Nucleotide substitutions in the long terminal repeat are not required for development of neurovirulence by simian immunodeficiency virus strain mac

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The question of whether consensus nucleotide substitutions in the long terminal repeat (LTR) region of simian immunodeficiency virus strain mac (SIVmac) are important for neurovirulence was investigated in this report. Brains and lymph nodes from two macaques that developed AIDS and encephalitis following inoculation with two strains of neurovirulent SIVmac and from one animal with AIDS but no neurological disease after inoculation with non-neurovirulent SIVmac239 were used. The 5' LTR regions from neurovirulent SIVmacR71/17E and SIVmac7F-Lu were amplified, cloned and sequenced and these sequences were compared to the LTRs amplified from three regions of the respective encephalitic brains and lymph nodes from macaques inoculated with each virus. The SIVmac7F-Lu and SIVmacR71/17E viruses had zero and three consensus substitutions, respectively, in the U3, R and U5 regions of the LTR compared to that of SIVmac239. The only consensus substitution in the LTR-gag region of the genome was a T to C change at position 829 within the tRNA binding site. The sequences amplified from the brain and lymph nodes of the two animals with AIDS and encephalitis were identical. This single common substitution in this region of the virus genome, the T to C substitution at position 829, was also found in the LTRs isolated from the brain and lymphoid organs from the macaque inoculated with SIVmac239. The virtual identity in nucleotide sequences in the LTR of the neurovirulent and non-neurovirulent viruses and in CNS and lymph tissues of animals inoculated with the viruses suggests that the LTR has no effect on the tissue tropisms of the viruses.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is best known for its ability to cause AIDS, which is characterized by infection and depletion of the CD4+ subset of T cells, resulting in gradual immunological impairment, increased opportunistic infections and increased incidence of tumours (Poli et al., 1993; Levy, 1993; Schnittman & Fauci, 1994; Pantaleo & Fauci, 1995). In addition to causing destruction of the immune system, HIV-1 crosses the blood–brain barrier early after infection, causing infection in the CNS (Hollander et al., 1987). At later stages of infection, when patients progress into AIDS, approximately 7–11% of HIV-1-infected patients and approximately 23% of children who acquire their HIV-1 infection perinatally (Janssen, 1997) develop clinical syndromes known as HIV-1-associated cognitive/motor complex and AIDS dementia complex (ADC) (McArthur, 1987; Price et al., 1988; American Academy of Neurology, 1991; Spencer & Price, 1992). In the brain, the salient pathological findings from people with or without ADC include encephalitis, multinucleated giant cells, diffuse white matter pallor, reactive astrocytosis and neuronal loss (Sharer et al., 1997). Virus infection of the neuropil appears to be confined to microglial cells and the viruses isolated from the brains of these patients are invariably macrophage-tropic in nature (Cheng-Mayer et al., 1989).

Similar to human HIV-1 infection, macaques infected with simian immunodeficiency virus (SIVmac) develop AIDS and, in some cases, SIV-induced encephalitis (Desrosiers, 1990; Letvin & King, 1990). SIVmac239 is a molecularly cloned lymphocyte-tropic (L-tropic) strain which causes AIDS in approximately

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50% of inoculated macaques within 2 years (Kestler et al., 1990). While this virus has been shown to invade the CNS early after inoculation (Lackner et al., 1991; Stephens et al., 1995a), it rarely causes encephalitis (Narayan et al., 1995, 1997). However, passage of SIVmac239 in macaques can result in the selection of macrophage-tropic strains that cause encephalitis (Desrosiers et al., 1991; Sharma et al., 1992; Zhu et al., 1995). Interviral recombinants whose env gene is derived from encephalitic brains and which are macrophage-tropic in culture (Anderson et al., 1993; Stephens et al., 1995b) fail to cause neurological disease after inoculation into macaques (Joag et al., 1995). Thus, by exchanging the env gene, the virus becomes macrophage-tropic but less pathogenic than the parental SIVmac239. These results suggest, firstly, that the molecular determinants of neurovirulence, if they exist, do not reside solely within the env gene and, secondly, that virus tropism for macrophages alone does not predict neurovirulence even though neurovirulent viruses are macrophage-tropic. In this study we investigated whether nucleotide sequences in the viral long terminal repeat (LTR) correlate with the neurovirulent phenotype of the viruses. The data showed that the LTRs of the two neurovirulent viruses were almost identical to that of non-neurovirulent SIVmac239. Virtual identity was also observed between encephalitic brain and lymph node tissue of the two animals infected with the two neurovirulent viruses and brain and lymph node tissues of the animal infected with non-neurovirulent SIVmac239.

Methods

- **Virus stocks.** SIVmac239 is a molecularly cloned, L-tropic strain whose pathogenic properties have been previously described (Kestler et al., 1990; Sharma et al., 1992). This virus causes AIDS in macaques and is generally considered not to be neurovirulent, regardless of the route of inoculation (Lackner et al., 1991; Sharma et al., 1992; Joag et al., 1994b). The stock of SIVmac239 was derived by transfection of the original molecular clone into CEMx174 cells using standard protocols (Milman & Herzberg, 1981). Ten days following transfection, the culture medium was harvested and titrated using the CEMx174 cells (Joag et al., 1994a, b). The SIVmac7F-Lu virus stock was prepared as a homogenate from the lung of macaque 7F that was inoculated with SIVmac239 and developed AIDS and severe interstitial pneumonia and was euthanized 51 weeks post-inoculation (Zhu et al., 1995). The virus stock prepared from the lung of this animal was shown to be neurovirulent when inoculated intratracheally into macaques. The neurovirulent SIVmacR71/17E virus was independently derived from SIVmac239 by a series of passages in rhesus macaques (Sharma et al., 1992). The SIVmacR71/17E was prepared from pooled brain homogenates from macaques R71 and 17E, both of which developed SIV-induced encephalitis during neuroadaptation of SIVmac239 (Sharma et al., 1992).

- **Inoculation of macaques.** A 3-year-old macaque, AQ43, was inoculated in the bone marrow with $10^5$ TCID$_{50}$ SIVmacR71/17E and became moribund with signs of paralysis at 6 weeks after inoculation. A second 3-year-old macaque, 18G, was inoculated with $10^5$ TCID$_{50}$ of SIVmac7F-Lu virus via the intratracheal route (Zhu et al., 1995). Macaque 18G became moribund with signs of neurological dysfunction at 9 weeks post-inoculation and was euthanized. Macaque M75 was inoculated intravenously with $10^4$ TCID$_{50}$ SIVmac239 and developed AIDS at 67 weeks post-inoculation with no obvious signs of neurological dysfunction.

- **Processing of blood and tissue samples.** Heparinized blood was centrifuged to separate the plasma from the buffy coat. The cells were centrifuged through Ficoll–Hypaque density gradients to isolate peripheral blood mononuclear cells (PBMC). Single cell suspensions of spleen cells (SPC) and lymph node cells (LNC) were prepared by passing the tissues through a fine mesh. The isolated PBMC, LNC and SPC suspensions were assayed for virus as described below.

  To enumerate the number of PBMC, LNC and SPC that were producing infectious virus, infectious centres assays (ICA) were performed on serial 10-fold dilutions of isolated PBMC, LNC and SPC from $10^5$ to $10^6$ cells. Cells were inoculated into 24-well plates along with 1 ml freshly prepared $10^8$ C8166 cells and cultures were maintained in sRPMI containing 10% FBS, as previously described (Joag et al., 1994a, b). The number of cells producing infectious virus was calculated as the number of infectious cells per 106 PBMC, LNC or SPC.

  To determine whether virus was produced from PHA/IL2-stimulated cultures, indicating the presence of virus in lymphoid tissues, approximately $10^6$ PBMC, LNC or SPC were cultured for 2 days in 1 ml sRPMI containing 10% FBS and 1 µg/ml PHA-P (Wellcome). Cultures were centrifuged and the cell pellets resuspended in sRPMI + 100 U/ml rhuIL2 (Cetus) and cultured for 5 days. The cultures were again centrifuged and the cell-free supernatant fluids assayed for virus infectivity titre. The titre was calculated by the Karber method (Lennette, 1969) and expressed as the log$_{10}$ TCID$_{50}$/ml.

  To determine whether macrophages were producing infectious virus, isolated cells were cultured in macrophage differentiation medium (MDM) as previously described (Sharma et al., 1992). Virus infectivity was assayed by harvesting the supernatant fluids at 7 days and co-culturing with C8166 indicator cell line.

  To determine whether cell-free virus was present, 10% (w/v) homogenates were prepared and assayed for infectivity in C8166 and CEMx174 cultures as previously described (Joag et al., 1994a, b).

  The presence of virus was assayed in brain tissue by cultivation of tissue explants in MDM to test for productive virus replication in tissue macrophages and by preparing 10% (w/v) homogenates and assaying for infectivity in C8166 and CEMx174 cultures as previously described (Joag et al., 1994a, b).

  Portions of tissue specimens were frozen at $-80 \, ^\circ$C for use as a source of genomic DNA, and were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) for histopathological examination.

- **Brain dissection and neuropathological methods.** Within 1 h after necropsy, the brain was bisected and the right half was transferred immediately into 10% buffered formalin. The left cerebral hemisphere, cerebellum, and brainstem were subdivided into 14 anatomically distinct regions based on a brain map generated in our laboratory from serial slices of normal macaque brains of the same size. Throughout the dissection procedure, care was taken to remove adherent leptomeninges and portions of choroid plexus from the relevant parts, to minimize contamination with DNA from the meningeal and choroidal vasculature. Samples were collected from the spinal cord (cervical, thoracic and lumbar) and thoracolumbar dorsal root ganglia. All subdivided tissue samples were snap-frozen over dry ice and saved at $-70 \, ^\circ$C for PCR analysis of viral DNA. The remaining portions of the left half of the brain and spinal cord were fixed in 10% buffered formalin for 3–4 days. Blocks taken from the fixed brain were embedded in molten paraffin wax and processed. A preliminary screening for morphological abnormalities was performed on 3–5 µm paraffin sections stained with H&E. Serial sections
were stained with Luxol fast blue and Sevier-Munger stains, respectively, for the assessment of suspected myelin and axonal abnormalities in relevant parts of the CNS. The remaining sections were set aside for immunostaining.

- **Immunohistochemistry.** Paraffin sections (5–7 μm thickness) were reacted overnight with primary antibodies, CD68 (KP1, Dako) mouse monoclonal (used at 1:50 dilution) or SIV Gag (FA2) mouse monoclonal (used at 1:100 dilution). After repeated washing, the sections were incubated with biotinylated goat anti-mouse (Dako) antibodies, which in turn were detected using avidin–biotin–peroxidase enzyme complex followed by the DAB substrate. The stained sections were lightly counterstained with haematoxylin (Fisher). Appropriate controls were used with each test antibody.

- **PCR analysis of CNS tissues for the presence of viral sequences.** The CNS from infected macaques AQ43, 18G and M75 were dissected into the following regions: frontal cortex, parietal cortex, occipital cortex, motor cortex, temporal cortex, hippocampus, thalamus, internal capsule, basal ganglia, midbrain, pons, medulla, cerebellum, cervical spinal cord, thoracic spinal cord and lumbar spinal cord. The tissues were homogenized in a tight-fitting Dounce homogenizer in the presence of 50 mM Tris–HCl pH 8.2, 10 mM EDTA and 0.5% SDS. The homogenate was digested at 37 °C for 2 h in the presence of 100 μg/ml proteinase K followed by extraction twice with an equal volume of phenol and once with chloroform–isoamyl alcohol. The DNA in the aqueous phase was precipitated with 2 vols ethanol, pelleted and resuspended in DNase-free H2O. We used nested PCR to determine whether viral sequences were present in the DNA isolated from the various tissues (Joag et al., 1994b). In the first round, SIV oligonucleotide primers used were 5′ GATGGGGTGAAGAACTCCGTCTT 3′ and 5′ CTCCTCTGTGATATGCTGCTGG 3′ which are complementary to bases 1052–1075 and 1423–1450 of the SIV mac 239 gag gene, respectively (Regier & Desrosiers, 1990). As an internal control, a separate PCR was performed in which the fourth exon of β-actin was amplified with oligonucleotide primers 5′ TCATGTTTTGAACCGTTC- AACACCCCAC 3′ and 5′ CCAAAGAAGCCTTGAAGAGTGC- CC 3′ (non-coding) complementary to the published sequence (Nakijima-Iijima et al., 1985). The PCR amplification was performed using the following conditions: denaturation at 92 °C for 1 min, annealing at 55 °C for 1 min and primer extension at 72 °C for 3 min. To increase the sensitivity of the reaction, 1 μl of the first PCR product was used as a template for a second amplification using the same conditions. The nested SIV primers used were 5′ GTACATGTTGAAGCACTGATATTAGGGCAGC 3′ and 5′ CACCACACTGTTGCTGACATCGT 3′, which are complementary to bases 1142–1165 and 1356–1362 of SIV mac 239, respectively. The nested β-actin primers used were 5′ CCCACACG- CATGACCGTGCATCC 3′ and 5′ GCCTCAAGGGCAGCCGA- CCCTCA 3′. Samples were amplified for another 35 cycles as described above. Following the second round of amplification, a 10 μl aliquot was removed and run on a 1.5% agarose gel and bands visualized by staining with ethidium bromide. All PCR results obtained using gag oligonucleotide primers were confirmed using oligonucleotides and PCR that would specifically amplify the env gene of SIV mac 239 (Stephens et al., 1995a). In all cases, amplification of the gag gene was confirmed by amplification of the env gene (data not shown).

- **Quantitative PCR to analyse the virus burdens in the CNS of macaques.** The virus burden in the CNS and lymphoid tissues was determined using a quantitative PCR assay modified from a PCR–infected cell assay described previously (Joag et al., 1994b). In this assay, 1 μg of total cellular DNA isolated from tissues was subjected to a series of 10-fold dilutions such that samples contained from 100 ng to 10 fg (less than 1 copy of chromosomal DNA). These samples were used in nested PCR reactions that amplified either the β-actin gene of the cell (a single copy gene) or the gag gene from SIV mac. Amplification of either gene using the primers previously described was shown to detect one copy of each gene (Joag et al., 1994b). Thus, amplification of the β-actin gene determined the number of genome equivalents in each sample, while amplification with the gag primers determined the number of virus copies per number of genome equivalents. The values were expressed as the number of virus copies per 109 genome equivalents.

- **Cloning and sequencing of the LTR region.** For analysis of the LTR region of the viral genome, we chose three regions at random that exhibited encephalitic lesions: the occipital cortex, basal ganglia and midbrain. For comparative purposes, we also analysed the LTR region of viral genomes in lymph node tissue from macaques AQ43 and 18G, and the LTR region of the SIV mac R71/17E and SIV mac 7F-Lu 48 h after inoculation of C8166 cells. For macaque M75, which was inoculated with SIV mac 239 and was devoid of CNS lesions, occipital cortex and lymph node tissues were examined. Genomic DNA (1 μg) was used in the PCR (Saiki et al., 1985, 1988) containing 1 mM MgSO4, 200 μM each of the four deoxynucleotide triphosphates, 100 pM each of oligonucleotide primer (5′ TGGAAAGGTATTTATCACTGCAAGAAG-AC 3′, sense strand; and 5′ TCTTATATCTGTCCTTCTCCTGTA 3′, reverse strand, which correspond to nucleotides 1–30 and 1077–1103 of the SIV mac 239 genome, respectively) and a mixture of Taq and Pyrococcus species GB-D polymerases (Elongase, GibcoBRL). The template was denatured at 94 °C for 2 min and PCR amplification performed with an automated DNA Thermal Cycler (Perkin-Elmer Cetus) for 35 cycles using the following profile: denaturation at 94 °C for 30 s, annealing at 65 °C for 1 min and primer extension at 68 °C for 5 min. Amplification was completed by incubation for 10 min at 68 °C. The amplified DNA was purified by electrophoresis through a 1% agarose gel, the 1 kb base pair of interest excised and purified from the agarose using GeneClean (Bio 101) according to the manufacturer’s instructions. The isolated DNA fragment was ligated into the pGEM-T vector (Promega) and ligated DNA used to transform competent E. coli (strain JM109) using standard cloning procedures. Recombinants were identified on the basis of the size of the insert and recombinants with the LTR DNA fragment sequenced using the deoxy chain termination technique and the Sequenase enzyme (US Biochemical). The sequences of the SIV mac LTRs were analysed using the PCGENE sequence analysis software and compared to the sequence of the SIV mac 239 LTR (Regier & Desrosiers, 1990).

**Results**

**Histopathological and virological findings**

Lymph node and thymus tissues from all three animals had undergone atrophy accompanied by severe depletion of lymphocytes and a lack of follicular activity, consistent with end-stage disease in SIV mac infection of macaques. At necropsy, several assays were performed to assess the virus burden in these animals. All three macaques had a productive SIV mac systemic infection as indicated by the presence of virus-producing cells (determined by ICA) and the recovery of virus from PHA/IL2 cultures prepared from PBMC, SPC and LNC. In addition, MDM cultures prepared from PBMC, SPC and LNC, and cell-free homogenates from spleen and lymph node, also yielded virus when co-cultured with C8166 cells.
The results of these assays are summarized in Table 1. Homogenates prepared from brain tissue of macaques 18G and AQ43, and MDM cultures prepared from brain explants of these macaques were positive for virus when co-cultured with C8166 or CEMx174 cells. On the other hand, virus was not recovered from homogenate and brain explant cultures prepared from macaque M75. This showed that productive virus replication had occurred in the brains of macaques AQ43 and 18G but not in macaque M75. The high virus burdens and the presence or absence of infectious virus in the brains of these animals were similar to previously reported values for animals inoculated with these viruses (Sharma et al., 1992; Zhu et al., 1995).

**Neuropathological findings**

Macaque AQ43, inoculated with the SIV<sub>mac</sub>R71/17E virus, had a severe meningoencephalomyelitis. The lesions were disseminated within the gray and white matter of the cerebral hemispheres, brainstem and cervical spinal cord. The cellular composition and immunological phenotype of the inflammatory cell infiltrate consisted of nodular or perivascular aggregates of CD68<sup>+</sup> monocyte–macrophages, microglia and multinucleated giant cells. A few CD3<sup>+</sup> T lymphocytes and GFAP<sup>+</sup> glial cells were also present within the lesions. Many neurons in the motor cortex had undergone degeneration. White matter lesions included focal axonal degeneration accompanied by perilesional myelin loss. Lesions observed in the occipital cortex, basal ganglia and midbrain are shown in Fig. 1 since these are the regions from which we amplified and sequenced the LTRs (see below). The SIV Gag antigen was clearly identified in the macrophage populations within the lesions.

The CNS of macaque 18G, inoculated with SIV<sub>mac</sub>7F-Lu, had similar lesions to macaque AQ43. The lesions were distributed within the leptomeninges, cerebral cortex, subcortical white matter, basal ganglia, thalamus, brainstem and spinal cord. The inflammatory pathology was extensive and involved extra-cerebral sites such as the cranial nerves, ocular, perineural and muscle tissues. These findings were consistent...
with SIV-induced multifocal meningoencephalomyelitis of considerable severity. Typical lesions in the occipital cortex, basal ganglia, and midbrain are shown in Fig. 2. Immuno-staining of sections with an anti-SIV Gag monoclonal antibody was positive for Gag within histological lesions.

Macaque M75, inoculated with SIV<sub>mac</sub>239, was devoid of any lesions in the CNS. Virus was not recovered from CEMx174 cultures inoculated with brain homogenates and explants from brain tissue co-cultured with CEMx174 cells failed to recover virus.

**Macaques inoculated with neuroadapted SIV<sub>mac</sub> have much higher virus burdens in the brain than macaques inoculated with L-tropic SIV<sub>mac</sub>239**

Nested PCR techniques were used to determine the number of regions of the CNS that were positive for SIV<sub>mac</sub> in the brains of macaques inoculated with neurovируlent SIV<sub>mac</sub>, or L-tropic SIV<sub>mac</sub>239. As shown in Table 2, the macaques inoculated with neurovirulent strains of SIV<sub>mac</sub> (AQ43, 18G) were positive for viral sequences in all 17 regions analysed, whereas the macaque inoculated with SIV<sub>mac</sub>239 (M75) had viral sequences in only four of the 12 regions analysed. We also determined the virus burden in selected regions of the brain tissue (i.e. the number of virus copies per 10<sup>6</sup> genome equivalents) using nested PCR techniques as described in Methods. As shown in Fig. 3, the virus burden in the occipital cortex, basal ganglia and midbrain of macaque AQ43 (inoculated with the SIV<sub>mac</sub>R71/17E virus) was 10<sup>4</sup>, 10<sup>5</sup> and 10<sup>4</sup> virus copies per 10<sup>6</sup> genome equivalents, respectively. Similarly, the provirus burden in macaque 18G (inoculated with SIV<sub>mac</sub>7F-Lu virus) was 10<sup>4</sup> virus copies per 10<sup>6</sup> genome equivalents in all three regions. These levels were then compared with macaque M75, which was inoculated with L-tropic SIV<sub>mac</sub>239 and developed AIDS but no neuro-pathological changes. Examination of the DNA isolated from the occipital cortex and basal ganglia of this animal revealed a virus burden of 10<sup>5</sup> virus copies per 10<sup>6</sup> genome equivalents. Thus, the macaques inoculated with neurovируlent SIV<sub>mac</sub> had brain virus burdens 100- to 1000-fold higher than in the macaque inoculated with SIV<sub>mac</sub>239.
Table 1. Evaluation of virus burden in macaques 18G, AQ43 and M75 at necropsy

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Tissue</th>
<th>ICA*</th>
<th>PHA/IL2†</th>
<th>MDM‡</th>
<th>Virus recovery from homogenates</th>
</tr>
</thead>
<tbody>
<tr>
<td>18G</td>
<td>Brain</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>100</td>
<td>4.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Lymph node</td>
<td>100</td>
<td>5.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>100</td>
<td>3.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AQ43</td>
<td>Brain</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>100</td>
<td>2.0</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Lymph node</td>
<td>1000</td>
<td>4.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>1000</td>
<td>4.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M75</td>
<td>Brain</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>10</td>
<td>1.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lymph node</td>
<td>100</td>
<td>3.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>100</td>
<td>3.0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ND, Not done.
* Number of infectious cells per 10^6 as determined by cytopathology assay.
† Expressed as log_{10} TCID_{50}/ml.
‡ MDM cultures of PBMC, lymph node and spleen were derived from single cell suspensions whereas MDM cultures of brain were derived from tissue explants.

Sequence analysis of the LTR region in virus stocks and animal tissues

We analysed the sequence of the LTR region from the neurovirulent virus stocks (SIV<sub>mac</sub>7F-Lu and SIV<sub>mac</sub>R71/17E) as well as from three regions of encephalitic brains and lymph node tissues in animals inoculated with these viruses. The nucleotide substitutions were compared to those in brain and lymph node tissues from the SIV<sub>mac</sub>239 inoculated macaque. Fig. 4 shows the sequence of the SIV<sub>mac</sub>239 5′ LTR and the consensus sequence changes that were observed in the LTR clones isolated from the occipital cortex, midbrain, basal ganglia and lymph node tissue from macaques AQ43 and 18G. We defined a consensus sequence change as one that was found in all tissues analysed and in over 50% of the clones analysed. However, it should be noted that all consensus nucleotide substitutions were observed in 100% of the clones analysed, with the exception of one substitution in the LTR from AQ43 (position 63), which was found in 90% of the clones analysed. Analysis of the LTR from the SIV<sub>mac</sub>R71/17E virus stock inoculated into C8166 cells and macaque AQ43 revealed three consensus nucleotide sequence substitutions in the clones derived from the virus stock and from the occipital cortex, basal ganglia, midbrain and lymph node tissue from macaque AQ43. These substitutions included two substitutions in the U3 region, a G to A substitution at position 63 and an A to T substitution at position 115, which led to a glutamic acid to phenylalanine change and a tyrosine to serine amino acid change in the Nef protein, respectively. An A to G substitution was observed in the TAR element of the U5 region of the LTR at position 602. In addition to the U3, R and U5 regions of the LTR, a T to C substitution occurred in the tRNA binding domain (and not in the LTR; Fig. 4) at position 829 and a G to A substitution in the leader sequence at position 927. Interestingly, all three consensus changes were found in the LTR analysed from the brain as well as the lymph node and thymus tissues from AQ43. The three consensus nucleotide substitutions observed in the LTRs from macaque AQ43 represented a 0.45% change in the nucleotide sequence from SIV<sub>mac</sub>239. Sequence analysis of the LTRs from the SIV<sub>mac</sub>R71/17E virus stock inoculated into C8166 cells and macaque 18G revealed no substitutions in the U3, R and U5 regions of the LTR. A single nucleotide substitution, the same T to C substitution at position 829 in the tRNA binding domain observed in the LTR–gag clones from macaque AQ43, was also observed in all clones analysed from the virus stock and from the brain and lymph nodes from macaque 18G. The single consensus nucleotide substitution at position 829 represented a 0.09% change in the nucleotide sequence from SIV<sub>mac</sub>239. In summary, the consensus nucleotide substitutions observed in LTRs isolated from the CNS of animals infected with neurovirulent virus were also observed in lymph node tissue and no consensus substitutions were found in macaque 18G.

Since sequence analysis revealed no consensus nucleotide substitution in the SIV<sub>mac</sub>7F-Lu or SIV<sub>mac</sub>R71/17E virus stocks or tissues from animals inoculated with these viruses, and only a single common nucleotide substitution at position
Table 2. Detection of SIV sequences in CNS from macaques AQ43, 18G and M75

DNA from different regions of the brain was used in nested PCR with oligonucleotides that amplified the gag region of the SIVmac genome.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Macaque 18G</th>
<th>Macaque M75</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQ43</td>
<td>18G M75</td>
<td></td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Motor cortex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thalamus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Internal capsule</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Midbrain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pons</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Medulla</td>
<td>+</td>
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</tr>
<tr>
<td>Cerebellum</td>
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<td>Lumbar spinal cord</td>
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ND, Not done.

In the tRNA binding domain, we assessed whether this substitution was selected following inoculation of macaques with SIVmac239 or whether it was specific for neurovirulent strains of SIVmac. We chose macaque M75, which was inoculated with SIVmac239 and euthanized at 67 weeks due to AIDS complications but which exhibited no neuropathologic changes. We examined the LTRs from one region of the brain (occipital cortex) of this animal as well as from the lymph node tissue. As shown in Fig. 4, the LTR clones isolated from occipital cortex and lymph node tissue of macaque M75 all had the T to C substitution at position 829.

Discussion

In this study, we compared the sequence of the LTR region of two neurovirulent strains of SIVmac with those of the parental but non-neurovirulent SIVmac239 in the hope of finding signature sequences for neurovirulence. The rationale for analysing the LTR came from previous studies on other retroviruses that demonstrated the importance of the LTR in cell tropism, gene regulation and disease specificity. For example, interval recombinants constructed between molecular clones of Moloney MuLV and Friend MuLV, which cause T cell lymphomas and erythroleukaemia in mice, respectively, defined sequences within the U3 region of the LTR as responsible for disease specificity (Chatis et al., 1984; Li et al., 1987) and transcriptional activity in various tissues (Short et al., 1987). Similarly, the expression of mouse mammary tumour virus (MMTV) in mammary cells is found to be regulated by sequences located in an enhancer element in the extreme 5' end of the LTR of this virus (Mink et al., 1992; Hartig et al., 1993). Further, among members of the avian sarcoma/leukosis virus group, changes within the U3 region result in a 10–30-fold increase in rate of replication of the exogenous lymphoma-inducing members of this group (Tsichlis et al., 1982; Cullen et al., 1983). In studies on lentiviruses, Kurth et al. (1996) showed that transgenic mice that carry the LTR from a CNS isolate of HIV-1 bind specific proteins from extracts prepared from the brainstem tissue of newborn mice whereas transgenic mice that carry the LTR from an L-tropic isolate do not have this activity. A similar report by Corboy et al. (1992) found that transgenic mice carrying the LTR derived from CNS isolates of HIV-1 support gene expression in neurons in brains of adult and young mice while transgenic mice carrying the LTR from a T cell tropic isolate (HIVIIIB) do not support virus gene expression in brains. A major caveat of these experiments is that gene expression was observed only in neurons and not in microglial cells, the only cell type shown to be reproducibly infected in humans and macaques infected with HIV-1 and SIV, respectively. Due to the lack of a suitable animal model, the significance of changes in the LTR of HIV-1 isolated from the brains of patients with HIV-associated encephalopathy is currently unknown. We asked, therefore, whether there might be signature sequence changes in the LTR of different SIVmac strains that cause neurological disease.

We analysed the predominant sequence changes that occurred in the LTR region of two neurovirulent virus strains

![Fig. 3. Viral burden in the occipital cortex (OC), basal ganglia (BG) and midbrain (MB) of macaques AQ43 (spotted bars), 18G (dark shaded bars) and M75 (light shaded bars). Total cellular DNA was extracted from the tissues and nested PCR was used to quantitate the number of virus copies per 10^6 genome equivalents as described in the Methods section.](image-url)
Fig. 4. For legend see page 1098.
Fig. 4. For legend see page 1098.
Fig. 4. Sequence analysis of the 5' LTR–gag region amplified from SIVmacR71/17E (R71/17E)- and SIVmac7F-Lu (7F-Lu)-infected C8166 cells and from occipital cortex (OC), basal ganglia (BG), midbrain (MB) and lymph node tissue (LN) from macaques 18G and AQ43 and OC and LN from macaque M75. The sequences shown represent the consensus sequences of five clones from three PCR reactions. SIVmac LTR sequences were compared to SIVmac239 using PCGENE sequence analysis. Dashes represent nucleotide sequence identity. All consensus sequence changes shown were represented in 100% of the clones with the exception of the substitution at position 63 in macaque AQ43 which was present in 90% of the clones.

before and after inoculation into macaques. We analysed the LTR–gag region from three regions of the brain from macaques that had severe lesions consistent with SIV-induced encephalitis as well as from lymph node tissue from the same animals. We found a total of three consensus nucleotide substitutions in the LTR region of the SIVmacR71/17E virus stock and in all four tissue samples from the macaque inoculated with this virus. There were no consensus nucleotide substitutions in the LTR region of the SIVmac7F-Lu virus stock or in the tissue samples analysed from the macaque inoculated with this virus. These finding were not surprising since the SIVmac7F-Lu virus stock was created by passage in one macaque whereas the SIVmacR71/17E virus stock was passaged in a total of four animals. Examination of tissue DNA showed a single consensus nucleotide substitution in the LTR–gag region of the viral genome isolated from encephalitic brains of both animals.
inoculated with the two viruses. We inquired whether this substitution was specific for neurovirulent strains of SIVmac, or whether this was a common change that also occurred following inoculation of macaques with non-neurovirulent SIVmac239 (i.e. an in vivo substitution). We found that this T to C substitution at position 829 in the tRNA binding site also occurred in the LTR–gag region amplified from the brain and the lymph node tissues of the macaque inoculated with SIVmac239 and developed AIDS but exhibited no neuropathology. Thus, this change was not a marker of neurovirulent SIVmac.

It is interesting to note that previous studies have shown that HIV-1, HIV-2, SIV and MMTV preferentially utilize the tRNA^Lys^ to prime DNA replication (Das et al., 1995). Examination of the molecular clone of SIVmac239 used to inoculate macaque M75 confirmed the presence of a thymidine at position 829 which is located in the primer binding site (PBS) (data not shown). The PBS of the molecular clone of SIVmac239 is complementary at 17 out of 18 residues of tRNA^Lys^, with the only mismatch being a thymidine at position 829. Because the T to C substitution at position 829 was found in the SIVmac7F-Lu and SIVmacR7117E virus stocks and in all LTRs amplified from tissues examined in this study (thus resulting in 18 out of 18 residues in the PBS complementary to tRNA^Lys^), this substitution probably represents an in vivo selection to utilize tRNA^Lys^ more efficiently.

The lack of consensus sequence changes in the LTRs isolated from the brain tissues of animals infected with these two neurovirulent strains of SIVmac (especially the one inoculated with SIVmac7F-Lu virus) suggests that LTR-mediated transcriptional regulation of viral gene expression in microglial cells of the brain is not a determinant of whether certain strains of SIVmac can cause neurological disease in macaques.

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