Genomic and biological alteration of a human immunodeficiency virus type 1 (HIV-1)–simian immunodeficiency virus strain mac chimera, with HIV-1 Env, recovered from a long-term carrier monkey

Tatsuhiko Igarashi, 1,2 Takeo Kuwata, 1 Jun Takehisa, 1 Kentaro Ibuki, 1 Riri Shibata, 1† Ryozaburo Mukai, 3 Toshihiko Komatsu, 4 Akio Adachi, 1 Eiji Ido 1 and Masanori Hayami 1

1 Institute for Virus Research, Kyoto University, Kyoto 606, Japan
2 Japanese Foundation for AIDS Prevention, Tokyo 105, Japan
3 Tsukuba Primate Center for Medical Science, National Institute of Health, Tsukuba 305, Japan
4 Division of Biosafety Control and Research, National Institute of Health, Tokyo 162, Japan

A macaque monkey infected with NM-3, a human immunodeficiency virus type 1 (HIV-1)–simian immunodeficiency virus strain mac (SIVmac) chimeric virus with env, rev, tat and vpu derived from HIV-1 and LTR, gag, pol, vif and vpx derived from SIVmac, became a long-term carrier (more than 2.8 years). This monkey produced neutralizing antibodies to the original NM-3 as well as to the parental HIV-1. The virus recovered at 116 weeks replicated more rapidly and productively in macaque peripheral blood mononuclear cells than the original virus. The recovered virus was not neutralized either by antibodies raised early in the monkey or by a neutralizing monoclonal antibody that recognizes the V3 loop of HIV-1 Env, whereas both the early antibodies and the monoclonal antibody neutralized the original NM-3. Analysis of the virus genomic population revealed a few common mutations in the V3 region that caused amino acid changes. These data are consistent with the hypothesis that the virus escaped from the early antibodies and that the observed mutations contributed to this, as with HIV-1-infected humans. The observed mutations could equally well be the result of adaptation to simian cells. These results suggest that the HIV-1–SIVmac chimeric virus will be useful for investigating genetic variation of HIV-1 env and alteration of biological properties in vivo in relation to the host immune response.

Introduction

Human immunodeficiency virus type 1 (HIV-1) causes a persistent infection during which phenotypic changes in the virus, such as increased cell tropism or syncytium-forming activity, have been observed to accompany disease progression (Cheng-Mayer et al., 1988; Kuiken et al., 1992). Some of these phenomena were explained as a consequence of mutations in the third hypervariable region, the V3 region, in the env gene (de Jong et al., 1992a, b; Grimaila et al., 1992). The V3 region is also known to be the major neutralizing epitope of HIV-1. There have been reports describing the emergence of escape mutants of HIV-1 from neutralizing antibodies raised in infected individuals (Albert et al., 1990; Arendrup et al., 1992; McKeating et al., 1989; Reitz et al., 1988; Tremblay & Wainberg, 1990). These observations indicate that genetic analyses of the V3 region in relation to the viral phenotypes and the host immune reactions may provide important information for understanding the pathogenesis of HIV-1. Such analyses require an experimental infection system, which would enable the use of well-characterized single clones of the virus strain as inoculum in order to follow genetic and phenotypic changes from the original virus. However, experimental infection of HIV-1 is difficult because few animal species are susceptible to HIV-1 infection; the only generally accepted animal, the chimpanzee, is rare and expensive.

Simian immunodeficiency virus strain mac (SIVmac), one of
the primate lentiviruses, has a genomic organization and biological properties that are similar to those of HIV-1 and induces an AIDS-like disease in infected macaque monkeys (Kestler et al., 1990). Therefore, the SIVmac-macaque system has been studied by many researchers. However, SIVmac is genomically closer to HIV-2 than to HIV-1 and the biological significance of the V3 region of SIVmac does not correspond to that of T cell-adapted strains of HIV-1, i.e. the V3 region of T cell-adapted strains of HIV-1 is the principal neutralizing epitope but that of SIVmac is not (Javaherian et al., 1992; Kent et al., 1992; Robert-Guroff et al., 1992). However, the V3 region of SIVmac was reported to be a determinant of cell tropism (Kirchhoff et al., 1994). Therefore, variations in the V3 region of T cell-adapted strains of HIV-1 which affect the viral phenotype and host immune reactions may not correspond with those in the SIVmac-macaque system.

We have developed HIV-1–SIVmac chimeric viruses that have HIV-1-derived genes, including env (Shibata et al., 1991). Previously, we reported the successful infection of a cynomolgus monkey with an HIV-1–SIVmac chimeric virus, designated NM-3, which has tat, rev, vpu and env from HIV-1 and LTR, gag, pol, vif and vpx from SIVmac (Sakuragi et al., 1992). This monkey became a long-term carrier, maintaining the viraemic stage in the presence of anti-HIV-1 Env antibody, which has neutralizing activity against NM-3, for over 2 years.

In this study, we analysed mutational changes in the HIV-1-derived env V3 region and biological properties of the virus recovered after lengthy persistence [116 weeks post inoculation (p.i.)] in the presence of the host immune reaction. We found that the virus recovered had escaped from antibodies present in the early sera and that the V3 region had mutated, resulting in amino acid changes. Furthermore, a monoclonal antibody that neutralizes the original NM-3 by binding to the V3 region failed to neutralize the mutant strain, indicating that the mutated V3 region had altered antigenicity. This suggests that these mutations contribute to the escape of the virus, as has been reported in HIV-1-infected humans. In addition, the virus replicated more rapidly and to higher titre in the monkey peripheral blood mononuclear cells (PBMCs) than the original NM-3. These data suggest that the HIV-1–SIVmac chimeric virus is useful for investigating the genetic variation of HIV-1 env in relation to the host immune reaction and the change in biological properties of the virus during the persistence.

**Methods**

- **An NM-3-infected monkey.** The construction and genomic organization of the HIV-1–SIVmac chimeric virus NM-3 has been described previously (Sakuragi et al., 1992). The genomic organization of NM-3 and the parental viruses are shown in Fig. 1. The monkey used in this study has been described previously (Sakuragi et al., 1992). Briefly, a
male cynomolgus monkey (*Macaca fascicularis*) was inoculated intravenously with $10^4 \text{TCID}_{50}$ of NM-3 virus grown in the CD4+ human T-lymphoid cell line M8166 (a subclone of C8166; Clapham et al., 1987).

**Virus recovery.** Samples of $2-4 \times 10^6$ PBMCs of the inoculated monkey were obtained periodically, stimulated with concanavalin A (ConA), and maintained for 3–7 days in the presence of IL-2. An equal number of M8166 cells was then added and the cocultures were maintained for another 6 weeks. During cocultivation, the appearance of virus antigens was monitored by an indirect immunofluorescence antibody assay, using serum from an HIV-1-infected human as reference serum; virus production was measured by a reverse transcriptase (RT) assay. CPE formation in the cultured cells was also monitored. At week 116 p.i., coculture was done with CEM × 174 cells (Hoxie et al., 1988) in addition to M8166 cells.

**Preparation and titration of stock viruses.** Stock viruses were prepared basically as described by Burns et al. (1993). Briefly, CEM × 174 cells transfected with proviral DNA or infected with the recovered virus were maintained at a density of $6 \times 10^6$ cells/ml and supernatants were collected daily (at 24 h intervals). The infectivity of the stock virus was titrated using M8166 cells as follows. Tenfold serially diluted virus solutions ($100 \mu l$) were mixed with $100 \mu l$ of $2 \times 10^4$ M8166 cells in RPMI-1640 containing 10% FCS (RPMI-10). After culturing for 7–14 days, the endpoint was determined by judging syncytium formation in quadruplicate at each dilution by microscopic observation, and the TCID$_{50}$ was calculated by the Behrens–Kärber method (Kärber, 1931).

**Western blotting.** Antibody production by the inoculated monkeys and the antigenicity of the virus recovered from the monkey were evaluated by a Western blotting assay using lysates of M8166 cells that were infected with parental viruses, the original inoculum of the chimeric virus NM-3 or virus recovered from the inoculated monkey. The lysates of infected cells were resolved in a 10% (w/v) SDS–polyacrylamide gel and transferred to a PVDF membrane (Immobilon; Millipore). After blocking with Block Ace (Snow Brand), the membrane was incubated at 4 °C overnight with the serum of the infected monkeys at a 100-fold dilution. After incubation with peroxidase-labelled anti-human Igs (Caltag) for 18 h at 4 °C, the samples were washed and examined using enhanced chemiluminescence detection reagents (Amersham).

**Infection of human cells and monkey PBMCs.** Appropriate volumes of stock viruses were inoculated onto M8166 cells ($5 \times 10^4 \text{TCID}_{50}$ per $2 \times 10^4$ cells) or macaque PBMCs ($4 \times 10^3 \text{TCID}_{50}$ per $1 \times 10^6$ cells) 3 days after ConA stimulation. The virion-associated RT activity of the cultures was then measured as described by Willy et al. (1988). PBMCs from cynomolgus monkeys (*M. fascicularis*) were separated from heparinized whole blood by Percoll (Pharmacia) density gradient centrifugation (density 1.072 g/ml), stimulated with ConA (10 μg/ml) for 24 h, and maintained in RPMI-1640 containing 20% FCS and IL-2 as described by Ohta et al. (1988). M8166 cells were maintained in RPMI-10.

**Virus neutralization assay.** Heat-inactivated sera were titrated at a twofold dilution. Serum samples (170 μl) were mixed with an equal volume of virus ($1 \times 10^3 \text{TCID}_{50}$ per 30 μl in M8166) and incubated at 37 °C for 90 min. Aliquots of the virus-serum mixtures (100 μl) were then inoculated onto $2 \times 10^5$ M8166 cells in triplicate. After 4–9 days, the RT activity of the supernatant was measured; a reduction in RT

---

Fig. 2. Antibody response of the NM-3-infected monkey. Serum samples collected at intervals were subjected to Western blotting using antigens of

(a) the parental HIV-1 (NL432) and (b) the parental SIVmac (MA239). Lanes 1–11: serum before infection and 10, 21, 32, 40, 100, 112, 121, 125, 138 and 149 weeks p.i., respectively (100-fold dilution).
activity of at least 50% was considered indicative of virus neutralization. To compare the sensitivity of NM-3 with the virus recovered at 116 weeks p.i., 170 µl of each virus stock (2 x 10⁶ TCID₅₀ per 50 µl) was mixed with an equal volume of serum, and the mixture was used to inoculate 8 x 10⁴ pooled normal cynomolgus PBMCs that had been stimulated with ConA and cultivated for 2 days in the presence of IL-2. The monoclonal antibody 0-5β (Matsushita et al., 1988), which recognizes the V3 loop and neutralizes the parental HIV-1, NL432, was kindly provided by T. Hattori (Kyoto University, Kyoto, Japan) for use as a reference antibody.

■ Infection of monkeys by blood transfusion from the long-term infected monkey. Four naive monkeys (two juvenile female cynomolgus monkeys, nos 12 and 13, and two adult male rhesus monkeys, nos 11 and 13) were infected by intravenous injection of heparinized blood (6 ml per monkey) from the long-term infected monkey (119 weeks p.i.). Blood was collected periodically from the inoculated monkeys for virus and antibody assays. All the monkeys used in this study were seronegative for simian retrovirus type D, simian T-cell leukaemia virus type I and foamy virus.

■ PCR amplification of the proviral sequence. Chromosomal DNAs of PBMCs and M8166 cells or CEM x 174 cells infected with the recovered virus were extracted using glass milk powder (Bio-Rad, Prep-A-Gene DNA purification kit). One-eighth of the extracted DNA was used for analysis of proviral DNA by PCR. For amplification of proviral DNA of the recovered virus, HIV-1 provirus region primers described by Sakuragi et al. (1992) were used. The sense primer was F1 (5’ TGGCTGTTAAATGCAGTCTAGC 3’, nt 6990–7011 in NL432) and the antisense primer was R1 (5’ CCCTCATATCTCCTCTCC- TCCAGG 3’, nt 7628–7649 in NL432), covering the latter half of the C4 region to the end of the V5 region, including V3. The buffer conditions were as described previously (Miura et al., 1990), except that the pH was 8.9 and the MgCl₂ concentration was 6 mM. PCR was carried out under the following conditions in a total volume of 50 µl: 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and elongation for 60 s at 72 °C.

For amplification of the viral DNA sequence from PBMCs, nested PCR was used. The sense primer used for the first PCR was MK650 (Oka et al., 1994) (nt 6935–6959 in NL432) and the antisense primer was V3BF1 (5’ CAGGCCAGTAGTATCAACTGC 3’, nt 6967–6992 in NL432) and the antisense primer was YT001 (Oka et al., 1994) (nt 7304–7329 in NL432), covering the V3 region. The buffer conditions were as described by Oka et al. (1994). PCR was carried out under the following conditions in a total volume of 50 µl: 35 cycles of denaturation for 60 s at 94 °C, annealing for 60 s at 55 °C and elongation for 60 s at 72 °C.

■ Nucleotide sequencing of PCR products covering the V3 region of HIV-1 env. PCR products were phosphorylated by polynucleotide kinase (Toyobo) and purified by 1.5% (w/v) agarose gel electrophoresis. The purified DNA fragments were inserted into the SmaI site of pUC119 using a DNA blunting kit (Takara) and sequenced with an automated DNA sequencer (ABI 373A auto sequencer).

Results

Recovery of virus and antibody response in the carrier monkey

We previously reported the successful infection of a cynomolgus monkey with the HIV-1-SIVmac chimeric virus NM-3, and described the results obtained up to 12 weeks p.i. (Sakuragi et al., 1992). Infectious virus was recovered from PBMCs at 2 and 6 weeks p.i., but recovery then ceased temporarily. Successful virus recovery started again at 94 weeks p.i. and continued through the observation period up to 149 weeks p.i. This indicated the establishment of a long-term carrier state in the monkey, though no symptoms associated with AIDS, such as emaciation, diarrhoea or generalized lymphadenopathy, were observed.

The monkey produced antibodies, as expected from the chimeric structure of the NM-3 virus. These were anti-HIV-1 Env antibodies (anti-gp120 and anti-gp41), which were detected from 10 weeks p.i., and anti-SIVmac Gag antibody (anti-p26), which was detected from 6 weeks p.i. by a Western blotting assay (Fig. 2). The antibodies were maintained and gave increasing intensities on Western blotting during the period of observation. The antibodies obtained 40 and 121 weeks p.i. showed neutralizing activity titres against NM-3 of 20480 (Table 1). In addition, they could neutralize the parental HIV-1, NL432, but not the parental SIVmac, MA239 (titre < 20). These results indicated that anti-HIV-1 Env antibody was involved in neutralization of NM-3 but that anti-SIVmac Gag was not.

Neutralization and growth kinetics of the virus recovered 116 weeks p.i.

The virus that was recovered from the monkey 116 weeks p.i. was shown by Western blotting to have the Env protein of HIV-1 and the Gag protein of SIVmac, indicating maintenance of the basic chimeric structure of NM-3 (Fig. 3). The recovered virus was not neutralized by sera obtained before 116 weeks p.i. but was weakly neutralized by sera at and after 110 weeks. All these sera had a high neutralizing activity against the original NM-3. Moreover, the monoclonal antibody 0-5β,

Table 1. Neutralizing antibody responses of the carrier monkey infected with NM-3 against NM-3, NL432 (HIV-1) and MA239 (SIVmac)

| Time p.i. when serum was obtained (weeks) | Neutralizing titre against: |  |
|-----------------------------------------|-------------------------------|
|                                         | NM-3                         | NL432 | MA239   |
| 0                                       | < 20                          | < 20   | < 20    |
| 10                                      | 10240                         | 640    | < 20    |
| 21                                      | 20480                         |        |         |
| 32                                      | 20480                         |        |         |
| 40                                      | 20480                         | 2560   | < 20    |
| 100                                     | 10240                         |        |         |
| 112                                     | 20480                         |        |         |
| 121                                     | 20480                         | 10240  | < 20    |
| 0-5β                                    | 1600                          | 1600   | < 20    |
which has neutralizing activity against the original NM-3 and the parental NL432, failed to neutralize the recovered virus (Tables 1 and 2). These results indicate that the recovered virus had escaped from the neutralizing antibody that was produced early on by the monkey and that some of the escape mutations altered the antigenicity of the V3 region, since 0-5β recognizes the V3 loop of NL432 and NM3.

Table 2. Neutralizing activities of sera from the monkey against NM-3 and the virus recovered at 116 weeks (116)

<table>
<thead>
<tr>
<th>Time p.i. when serum was obtained (weeks)</th>
<th>Neutralization titre against:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NM-3</td>
</tr>
<tr>
<td>0</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>40</td>
<td>20480</td>
</tr>
<tr>
<td>116</td>
<td>20480</td>
</tr>
<tr>
<td>149</td>
<td>20480</td>
</tr>
<tr>
<td>0-5β</td>
<td>800</td>
</tr>
</tbody>
</table>

in cynomolgus monkey PBMCs, but in human M8166 cells the recovered virus grew similarly, although the rise in RT activity was slower.

Thus, the virus recovered 116 weeks p.i. had acquired a higher growth potential than the original NM-3 virus in monkey PBMCs.

Infection of monkeys by transfusion of blood from the persistently infected monkey

Since the enhanced growth potential of the recovered virus in monkey PBMCs suggested adaptation of the NM-3 virus to macaque monkeys during persistent infection, we inoculated blood obtained from this carrier monkey 119 weeks p.i. into four naive macaque monkeys (two cynomolgus and two rhesus monkeys). As shown in Table 3, infectious virus was recovered from all four inoculated monkeys 2–6 weeks p.i. Thereafter, the virus was recovered from each of the monkeys, except no. 12, throughout the observation period (36 weeks). The virus appeared to grow better and induce higher antibody levels in rhesus monkeys than in cynomolgus monkeys. All four monkeys produced anti-SIVmac Gag p26 antibodies. Anti-HIV-1 Env gp120 antibody was produced in two rhesus monkeys (nos 11 and 13), but not in two cynomolgus monkeys (nos 12 and 15), judged by Western blotting using a parental NL432-infected cell lysate as antigen. It seemed that, after long-term infection, the virus acquired a higher infectivity than that of the original NM-3 virus, because, in the case of the original NM-3 infection of two cynomolgus monkeys, one showed viremia at 2 and 6 weeks p.i. (Sakuragi et al., 1992). That monkey became the long-term carrier in this study. These virus-infected monkeys did not show any symptoms associated with AIDS.

Genetic variation of the NM-3 virus that persisted in the monkey

The virus recovered from the carrier monkey 116 weeks p.i. was resistant to the neutralizing antibodies raised early in the
Fig. 4. Growth kinetics of NM-3 and the virus recovered 116 weeks p.i. (116) in human T-lymphoid M8166 cells (a) and the PBFs of two normal cynomolgus monkeys (b, c). ○, NM-3; ●, 116; □, mock-infected.

Table 3. Infection of four macaque monkeys by blood transfusion from the carrier monkey 119 weeks p.i.

<table>
<thead>
<tr>
<th>Virus recovery and antibody</th>
<th>Monkey no.</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>10</th>
<th>12</th>
<th>17</th>
<th>20</th>
<th>24</th>
<th>28</th>
<th>32</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus recovery</td>
<td>12 MF</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>15 MF</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>11 MM</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>13 MM</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-HIV-1 Env</td>
<td>12 MF</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>15 MF</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>11 MM</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>13 MM</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-SIVmac Gag</td>
<td>12 MF</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>15 MF</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>11 MM</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>13 MM</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

post-inoculation period and to monoclonal antibody O-5β, which recognizes the V3 loop in the Env protein. To examine the molecular basis of this phenomenon, we amplified and sequenced the env gene V3 region of the integrated proviral sequence of the recovered virus-infected cells and PBMCs 116 weeks p.i. Fig. 5 (a) shows the nucleotide alignment of nine clones in PBMCs and Fig. 5 (b) shows the deduced amino acid sequences of proviruses in the PBMCs (nine clones) and the virus recovered by cocultivation with M8166 cells (16 clones) or CEM × 174 cells (16 clones). Each V3 clone had three sequential base deletions (CAA, nt 7170–7172) and consisted of 105 bases. There were 38 nucleotide substitutions out of 945 bases in a total of nine V3 sequences from PBMCs, the frequency of the A-to-G transition (22/38, 57.9%) being highest. Of the nucleotide substitutions, 89.5% (34/38) resulted in amino acid changes. Two amino acid substitutions and one deletion were commonly observed in all the clones sequenced: asparagine-298 was changed to serine (caused by an A-to-G transition), asparagine-323 was changed to aspartate (caused by an A-to-G transition) and threonine-317 was deleted. In addition, nucleotide substitutions in the crown of the V3 loop were observed in some of the clones. They were the substitution of histidine for glutamine-308 (G-to-T transversion), observed in five of nine clones, and the substitution of leucine for proline-311 (C-to-T transversion) in two of nine clones (Fig. 5 b).

As shown in Fig. 5 (b), the infectious viruses recovered from PBMCs were more homogeneous populations than the proviral populations in PBMCs. They showed similar mutations, i.e. the changes of asparagine-298 to serine and asparagine-323 to aspartic acid and deletion of threonine. The substitution of glutamine-308 for histidine was observed in the
virus recovered in M8166 cells and that of proline-311 for leucine in the viruses recovered in M8166 and CEM x 174 cells. These results showed that the virus population persisting in this carrier monkey was rather homogeneous, having common mutation sites irrespective of the detection system.

**Discussion**

In this report, we demonstrated that an HIV-1-SIVmac chimeric virus, NM-3 (Shibata et al., 1991), which had tat, rev, env and vpu derived from HIV-1, and LTR, gag, pol, vif and vpx from SIVmac, persistently infected a cynomolgus monkey for about 3 years. The infected monkey produced anti-HIV-1 Env and anti-SIVmac Gag antibodies, which were maintained throughout the observation period. These antibodies included virus-neutralizing antibodies that could neutralize NM-3 and the parental HIV-1, NL432, but not the parental SIVmac, MA239. These observations indicated that the Env protein of HIV-1, but not the Gag protein of SIVmac, was involved in this neutralization, and that the neutralizing epitopes of NM-3 are similar to those of the parental HIV-1. The V3 loop of HIV-1 was also considered to be the same as that in the parental HIV-1 because the monoclonal antibody 0-5β, which can bind to the V3 loop and neutralize the parental HIV-1, also neutralized the NM-3 virus (Table 1). Although NL432 and NM-3 were neutralized by 0-5β at the same titre, early sera from the monkey (at 10 and 40 weeks p.i.) neutralized NM-3 more effectively than NL432. We cannot attribute this discrepancy to the involvement of SIVmac Gag-directed neutralizing antibody, because these sera could not neutralize SIVmac. Further study will be needed to clarify this.

Even though the virus-infected monkey produced and maintained anti-viral antibodies possessing virus-neutralizing activity, the virus was still circulating in the peripheral blood, as is commonly seen in HIV-1-infected humans. One possible explanation is that the NM-3 virus persistently infecting the monkey mutated to escape from the host immune reaction. In fact, serum samples taken from the monkey early in infection failed to neutralize the virus recovered after lengthy persistence (116 weeks p.i.). On the other hand, the later serum samples could neutralize the virus, although all the serum samples tested neutralized the original NM-3.

Furthermore, the recovered virus was resistant to neutralization by the monoclonal antibody 0-5β, which recognizes the V3 loop and neutralizes the original NM-3 virus (Table 1), indicating that some mutations that occurred in the V3 region made the virus resistant to 0-5β. Therefore, to elucidate the genetic background underlying the escape phenomenon, we determined proviral sequences of the env V3 region. In HIV-1_LAI strains, the V3 loop is the principal neutralizing epitope and is highly variable during the course of infection (Holmes et al., 1992; Wolfs et al., 1991). There are reports that amino acid changes in the V3 loop resulted in escape from anti-V3 neutralizing antibody (Ivanoff et al., 1991; Wolfs et al., 1991). Nevertheless, our results showed that the sequence of the virus recovered in M8166 cells and that of proline-311 for leucine in the viruses recovered in M8166 and CEM x 174 cells. These results showed that the virus population persisting in this carrier monkey was rather homogeneous, having common mutation sites irrespective of the detection system.

**Discussion**

In this report, we demonstrated that an HIV-1-SIVmac chimeric virus, NM-3 (Shibata et al., 1991), which had tat, rev, env and vpu derived from HIV-1, and LTR, gag, pol, vif and vpx from SIVmac, persistently infected a cynomolgus monkey for about 3 years. The infected monkey produced anti-HIV-1 Env and anti-SIVmac Gag antibodies, which were maintained throughout the observation period. These antibodies included virus-neutralizing antibodies that could neutralize NM-3 and the parental HIV-1, NL432, but not the parental SIVmac, MA239. These observations indicated that the Env protein of HIV-1, but not the Gag protein of SIVmac, was involved in this neutralization, and that the neutralizing epitopes of NM-3 are similar to those of the parental HIV-1. The V3 loop of HIV-1 was also considered to be the same as that in the parental HIV-1 because the monoclonal antibody 0-5β, which can bind to the V3 loop and neutralize the parental HIV-1, also neutralized the NM-3 virus (Table 1). Although NL432 and NM-3 were neutralized by 0-5β at the same titre, early sera from the monkey (at 10 and 40 weeks p.i.) neutralized NM-3 more effectively than NL432. We cannot attribute this discrepancy to the involvement of SIVmac Gag-directed neutralizing antibody, because these sera could not neutralize SIVmac. Further study will be needed to clarify this.

Even though the virus-infected monkey produced and maintained anti-viral antibodies possessing virus-neutralizing activity, the virus was still circulating in the peripheral blood, as is commonly seen in HIV-1-infected humans. One possible explanation is that the NM-3 virus persistently infecting the monkey mutated to escape from the host immune reaction. In fact, serum samples taken from the monkey early in infection failed to neutralize the virus recovered after lengthy persistence (116 weeks p.i.). On the other hand, the later serum samples could neutralize the virus, although all the serum samples tested neutralized the original NM-3.

Furthermore, the recovered virus was resistant to neutralization by the monoclonal antibody 0-5β, which recognizes the V3 loop and neutralizes the original NM-3 virus (Table 1), indicating that some mutations that occurred in the V3 region made the virus resistant to 0-5β. Therefore, to elucidate the genetic background underlying the escape phenomenon, we determined proviral sequences of the env V3 region. In HIV-1_LAI strains, the V3 loop is the principal neutralizing epitope and is highly variable during the course of infection (Holmes et al., 1992; Wolfs et al., 1991). There are reports that amino acid changes in the V3 loop resulted in escape from anti-V3 neutralizing antibody (Ivanoff et al., 1991; Wolfs et al., 1991). Nevertheless, our results showed that the sequence of the virus recovered in M8166 cells and that of proline-311 for leucine in the viruses recovered in M8166 and CEM x 174 cells. These results showed that the virus population persisting in this carrier monkey was rather homogeneous, having common mutation sites irrespective of the detection system.

**Discussion**

In this report, we demonstrated that an HIV-1-SIVmac chimeric virus, NM-3 (Shibata et al., 1991), which had tat, rev, env and vpu derived from HIV-1, and LTR, gag, pol, vif and vpx from SIVmac, persistently infected a cynomolgus monkey for about 3 years. The infected monkey produced anti-HIV-1 Env and anti-SIVmac Gag antibodies, which were maintained throughout the observation period. These antibodies included virus-neutralizing antibodies that could neutralize NM-3 and the parental HIV-1, NL432, but not the parental SIVmac, MA239. These observations indicated that the Env protein of HIV-1, but not the Gag protein of SIVmac, was involved in this neutralization, and that the neutralizing epitopes of NM-3 are similar to those of the parental HIV-1. The V3 loop of HIV-1 was also considered to be the same as that in the parental HIV-1 because the monoclonal antibody 0-5β, which can bind to the V3 loop and neutralize the parental HIV-1, also neutralized the NM-3 virus (Table 1). Although NL432 and NM-3 were neutralized by 0-5β at the same titre, early sera from the monkey (at 10 and 40 weeks p.i.) neutralized NM-3 more effectively than NL432. We cannot attribute this discrepancy to the involvement of SIVmac Gag-directed neutralizing antibody, because these sera could not neutralize SIVmac. Further study will be needed to clarify this.

Even though the virus-infected monkey produced and maintained anti-viral antibodies possessing virus-neutralizing activity, the virus was still circulating in the peripheral blood, as is commonly seen in HIV-1-infected humans. One possible explanation is that the NM-3 virus persistently infecting the monkey mutated to escape from the host immune reaction. In fact, serum samples taken from the monkey early in infection failed to neutralize the virus recovered after lengthy persistence (116 weeks p.i.). On the other hand, the later serum samples could neutralize the virus, although all the serum samples tested neutralized the original NM-3.

Furthermore, the recovered virus was resistant to neutralization by the monoclonal antibody 0-5β, which recognizes the V3 loop and neutralizes the original NM-3 virus (Table 1), indicating that some mutations that occurred in the V3 region made the virus resistant to 0-5β. Therefore, to elucidate the genetic background underlying the escape phenomenon, we determined proviral sequences of the env V3 region. In HIV-1_LAI strains, the V3 loop is the principal neutralizing epitope and is highly variable during the course of infection (Holmes et al., 1992; Wolfs et al., 1991). There are reports that amino acid changes in the V3 loop resulted in escape from anti-V3 neutralizing antibody (Ivanoff et al., 1991; Wolfs et al., 1991). Nevertheless, our results showed that the sequence of the virus recovered in M8166 cells and that of proline-311 for leucine in the viruses recovered in M8166 and CEM x 174 cells. These results showed that the virus population persisting in this carrier monkey was rather homogeneous, having common mutation sites irrespective of the detection system.
Masuda et al., 1990). When proviral V3 sequences in the PBMCs and those of the virus recovered from the PBMCs were determined, these virus populations showed rather homogeneous sequences, and some mutations common to all the clones were observed. These included the changes of asparagine-298 to serine and asparagine-323 to aspartic acid and the deletion of threonine-317. Although these mutations were outside the known epitope of 0'5f1 (Masuda et al., 1990), it is possible that the mutations influenced the conformation of the loop and inhibited binding of 0'5f1 to the loop, since these observed mutations resulted in the substitution of amino acids with different charges. It is probable that mutations in the V3 loop of the recovered virus were responsible for its resistance to neutralization by 0'5f1, and that the same mutations were probably partly responsible for its resistance to the early sera.

Rudensey et al. (1995) reported that the predominant variants in pig-tailed macaque monkeys experimentally infected with SIVmne replicated and persisted in CEM x 174 cells, as well as in cultured macaque lymphocytes, more efficiently than in C8166 cells. Based on their report, we used CEM x 174 cells in addition to M8166 cells to obtain virus populations that closely reflect populations in the monkey. However, we found that the viruses recovered using M8166 and CEM x 174 cells had similar sequences in the env V3 region, although the virus from M8166 had slightly more mutations than that from CEM x 174 (Fig. 5b).

We sequenced PBMC proviral populations to determine whether the recovered virus represented the major population in the monkey, since virus isolation, especially using established cell lines, often selects some specific population of an in vivo quasi-species. The proviral populations in the PBMCs were more variable, but common mutations observed in all the clones were also observed in viruses recovered from M8166 and CEM x 174 cells. Therefore, the mutations observed in the recovered virus were considered to reflect those that actually occurred in vivo, at least in the V3 region. Two mutations in the crown of the V3 loop, i.e. changes of glutamine-308 to histidine and proline-311 to leucine, were observed in proviruses in the PBMCs and in the virus recovered from M8166 cells. In an in vitro culture system, Masuda et al. (1990) reported the generation of an escape mutant derived from HIV-1 IIIb (asparagine-299 to lysine, asparagine-300 to isoleucine, proline-311 to glutamine and alanine-314 to threonine) that escaped from 0'5f1. In addition, Ivanoff et al. (1991), in an in vitro study of HIV-1, reported that site-directed mutation of proline-311 to alanine resulted in resistance to neutralization by 0'5f1. It is noteworthy that proline-311 was also found to be substituted in our persistently infected monkey, although the substitution was to leucine in this case (Fig. 5b). Therefore, the NM-3 mutation at proline-311 observed in the persistently infected cynomolgus monkey might reflect the HIV-1 env mutation in vivo in humans in the presence of antibody. The high frequency of A-to-G transition of the proviral env sequences observed in this study (Fig. 5a) matched the observation by Burns & Desrosiers (1991) that A-to-G transitions as well as G-to-A transitions occurred at high frequencies in their SIVmac in vivo system.

As mentioned above, mutations in the V3 loop appear to be responsible for the escape from 0'5f1. In this study, we focused on the V3 loop, since in T-cell adapted strains of HIV-1 it is the principal neutralizing epitope and since NM-3 has an HIV-1-derived env gene. However, it is possible that mutations in other regions are involved in the observed escape phenomenon. Li et al. (1995) recently reported that non-synonymous mutations occurred broadly in env, including the V3 region in the virus recovered from an HIV-1–SIVmac-infected monkey. It is unlikely that the SIVmac-derived Gag protein of NM-3 is involved in the escape, because the monkey studied showed no detectable neutralizing activity against parental SIVmac239, although anti-SIVmac Gag antibody was detected by Western blotting (Fig. 2).

In addition to the escape phenomenon, another major observation in this study was that the virus recovered 116 weeks p.i. grew better than the original NM-3 in monkey PBMCs. Perhaps during infection the NM-3 may have mutated to replicate better in the monkey. The efficient infection achieved by blood transfusion from the monkey to other normal monkeys is consistent with this possibility. In this study, all the virus recoveries were performed using almost equal numbers of PBMCs (2–3 x 10⁶ cells), although we did not determine cell-associated virus load. Therefore, the frequency of successful virus recovery represents a rough estimate of virus load. In the original NM-3 infection, successful virus recovery was achieved from only one of two monkeys at 2 and 6 weeks p.i.; thereafter, virus was not recovered until 94 weeks. On the other hand, blood transfusion rendered all four monkeys viraemic by 2–6 weeks. There is a possibility that the observed mutations in the HIV-1 env V3 region are responsible for the higher infectivity of the virus. In order to confirm this possibility, it will be necessary to construct NM-3 chimeric viruses with the mutated HIV-1 env V3 region and other regions that have not yet been sequenced. Regions from SIVmac might not be involved in this phenomenon, even if the mutation occurs in the SIVmac part of the genome of this chimeric virus, since the parental SIVmac, MA239, is considered to have the highest competence for replication in monkeys. At present, we cannot tell definitely whether the observed mutations in the V3 loop were a consequence of immune selection or a process of adaptation of NM-3 to the monkey. We believe that immune selection might drive the V3 to mutate, because the recovered virus, which has these mutations, was not neutralized by the early sera, although killer cell activity was not examined in this study, and also because the HIV-1 env V3 mutations frequently observed in this study were generally seen in HIV-1, which escaped neutralizing antibodies as discussed previously. In conclusion, this macaque infection system, which, unlike the SIVmac–macaque system, consists of a chimeric virus with HIV-1 Env,
is useful for analysing genetic variation of the HIV-1 env region in relation to the host immune mechanism in vivo. In humans, experimental infection using a cloned virus and experimental analysis in the course of the infection are impossible.

This work was supported by Grants-in-Aid for AIDS Research and from the Japanese Ministry of Health and Welfare, and Ministry of Education, Science and Culture of Japan.

References


Received 18 December 1995; Accepted 18 March 1996