Alterations in RANTES Gene Expression and T-Cell Prevalence in Intestinal Mucosa during Pathogenic or Nonpathogenic Simian Immunodeficiency Virus Infection

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RANTES, a β-chemokine, can suppress human immunodeficiency virus (HIV) as well as simian immunodeficiency virus (SIV) infections in T-lymphocyte cultures *in vitro.* However, the association of RANTES levels in peripheral blood with viral loads and disease outcome in HIV infection has been inconclusive. SIV-infected rhesus macaques were evaluated to determine whether RANTES gene expression correlated with suppression of viral infection in intestinal lymphoid tissues. Intestinal tissues were obtained from rhesus macaques infected with either pathogenic or nonpathogenic SIVmac variants at various stages of infection (primary acute, asymptomatic, and terminal). We examined the level of SIV infection (*in situ* hybridization), RANTES expression (quantitative competitive RT-PCR), and T-cell counts (immunohistochemistry). The most pronounced increase in RANTES gene expression in intestinal tissues was observed in primary SIV infection, which correlated with the pathogenicity of the infecting virus and not the tissue viral loads. Our results demonstrated that in contrast to the occurrence of viral suppression by RANTES *in vitro,* there was no direct correlation between high RANTES gene expression and suppression of viral loads in intestinal lymphoid tissues. Thus RANTES expression in the gut lymphoid tissue may not be a correlate for viral suppression. However, RANTES gene expression in primary SIV infection may be part of early host immune response to viral infection. © 1999 Academic Press

INTRODUCTION

The variable disease course characteristic of human immunodeficiency virus (HIV) infection can be attributed to a combination of both viral and host factors (Fauci, 1996). The β-chemokines, namely regulated-upon-activation normal T-cell-expressed and -secreted (RANTES), macrophage inflammatory protein-1α and -β (MIP-1α and MIP-1β) were shown to suppress human immunodeficiency virus (HIV-1 and -2) and simian immunodeficiency virus (SIV) infections in CD4$^+$ T lymphocyte cultures *in vitro* (Castro *et al.,* 1991; Cocchi *et al.,* 1995, 1996; Paliard *et al.,* 1996). β-chemokines are known to induce selective recruitment, migration, and activation of specific subsets of leukocytes to sites of inflammation induced by various pathogenic infections (Adams and Lloyd, 1997). Therefore, β-chemokines may be an important component in the induction of efficient cell-mediated and humoral immune responses to infections by modulation of cellular trafficking (Schall and Bacon, 1994). However, the suppression of HIV and SIV infections by these chemokines could be largely attributed to their ability to bind to chemokine receptors CCR4 and CCR5. Based on these observations, it was assumed that the chemokine levels may be one of the correlates of the long-term survival in HIV infection. HIV-1-infected non-

progressors had levels of RANTES, MIP-1α, and MIP-1β similar to those of uninfected controls, whereas other investigators found lower levels of circulating β-chemokines in HIV-1-infected patient populations whose viral loads ranged from high to low (Kakkanaiah *et al.,* 1998; Kinter *et al.,* 1998; Paxton *et al.,* 1996). *In vitro* cultures of T-cell clones derived from PBMC of long-term nonprogressors were shown to secret high levels of RANTES, MIP-1β, and MIP-1α (Scala *et al.,* 1997). Thus studies on chemokine levels in peripheral blood samples in HIV infection have not been conclusive.

Although most of the studies on chemokine expression in HIV infection have been performed in peripheral blood, blood represents only 2% of all the lymphocytes in the body. The gut-associated lymphoid tissue (GALT), being the largest lymphoid organ in the body, harbors more than 90% of the lymphocytes. Therefore it can be an important early target organ and a site for initial host–virus interactions as well as for persistent viral infection. The GALT provides a microenvironment conducive for active viral replication since it harbors a large proportion of activated lymphocytes and can be a significant viral reservoir. The substantial involvement of the intestinal mucosa in HIV infection is evidenced by high viral loads in intestinal tissues and a high frequency of gastrointestinal complications in HIV-infected patients as well as SIV-infected rhesus macaques (Heise *et al.,* 1991; Pantaleo *et al.,* 1991; Schneider, *et al.,* 1997). There is no information available on intestinal chemokine expres-

sion in early stages of HIV infection. Alterations in chemokine expression in peripheral blood may not adequately reflect changes in the intestinal mucosa following HIV infection. These compartments differ in the numbers and phenotypes of activated and differentiated T-cell subpopulations and thus necessitate examination of viral–host interactions in these compartments independently. Studies of this nature have been limited due to the difficulty in obtaining intestinal tissue samples in sufficient quantities in early stages of HIV infection.

The SIV-infected rhesus macaques provide a suitable animal model for studies on the early host–viral interactions and immunopathogenic studies on the mucosal lymphoid tissue. Availability of molecularly cloned, pathogenic SIVmac 239 and nonpathogenic SIVmac 1A11 variants, as well as the biologic isolate SIVmac 251, has facilitated these studies (Kestler *et al.,* 1990; Marthas *et al.,* 1989, 1990). Both SIVmac 251 and SIVmac 239 cause simian AIDS in rhesus macaques, whereas SIVmac1A11 does not cause any disease (Gardner *et al.,* 1996; Lohman *et al.,* 1994; Otsyula *et al.,* 1996; Van Rompay *et al.,* 1996).

We sought to examine whether RANTES gene expression correlated with viral suppression and CD3$^+$ T-cell prevalence in intestinal mucosa during the course of pathogenic or nonpathogenic SIV infection. We sought to examine RANTES expression, since RANTES alone is capable of suppressing HIV infection while the other members of the β-chemokine subfamily, MIP-1α and MIP-1β, exert HIV suppressor activity only in combination with RANTES (Cocchi *et al.,* 1996). We hypothesized that high RANTES gene expression in intestinal mucosa may correlate with low viral load and high CD3$^+$ T-cell counts in the intestine and the pathogenicity of the infecting virus. We tested this hypothesis by examining viral infection (*in situ* hybridization), RANTES gene expression (quantitative competitive RT-PCR), and prevalence of CD3$^+$ T lymphocytes (immunohistochemistry) in intestinal mucosa of rhesus macaques infected with either pathogenic SIVmac 239/SIVmac 251 or nonpathogenic SIVmac 1A11 during the primary acute, asymptomatic, and terminal phases of disease progression and compared them with results from uninfected healthy animals. Our studies showed that the magnitude of increase in RANTES gene expression correlated with the pathogenicity of the infecting virus and not with either viral suppression or CD3$^+$ T-cell prevalence in the GALT. These results demonstrated that RANTES gene expression in SIV-infected intestinal lymphoid tissues may not be a correlate of viral suppression *in vivo.*

RESULTS

Detection of SIV infection in intestinal mucosa

Dissemination and localization of SIV-infected cells in intestinal mucosa were determined by *in situ* hybridiza-
tion. The number of SIV-infected cells and their localization varied depending on the pathogenicity of the infecting SIV isolate and the stage of infection. Animals infected with pathogenic SIV isolates (SIVmac 239 or SIVmac 251) had high numbers of infected cells (5 SIV infection scores) during the primary acute and SAIDS phases of infection. However, relatively low numbers of infected cells (less than 2 SIV infection scores) were observed during the asymptomatic phase (4 and 8 weeks postinfection (p.i.)) in all asymptomatic stage animals. The SIV mac 1A11-infected animals displayed low SIV infection scores (0–2 SIV infection scores). The localization of SIV-infected cells in the jejunal mucosal lymphoid tissue differed between pathogenic and nonpathogenic SIV isolates. In SIVmac 251- and SIVmac 239-infected animals, SIV-positive cells were widely disseminated in the lamina propria, in Peyer's patches, and in lymphoid aggregates. Infected cells were also observed in germinal centers and paracortical regions of Peyer's patches. In the SAIDS animals, SIV-infected cells were found to be widely distributed in the intestinal mucosa. In the SIVmac 1A11-infected animals, SIV-positive cells were primarily localized at germinal centers.

RANTES gene expression during various stages of infection

Expression of RANTES mRNA in jejunal tissues during primary acute, asymptomatic, and SAIDS stages of SIV infection was determined by the quantitative-competitive RT-PCR (QC-RT-PCR) assay and compared with the levels observed in uninfected healthy controls. The expression of GAPDH, a housekeeping gene, was also determined in the same RNA samples. GAPDH mRNA was found to be constitutively expressed at $1-2 \times 10^{17}$ copies per microgram of tissue RNA irrespective of the disease status of the animals (Figs. 1B and 1C). The level of RANTES mRNA in all three uninfected controls was determined to be $1-6 \times 10^4$ copies per microgram of jejunal RNA. A consistent increase in RANTES gene expression in the intestinal tissue was observed in primary SIV infection with either pathogenic or nonpathogenic SIV compared to uninfected controls. The magnitude of increase in RANTES expression correlated with the pathogenicity of the infecting virus. The RANTES mRNA levels were consistently higher (a 500-fold increase or 5 log difference) in the SIVmac 251- or SIVmac239-infected animals during the primary acute stage (1–2 weeks p.i.) compared to uninfected controls and SIVmac 1A11-infected animals (Figs. 2C and 3C). In the asymptomatic stage, a decline in RANTES expression was observed in both pathogenic and nonpathogenic SIV infection. Animals with SAIDS also showed high levels of RANTES expression.
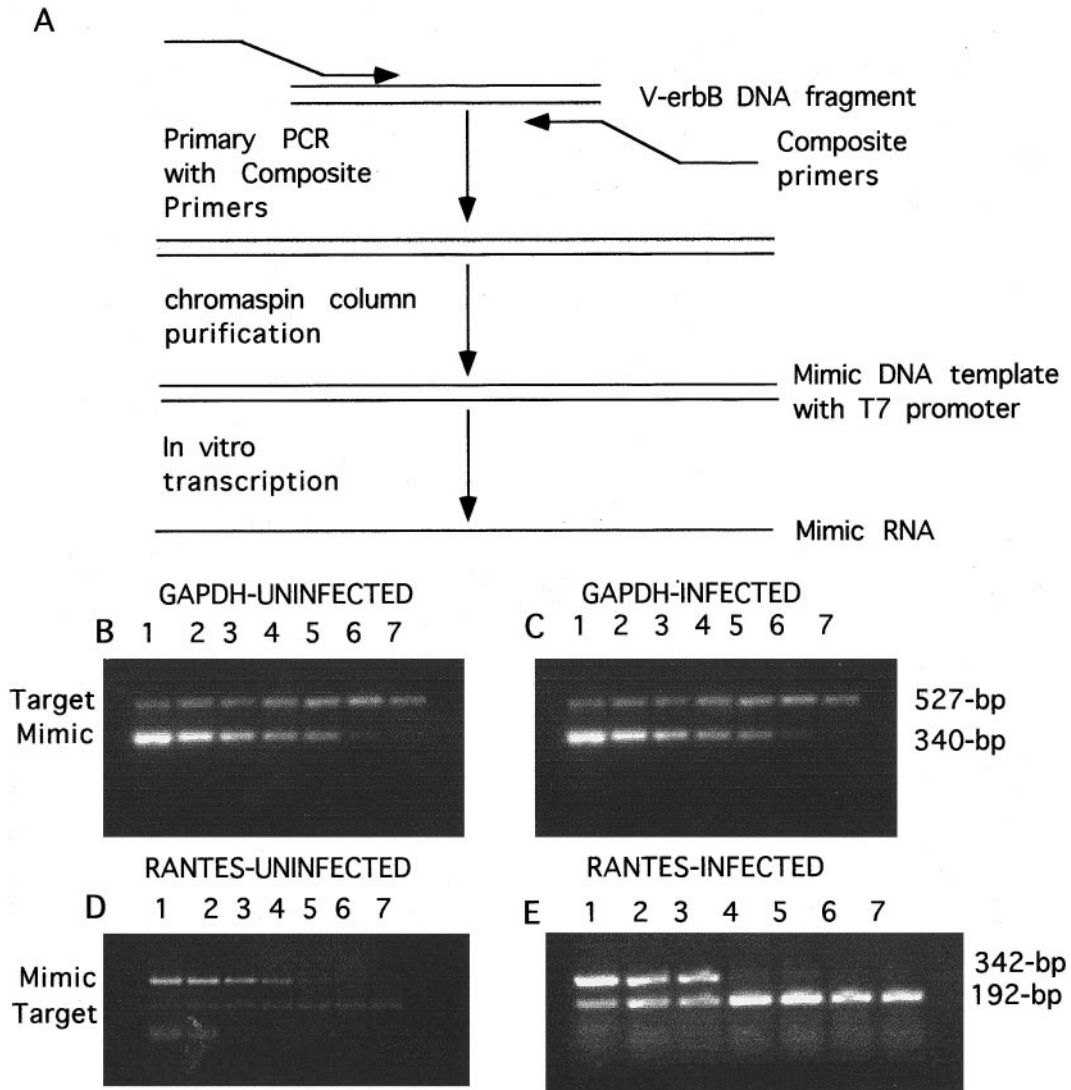
FIG. 1. Quantitation of RANTES and GAPDH mRNA by QC-RT-PCR. (A) Schematic presentation of the construction of mimic RNA. Composite, RANTES-specific and GAPDH-specific primers were designed and used to construct the internal competitor or mimic RNA as described under Materials and Methods. Expression of GAPDH mRNA in jejunal tissues of uninfected (B) and SIV-infected rhesus macaques (C) as determined by QC-RT-PCR analysis. Total RNA was aliquoted into seven replicates (0.1 μg each) and respective reactions were spiked with serial dilution competitor mimic RNA (lane 1, 500,000; lane 2, 50,000; lane 3, 25,000; lane 4, 10,000; lane 5, 5000; lane 6, 1000; lane 7, 500 copies). PCR products were resolved on a 3% ethidium agarose gel. The 527- and 340-bp bands corresponding to GAPDH and mimic PCR products, respectively, were quantified using NIH imaging and Collage software. The expression of GAPDH mRNA remained unaltered in both infected and uninfected animals. Expression of RANTES mRNA in jejunal tissues of uninfected (D) and SIV-infected (E) rhesus macaques. For RANTES quantitation, competitor mimic RNA was added to the uninfected macaque RNA samples (D) (lane 1, 500,000; lane 2, 50,000; lane 3, 25,000; lane 4, 10,000; lane 5, 5000; lane 6, 1000; lane 7, 500 copies) and SIV-infected RNA samples (E) (lane 1, 7×10^7; lane 2, 6×10^7; lane 3, 5×10^7; lane 4, 4×10^7; lane 5, 3×10^7; lane 6, 3×10^7; lane 7, 1×10^7 copies). The 342- and 192-bp bands correspond to RANTES mimic and RANTES, respectively. Enhanced RANTES mRNA expression was observed in jejunal tissues of SIV infected animals compared to uninfected controls.

RANTES expression and SIV infection in intestine

Animals infected with SIV mac 1A11 had consistently lower numbers of SIV-infected cells (0–2 SIV infection scores) during primary viral infection than animals infected with the pathogenic isolates as determined by *in situ* hybridization (Fig. 2A). These animals also had relatively low levels of RANTES mRNA expression ranging from 10^4 to 10^5 copies per microgram of total RNA. SIV-positive cells were not detectable in the intestinal tissues by *in situ* hybridization in two animals at 1 week p.i. (primary acute) and 23 weeks p.i. (asymptomatic stage). The RANTES mRNA levels in these animals were comparable to those in the uninfected controls. Thus a low SIV infection in nonpathogenic SIV infection did not lead to any significant increase in the levels of RANTES mRNA. In animals infected with pathogenic SIV isolates, many SIV-infected cells were detected at 1 week p.i. The level of SIV infection remained similar or increased in
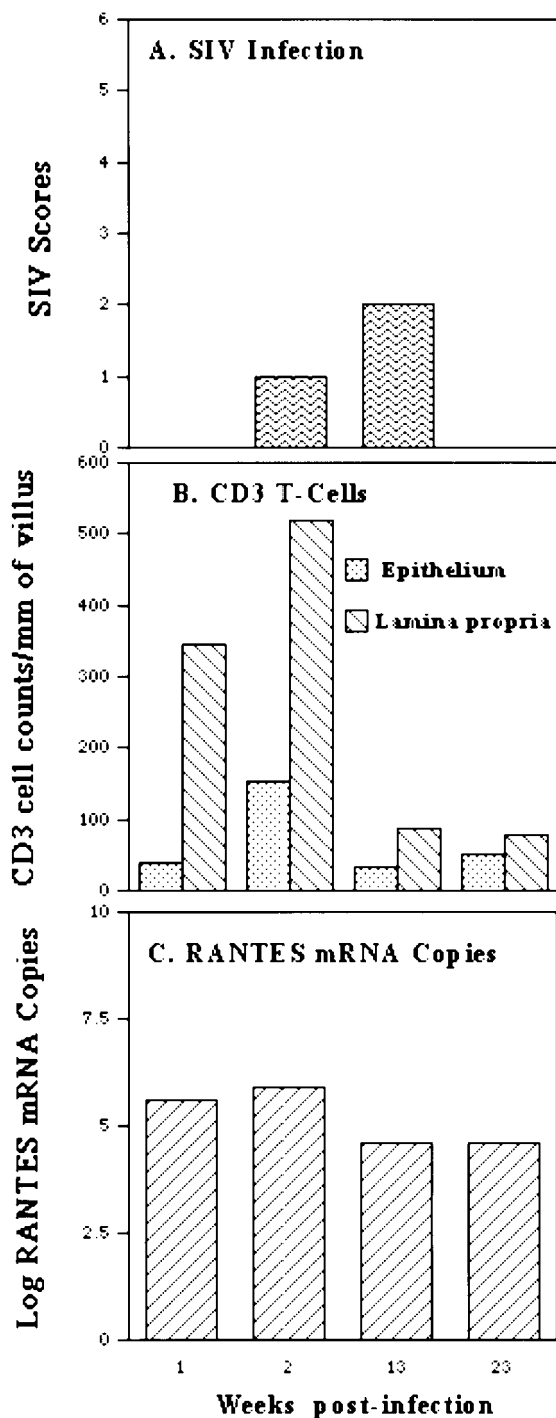
FIG. 2. The level of SIV infection (A), CD3^{+} T-cell counts (B), and RANTES mRNA expression (C) in jejunal tissues of rhesus macaques infected with nonpathogenic SIV isolate. *In situ* hybridization, immunostaining, and QC-RT-PCR were used to determine SIV infection scores, cell counts, and RANTES mRNA expression, respectively.

animals at 2 weeks p.i. (Fig. 3A). Animals with high SIV infection scores at both 1 and 2 weeks p.i. during primary SIV infection also showed high RANTES mRNA levels. In one of the six animals with primary pathogenic SIV infection, a high level of RANTES mRNA expression, 10^8

copies per microgram of RNA, accompanied by a low SIV infection score (1 SIV infection score) was detected in one animal at 1 week p.i. Animals in the asymptomatic stage (4 and 8 weeks p.i.), showed low to moderate SIV infection scores but had elevated RANTES mRNA expression, 10^6–10^8 copies compared to 10^4 copies in uninfected controls and SIV mac 1A11-infected animals at 13 and 23 weeks p.i. Only one of the four animals with SAIDS showed enhanced RANTES mRNA expression (10^9 copies) and a moderate SIV infection score (<2 SIV infection scores). Another animal with SAIDS had low SIV infection score and RANTES mRNA levels comparable to those of the uninfected controls. The remaining two animals with SAIDS displayed high SIV infection scores and high levels of RANTES mRNA (Fig. 3A).

RANTES expression and CD3^{+} T-cells in intestinal epithelium and lamina propria at different stages of disease progression

To determine the relationship between RANTES gene expression and CD3^{+} T-cell prevalence in the intraepithelial and lamina propria compartments of intestinal mucosa during primary SIV infection, immunohistochemical analysis was performed on jejunal tissues from animals infected with pathogenic or nonpathogenic SIV isolates and uninfected controls. In uninfected control animals, 100–200 CD3^{+} T-cells/mm of villus were observed in lamina propria while less than 100 CD3^{+} T-cells/mm of villus were scored in the villus epithelium. During the primary SIV infection, a significant increase in the number of CD3^{+} T cells in the lamina propria (345 and 518 CD3^{+} cells/mm of villus) was seen in the jejunal mucosa of SIVmac 1A11-infected animals at 1 and 2 weeks p.i., respectively (Fig. 2B). However, CD3^{+} T-cell counts in the asymptomatic stage (13 and 23 weeks p.i.) were similar to those in uninfected controls. In the pathogenic SIV-infected animals, relatively low CD3^{+} T-cell numbers were seen in both the intraepithelial and the lamina propria compartments in the primary infection starting at 1 week p.i. CD3^{+} T-cell counts in both compartments remained low during the asymptomatic and terminal stages of SIV infection. Thus an overall decrease in CD3^{+} T-cells was observed throughout the entire course of pathogenic SIV infection.

DISCUSSION

The gastrointestinal tract is an important portal of entry and early dissemination of HIV and SIV (Pantaleo *et al.,* 1991; Schneider *et al.,* 1997; Stone *et al.,* 1995). Since GALT harbors the majority of lymphoid cells in the body, it provides a microenvironment rich in activated cells for active viral replication (Nabel and Baltimore, 1987). The intestinal mucosa is substantially involved in the initial early events of the viral–host interaction and disease progression (Schneider *et al.,*
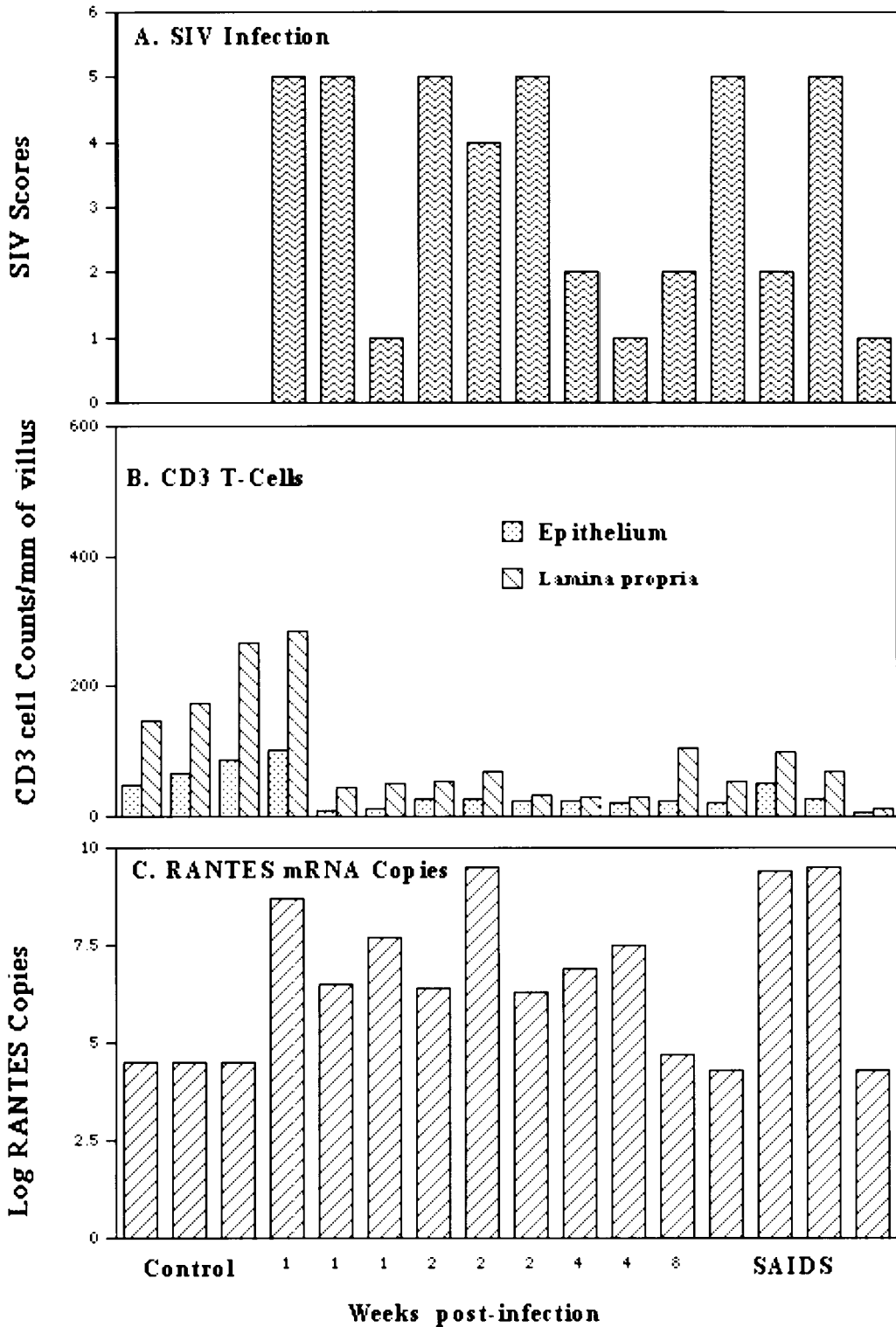
FIG. 3. The level of SIV infection (A), CD3^{+} T-cell counts (B), and RANTES mRNA expression (C) in jejunal tissues of uninfected controls and rhesus macaques infected with the pathogenic SIV isolate. *In situ* hybridization, immunostaining, and QC-RT-PCR were used to determine SIV infection scores, cell counts, and RANTES mRNA expression, respectively.

1997). High viral loads have been reported in the intestinal tissues of SIV-infected macaques early in the primary SIV infection (Heise *et al.,* 1991, 1993a,b; Stone *et al.,* 1995). The gastrointestinal tract has also

been shown to be a major site for early severe CD4^{+} T-cell depletion and dynamic immunophenotypic and functional changes in mucosal immune cells, emphasizing the significance of early viral–immune interac-

tions in this lymphoid tissue (Mattapallil *et al.,* 1998; Smit-McBride *et al.,* 1998; Veazey *et al.,* 1998).

Our study examined RANTES gene expression and the prevalence of SIV-infected cells and CD3^{+} T-cells in intestinal mucosa of rhesus macaques infected with either pathogenic or nonpathogenic SIV isolates. We examined the correlation between these parameters during the primary acute, asymptomatic, and SAIDS stages of viral infection. We observed enhanced RANTES expression in rhesus macaques infected with either pathogenic or nonpathogenic SIV isolates at various stages of disease progression. No clear correlation was observed between RANTES levels and viral loads in the intestinal mucosa during the primary SIV infection and SAIDS stages. Contradictory results have been reported on the RANTES levels in peripheral blood samples of HIV-1-infected asymptomatic individuals and long-term nonprogressors. Both high and low levels of RANTES were reported in blood samples of HIV-1-infected nonprogressors. A correlation between RANTES levels and low viral loads has not clearly been established in these studies (Scala *et al.,* 1997). In other studies, comparable RANTES levels were detected in blood samples of HIV-1-infected asymptomatic individuals and AIDS patients (Kakkanaiah *et al.,* 1998; Kinter *et al.,* 1998; Krowka *et al.,* 1997). Our results suggest that alterations in RANTES gene expression in SIV-infected animals correlated with the pathogenicity of the infecting virus. No correlation between high RANTES expression and low viral loads in intestinal tissues was found in these animals that could be indicative of RANTES-mediated viral suppression *in vivo.* Although the mechanisms for the lack of such correlation are not understood, various explanations may exist. The microenvironment of gut tissue is heterogeneous in nature, harbors a variety of cells, and is distinct from the pure cell cultures *in vitro.* Thus, the viral infection may involve a variety of coreceptors other than CCR5 and mechanisms of viral entry. Therefore, RANTES levels may not be able to inhibit *de novo* infections in gut as efficiently as in T-cell lines *in vitro.* The mechanisms of viral suppression may be more complex and multifactorial in gut tissue than in T-cell and macrophage cultures *in vitro.*

The alterations in CD3^{+} T-cells varied depending on the pathogenicity of the SIV isolate and disease stage. However, no correlation was observed between the expression of RANTES and CD3^{+} T-cells in the intestinal mucosa. In particular, high RANTES expression and high CD3^{+} T-cells counts were observed in the intraepithelial and lamina propria compartments at 1 and 2 weeks p.i. during the primary acute stage in animals infected with nonpathogenic SIV (Figs. 2B and 2C). The asymptomatic stage in these animals was characterized by moderate RANTES levels and low CD3^{+} T-cell counts despite low numbers of SIV-infected cells. Thus no correlation could be deduced from these data between RANTES expres-

sion, CD3^{+} T-cell prevalence, and number of SIV-infected cells. The chemoattractant property of RANTES during early stages of acute inflammation induced by viral infection may account for the increase in CD3^{+} T-cells at 1 and 2 weeks p.i. observed in nonpathogenic SIV infection. Lower numbers of CD3^{+} T-cells were observed during the asymptomatic stage despite high RANTES expression. The role of increased levels of RANTES in T-cell recruitment at the site of viral infection in intestine cannot be explained. Similarly, in pathogenic SIV animals the decline in CD3^{+} T-cells observed starting at 2 weeks p.i. and progressing to the asymptomatic and terminal phases did not corroborate previous data on the chemoattractant and viral suppressor properties attributed to high RANTES expression. Recently reported flow cytometric analyses of isolated intraepithelial and lamina propria lymphocytes have shown a dramatic depletion of CD4 T-cells in gastrointestinal tissues of SIV-infected rhesus macaques (Mattapallil *et al.,* 1998; Smit-McBride *et al.,* 1998; Veazey *et al.,* 1998). Disruption of the lymphoid structure previously reported in SIV infections at the terminal phase may account for the decline in T cells and low RANTES expression in animals with SAIDS.

Several mechanisms have been proposed to elucidate RANTES-mediated control of HIV/SIV infection *in vivo.* RANTES may directly suppress HIV/SIV infection through competitive inhibition of CCR5 receptor (Cocchi *et al.,* 1995, 1996). Second, the chemoattractant activity of RANTES has been shown to modulate the recruitment of T-cells and macrophages at sites of viral infection (Adams and Lloyd, 1997). RANTES expression has also been detected along with the expression of Th1 cytokine response (IFN-γ, IL-2) to infections. Thus an increase in RANTES gene expression in intestine during primary SIV infection may be a part of early host responses to infection at the site. A slight increase in CD3^{+} T-cells in both intraepithelial and lamina propria compartments was observed in two animals at 2 weeks following SIVmac 1A11 infection. This could partly be attributed to an influx of peripheral T-cells into the intestinal mucosa due to viral infection, suggesting that RANTES may be one of the early host factors to mediate altered leukocyte trafficking in the intestinal mucosa observed in HIV/SIV infections. This is supported by our previous studies that reported increased cell adhesion molecule expression in intestinal tissue during SIV infection correlating with the pathogenicity of the infecting virus (Stone *et al.,* 1995).

Taken together our results demonstrate that in contrast to the viral suppression by RANTES observed *in vitro,* there was no direct correlation between high RANTES gene expression and suppression of viral loads in intestinal lymphoid tissue. Thus RANTES expression in the gut lymphoid tissue may not be a correlate for viral suppression. However, the consistently high levels of RANTES gene expression in primary SIV infection sug-

TABLE 1

Disease Stages and Necropsy Time Points of Rhesus Macaques Infected with either Pathogenic or Nonpathogenic SIV Isolates

Animal No.	SIV isolate	Necropsy time point (weeks p.i.)	Disease stage
1	Control	N/A	N/A
2	Control	N/A	N/A
3	Control	N/A	N/A
4	SIVmac 1A11	1	Primary acute
5	SIVmac 1A11	2	Primary acute
6	SIVmac 1A11	13	Asymptomatic
7	SIVmac 1A11	23	Asymptomatic
8	SIVmac 251	1	Primary acute
9	SIVmac 251	1	Primary acute
10	SIVmac 251	1	Primary acute
11	SIVmac 251	2	Primary acute
12	SIVmac 251	2	Primary acute
13	SIVmac 239	2	Primary acute
14	SIVmac 251	4	Asymptomatic
15	SIVmac 251	4	Asymptomatic
16	SIVmac 251	8	Asymptomatic
17	SIVmac 251	12	SAIDS
18	SIVmac 239	23	SAIDS
19	SIVmac 251	27	SAIDS
20	SIVmac 251	35	SAIDS

Note. Three uninfected control animals were sacrificed and jejunal tissues were used in this study.

gest that it may be part of the early host immune response to viral infection.

MATERIALS AND METHODS

Animals, virus, and tissue collection

Twenty rhesus macaques (*Macaca mulatta*) housed at the California Regional Primate Research Center, Davis, were used in this study. Animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. These animals were seronegative for simian retrovirus-1 (SRV-1) and simian T-cell leukemia virus-1 (STLV-1). The rhesus macaques were intravenously inoculated with either the pathogenic SIV isolates SIVmac 251 or SIVmac 239 ($n = 13$) or with nonpathogenic SIVmac 1A11 ($n = 4$). Animals were necropsied at various time points following SIV infection and intestinal tissue samples were either immediately frozen in liquid nitrogen for RNA isolation or fixed in buffered formalin and embedded in paraffin for *in situ* hybridization and immunohistochemistry (Table 1). Tissue sections from the same intestinal tissue blocks were utilized for both *in situ* hybridization and immunohistochemistry. The animals infected with the pathogenic isolates were euthanized during the primary acute stage at 1 ($n = 3$) and 2 weeks p.i. ($n = 3$) and at the asymptomatic stage at 4 ($n = 2$), 8 ($n = 1$), and 23 weeks p.i. ($n = 1$). Animals with SAIDS ($n = 3$) were sacrificed at 12, 27, and 35

weeks p.i. The SIVmac 1A11-infected animals were similarly sacrificed during the primary SIV infection at 1 ($n = 1$) and 2 weeks ($n = 1$) p.i. and during the asymptomatic stage at 13 ($n = 1$) and 23 weeks p.i. ($n = 1$). Jejunal tissues from three uninfected healthy animals ($n = 3$) were included for baseline levels of CD3^{+} T-cell counts and RANTES mRNA expression.

Detection and localization of SIV-infected cells by *in situ* hybridization

Intestinal tissue samples were examined for SIV infection by *in situ* hybridization as previously reported (Heise *et al.,* 1994). Briefly, SIV-infected cells in intestinal tissues were detected by *in situ* hybridization of SIV nucleic acids with radiolabeled SIV-specific nucleic acid probes. The slides were examined for the presence of SIV-positive cells. Each tissue section was scored semiquantitatively from 0 to 5 for SIV viral load as following: 0 (no positive cells), 1 (1 to 5 cells per a tissue section), 2 (1–5 cells/10× field), 3 (6–10 cells/10× field), 4 (11–15 cells/10× field), and 5 (>15 cells/10× field).

Construction of RANTES and GAPDH mimics

Composite primers were designed and used to construct the internal competitors or mimics for the quantitative-competitive PCR assay for RANTES and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) based on a previously reported method (Heuvel *et al.,* 1993). The forward composite primer of 59 nt was composed of the 5′-T7 promoter sequence, the v-erbB, and either RANTES- or GAPDH-3′-specific sequences respectively. The reverse composite primer contained 5′-RANTES or GAPDH and v-erbB sequences and oligo(dT)$_{19}$-3′. Second, the gene-specific primer pairs were composed of 22-nt forward and reverse primers specific for GAPDH and RANTES, respectively (Fig. 1A and Table 2). PCRs were performed essentially as described previously (Siebert and Larrick, 1993) with the following modification. A *Bam*HI/*Eco*RI fragment of v-erbB DNA was amplified in a final volume of 50 μl containing 1× PCR buffer, 2 mM MgCl$_2$, 0.4 μM each of forward and reverse composite primers, a 0.2 mM concentration of each deoxyribonucleoside triphosphate, and 2.5 U *Taq* DNA polymerase (Promega, Madison, WI). The following cycle parameters were applied: an initial denaturation step 94°C for 3 min; 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s. The PCR products were purified using Chromaspin-100 columns (Clontech, Palo Alto, CA) and used as a template for *in vitro* RNA transcription using T7 RNA polymerase and a Ribomax *in vitro* transcription kit (Promega) according to the manufacturer's instructions. The RNA was quantified by spectrophotometry and the number of mRNA copies per microliter was determined.

TABLE 2

Composite and Gene-Specific Primer Sequences Used for Mimic Construction and QC-RT-PCR Assays

Forward RANTES mimic composite primer: 5′-TAATACGACTCACTATAGGGCATATTCCTCGGACACCACACCAAGTGAAATCTCCTCCG-3′
Reverse RANTES mimic composite primer: 5′-TTTTTTTTTTTTTTTTTCTCCAAGAGTTGATGTACTCCCACAAGATACTCATCTGCATC-3′
Forward GAPDH mimic composite primer: 5′-TAATACGACTCACTATAGGGACCACCATGGAGAAGGCTGGAAGTGAAATCTCCTCCGTC-3′
Reverse GAPDH mimic composite primer: 5′-TTTTTTTTTTTTTTTTTCTCAGTGTAGCCCAGGATGCACAAGATACTCATCTGCATC-3′
Forward RANTES gene-specific primer: 5′-CATATTCCTCGGACACCACACC-3′
Reverse RANTES gene-specific primer: 5′-CTCCAAGAGTTGATGTACTCCC-3′
Forward GAPDH gene-specific primer: 5′-ACCACCATGGAGAAGGCTGG-3′
Reverse GAPDH gene-specific primer: 5′-CTCAGTGTAGCCCAGGATGC-3′

Construction of plasmids containing simian GAPDH or RANTES cDNA

Total RNA was isolated from jejunal tissue using standard procedures. GAPDH cDNA was generated by reverse transcription. A 527-bp PCR product was amplified using primers specific for a highly conserved region of the GAPDH gene. The PCR product was subcloned into PCR 3.1 TA vector (Invitrogen) downstream of the T7 promoter. RNA transcripts were synthesized and used as standards in RT-PCRs according to the manufacturer's instructions. A plasmid construct containing monkey RANTES cDNA subcloned in pGEM vector (Promega) was a generous gift from Francois Villinger (Emory University).

Preparation of GAPDH and RANTES RNA for internal standards

Standard RNA transcripts were generated as run-off products by *in vitro* transcription of *Not*I linearized plasmids. The reaction mix was treated with RNase-free DNase to digest template DNA. RNA transcripts were extracted once with Trizol (Gibco BRL, Grand Island, NY) to remove any remaining DNA template and purified using Chromaspin columns as previously described.

Measurement of RANTES mRNA by QC-RT-PCR

Reverse transcription was performed in a 20-μl final reaction volume containing 1× PCR buffer, 5 mM MgCl$_2$, 1 mM each deoxyribonucleotide triphosphate, 50 mM random primers, 1 U RNase inhibitor, and 1.25 U MMLV reverse transcriptase. A reverse transcription master mix was prepared and aliquoted into seven replicates. One microgram of sample RNA per reaction and serial dilutions (500,000 to 500 copies) of competitor mimic RNA were spiked into respective reactions. Samples were incubated at 42°C for 1 h. Reverse transcriptase was inactivated by heating at 95°C for 5 min. After the inactivation step, 30 μl of a PCR master mix containing 1× PCR buffer, 2 mM MgCl, 0.2 mM deoxyribonucleotides, 0.8 mM forward and reverse primers, and 2.5 U *Taq* DNA polymerase were added to each of the cDNA samples, bringing the final volume to 50 μl. The reactions were denatured at 94°C for 3 min and subjected to 25 thermal cycles as follows: a denaturing step at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s using a GeneAmp PCR system 9600 Thermal cycler (Perkin–Elmer). A 10-μl aliquot from each PCR product was resolved on a 3% metaphor agarose gel (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. Relative intensities of the 192- and 342-bp PCR product bands corresponding to RANTES and mimic as well as the 527- and 340-bp products for GAPDH target and mimic, respectively, were captured, corrected for the difference in size, and quantified by computer imaging using the NIH image analysis software. The log ratio of target to mimic was plotted against the log ratio of mimic copies to determine the corresponding number of RANTES or GAPDH mRNA copies in 1 μg total RNA.

Detection and localization of CD3$^+$ T-cells by immunohistochemistry

For the detection of CD3$^+$ T-cells, formalin-fixed paraffin-embedded jejunal tissue sections were immunostained using anti-CD3 antibody (Dako) as previously described (Heise *et al.,* 1993b). To determine the CD3$^+$ T-cell counts in the epithelial and lamina propria compartments, slides were microscopically examined and images were digitally scanned and captured using Adobe Photoshop. Villus sizes were determined by superimposing the selected images on a prescanned hemocytometer grid (0.05 mm^2). The number of CD3$^+$ T-cells in the intraepithelial and lamina propria compartments was counted in a 4.4-mm villus length for the intraepithelial compartment and in a 2.05-mm villus length for the lamina propria compartments, respectively. At least 10 villi were counted per animal and the average numbers of cells were expressed as the number of CD3$^+$ T-cells in the respective compartments per linear length (in millimeters) of villus.

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