limit of the reaction was estimated to be 10 copies of SIV DNA by serial dilution of a pUC plasmid containing the entire gag gene.

In some cases, a nested PCR was performed using env-specific and pol-specific primers as described by Wakrim and coworkers (1996).

**In situ hybridization (ISH)**

ISH was performed with antisense RNA digoxigenin probes obtained by *in vitro* transcription (Riboprobe *in vitro* Transcription Systems, Promega, Madison, WI) of the entire SIV * nef* gene cloned into pBSK (Stratagene, Cambridge, UK). Tissues were fixed in 4% buffered Formalin and embedded in paraffin. Five-micrometer sections were first permeabilized with pepsin and then hybridized overnight at 70°C with antisense or sense probes (2 μg/ml). After hybridization, several washes with increasing stringency were performed and the hybridized probe was detected with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer, Mannheim, Germany), followed by NBT–BCIP (Gibco). Negative controls included noninfected tissues hybridized with the antisense riboprobe. Positive controls included tissue samples previously shown to contain SIV-infected cells.

Productively infected cells were identified as small dark spots at low magnification. At high magnification, the dark blue precipitate was visible throughout the cytoplasm and most often also filled the nucleus. For quantitation, entire LN sections were reconstituted in a montage of low magnification micrographs. Surface was estimated by overlaying the montage with a square grid. The spacing of the grid used to measure GC and follicular mantle was five times finer than that used for other areas of the LN. Cells were manually counted over the entire section. For a given animal, quantitation was always performed on sections from different lymph nodes subjected to ISH in the same experiment. Average values for LN for each were used for Table 3 and Fig. 2. The detailed quantitative data are presented for individual LN in the Appendix (Tables 4 and 5).
Combined *in situ* hybridization and immunohistochemistry

*In situ* hybridization was performed as described above up to the antibody step. Paraffin sections were then incubated with polyclonal rabbit anti-human CD3 antibody (Dako, Trappes, France) and alkaline phosphatase conjugated antidigoxigenin antibody for 45 min at room temperature. After several washes in PBS, a second incubation of 45 min with antibodies was performed using FITC-conjugated goat anti-rabbit and alkaline phosphatase-conjugated sheep antidigoxigenin antibodies. Alkaline phosphatase was detected using HNPP-Fast red (Boehringer) as substrate. Slides were counterstained with Hoescht's stain and mounted in Fluoromount medium.

**Immunohistochemistry**

For CD68 immunohistochemistry, tissues were fixed in 4% buffered Formalin and embedded in paraffin. Five-micrometer sections were deparaffinized, rehydrated, and then microwave treated in 0.01 M citric acid, pH 6 buffer. Sections were then incubated with monoclonal anti-human CD68 antibody (Dako) or anti-p55 antibody (obtained through the NIAID reference reagent program) for 45 min at room temperature. After several washes in PBS, a second incubation of 45 min was performed using FITC-conjugated goat anti-mouse antibodies. Slides were counterstained with Hoescht's stain and mounted in Fluoromount medium.

For DRC1 immunohistochemistry, tissues were snap-frozen in OCT compound by immersion in liquid nitrogen cooled isopentane. Ten-micrometer cryostat sections were obtained and fixed in acetone at room temperature after air drying. Sections were then incubated with DRC1 monoclonal antibody (Dako) for 60 min at room temperature. After several washes in PBS, a second incubation of 60 min was performed using FITC-conjugated goat anti-mouse. Slides were counterstained with Hoescht's stain and mounted in Fluoromount medium.

**APPENDIX**

**TABLE 4**

Quantitation of Infected Cells in Lymph Nodes in Intravenously-Infected Animals

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Location of LN</th>
<th>Surface of GC (% of follicle)</th>
<th>GC</th>
<th>Follicle outside of GC</th>
<th>Extral follicular cortex + paracortex</th>
<th>Medulla</th>
<th>Whole LN</th>
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<td>&lt;1</td>
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*Note.* Infected cells were identified by *in situ* hybridization and counted for each site. Surface areas were estimated by overlaying the micrographs with a grid. ND, not determined, NA not applicable (this structure was not present on the section).
### TABLE 5
Quantitation of Infected Cells in Lymph Nodes in Rectally-Infected Animals

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ACKNOWLEDGMENTS

We thank A-M. Aubertin for producing the stock of SIVmac251, O. Duquenne for technical advice for the in situ hybridization procedure, S. Delignat, M. Dupuis, N. Barget, and P. Villefroy for excellent technical assistance during some of these experiments, the EVA program management for gift of the SIV-specific primers, R. Le Grand for help and advice with the PCR and cellular viral load analysis, and the staff of the P3 macaque facility of the Pasteur Institute for assistance with animal work.

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<td>11</td>
<td>22</td>
</tr>
</tbody>
</table>

Note. Infected cells were identified by in situ hybridization and counted for each site. Surface areas were estimated by overlaying the micrographs with a grid. ND not determined, NA not applicable (this structure was not present on the section).
cency virus infection in lymph nodes. J. Virol. 68, 6634–6642.


Lackner, A., Vogel, P., Ramos, R., Kluge, J., and Marthas, M. (1994). Early events in tissues during infection with pathogenic (SIVmac239) and nonpathogenic (SIVmac1A11) molecular clones of simian immuno


