Construction of human immunodeficiency virus 1/simian immunodeficiency virus strain mac chimeric viruses having vpr and/or nef of different parental origins and their in vitro and in vivo replication

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We constructed a series of human immunodeficiency virus 1 (HIV-1)/simian immunodeficiency virus strain mac (SIVmac) chimeric viruses having vpr and/or nef genes of either HIV-1 or SIVmac based on a chimeric virus with LTRs, gag, pol, vif and vpx derived from SIVmac and tat, rev, vpu and env from HIV-1. All of the chimeric viruses replicated in human and macaque peripheral blood mononuclear cells (PBMCs) and in several CD4+ human cell lines, though their growth potentials were slightly different depending on whether vpr and nef were from HIV-1 or SIVmac, or were defective. The presence of nef accelerated replication in all the cells used and the replication of each chimera appeared to reflect that of the parental virus from which nef was derived. The presence of vpr had no clear effect in human and monkey PBMCs, but the replication of each chimera was influenced by the origin of vpr in H9 and A3.01 cells. NM-3rN, which carries HIV-1 vpr and SIVmac nef, was inoculated intravenously into three rhesus monkeys, three cynomolgus monkeys and two pig-tailed monkeys. From 2 to 14 weeks after inoculation, viruses were consistently re-isolated from all the monkeys and virus loads were as high as that of SIVmac reported previously. The results indicate that infection with NM-3rN is more efficient than any of our previous chimeric viruses and suggest that NM-3rN, having HIV-1 Env, will be a useful challenge virus for evaluating AIDS vaccines based on HIV-1 Env in macaque monkeys instead of chimpanzees.

Introduction

Human immunodeficiency virus 1 (HIV-1) can infect only a limited range of animal species other than humans, such as chimpanzees (Pan troglodytes) (Alter et al., 1984; Fultz et al., 1986; Gajdusek et al., 1985; Nara et al., 1987) gibbons (Hylobates lar) (Lusso et al., 1988) pig-tailed monkeys (Macaca nemestrina) (Agy et al., 1992), SCID-hu mice (Leonard et al., 1988) and rabbits (Filice et al., 1988). Chimpanzees infected with HIV-1 are the currently favoured animal model of HIV-1 infection. However, a limitation of this system is that HIV-1 does not induce AIDS-like symptoms in infected chimpanzees even during long observation periods.

Simian immunodeficiency viruses (SIVs) are closely related to HIV-1 and HIV-2 and can infect macaque monkeys, which are readily available for experimental use. Some SIV strains of SIVmac, SIVmne and SIVsmm are known to cause a fatal disease in macaque monkeys similar to human AIDS (Letvin et al., 1985; Benveniste et al., 1988; Fultz et al., 1989). Thus, macaque monkeys infected with these pathogenic SIVs are now considered to be the most appropriate system for studying AIDS pathogenesis. Nevertheless, the immune response to SIV envelope glycoproteins has been reported to be different from that in the case of HIV-1 (Weiss et al., 1986). Thus, for developing anti-HIV-1 drugs and vaccines and evaluating their efficacy and safety, a new animal model that utilizes the HIV-1 genes would be of value.

To establish a useful experimental infection system using HIV-1 and macaque monkeys, we previously generated two HIV-1/SIVmac chimeric viruses. One of these, named NM-3 (Shibata et al., 1991) contained tat, rev, vpu and env of HIV-1 (NL432) and LTRs, gag, pol, vif and vpx of SIVmac (MA239) and the other, named NM-3n (Igarashi et al., 1994) contained nef of HIV-1 in addition to the genome structure of the former chimera. Two cynomolgus monkeys (Macaca fascicularis) each were infected with NM-3 and with NM-3n, but in each

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case infection resulted in induction of a carrier state in only one monkey. In addition, one rhesus monkey (Macaca mulatta) each was infected with NM-3n or with the recovered virus from the NM-3n-infected cynomolgus monkey, and in each case infection resulted in induction of a carrier state. None of the animals, however, showed any clinical symptoms. (Sakuragi et al., 1992; Igarashi et al., 1994). The results indicate that these chimeras were still not suitable for practical use and that a more efficient infection system was required.

Recently, the importance of both the vpr and nef genes of SIVmac for viral pathogenesis in rhesus monkeys was reported (Kestler et al., 1991; Lang et al., 1993). For the construction of NM-3 and NM-3n, viral genomes from two different origins were joined using restriction enzyme sites within the vpr and nef genes and consequently NM-3 did not have intact vpr and nef whereas NM-3n did not have intact vpr. The lower infectivity and absence of pathogenicity of these chimeric viruses might be due to defects in vpr and/or nef. Therefore, in this study, we modified the genetic structure of the former chimeras to generate a new series of HIV-1/SIVmac chimeric viruses containing various combinations of intact vpr and/or nef from either HIV-1 or SIVmac and demonstrated that one of the new chimeras infected macaque monkeys very efficiently.

Methods

**DNA constructs.** The infectious molecular clones NL432 (HIV-1) (Adachi et al., 1986), MA239 (SIVmac), NM-3 (Shibata et al., 1991) and NM-3n (Igarashi et al., 1994) have been described previously.

To generate new chimeric viruses, we first produced several plasmids with various combinations of junction sequences. To create an MA239 vpx/HIV-1 vpr junction sequence, for example, we constructed a plasmid called pPR-12. First, the stop codon in the middle of nef (codon 93, nt 9353) of MA239 was changed by PCR mutagenesis from a stop (TAA) to a Glu (GAA) codon. Then, a BamHI-SacI fragment of NL432 which carries HIV-1 vpr was subcloned into pUC119 and an Nhel-PstI fragment of MA239/nef-open which carries SIVmac nef was subcloned into pUC18. The sequence of the NL432 env was modified by PCR to introduce an EcoRV site near the 3' terminus of its gene using a reverse primer 5' CAGATATCTTATAGCAAAATCCTTTCCAAGCC 3' (nt 5547-5587 of MA239) and an M13 forward primer and a reverse primer 5' CTCCCTCAAGGGTGTGTCTCCGTGTCGATTATAG 3' (nt 6322-6291 of MA239) and an M13 reverse primer. The two PCR products were then ligated and subcloned after digestion with Sphi (nt 6446 of MA239) and appropriate restriction enzymes, yielding the junction plasmid pPR-91011. Care was taken in the codon selection not to cause any amino acid substitutions in MA239 vpr by incorporation mutations. To generate NM-3R, which carries the intact SIVmac vpr upstream of HIV-1 tat (Fig. 1 a), the fragment was excised from pPR-91011 and inserted into the corresponding position of NM-3.

To create an HIV-1 env/SIVmac nef junction sequence [see Fig. 1 b (iv)] we constructed a plasmid called pPR-12. First, the stop codon in the middle of nef (codon 93, nt 9353) of MA239 was changed by PCR mutagenesis from a stop (TAA) to a Glu (GAA) codon. Then, a BamHI-SacI fragment of NL432 which carries HIV-1 env was subcloned into pUC119 and an Nhel-PstI fragment of MA239/nef-open which carries SIVmac nef was subcloned into pUC18. The sequence of the NL432 env was modified by PCR to introduce an EcoRV site near the 3' terminus of its gene using a reverse primer 5' CAGATATCTTATAGCAAAATCCTTTCCAAGCC 3' (nt 8793-8762 of NL432) and an M13 reverse primer. The sequence of MA239 nef was modified to have a SmaI site near the 5' terminus of the gene using a forward primer 5' AACCCGGTGGAGCTATTTCCTC- ATGAGGGCGTTC 3' (nt 9074-9105 of MA239) and an M13 reverse primer. The two PCR products were then ligated and subcloned after digestion with EcoRV or SmaI (both enzymes produce blunt ends) and appropriate restriction enzymes, and the junction plasmid pPR-12 was made [see Fig. 1 b (iv)]. To generate NM-3N, which carries the intact SIVmac nef downstream of HIV-1 env (Fig. 1 a), the junction fragment was excised from pPR-12 and inserted into the corresponding position of NM-3.

NM-3R, NM-3Rn and NM-3N were constructed from MA239, NL432 and NM-3 and the above-mentioned junction plasmids by standard recombinant DNA techniques using appropriate restriction sites. NM-3n, NM-3Rn, NM-3N and NM-3RN were constructed by recombination of NM-3r, NM-3N, NM-3R and NM-3n.

**Cells.** CD4+ human cell lines CEMx174 (Salter et al., 1985) M8166 (a subclone of C8166; Clapham et al., 1987) H9 (Popovic et al., 1984) and A3.01 (Folks et al., 1985) were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS). Peripheral blood mononuclear cells (PBMCs) from humans and monkeys were separated from heparinized whole blood, stimulated with 25 μg of concanavalin A/m for 24 h, and maintained in RPMI 1640 medium containing 10% FCS and crude or recombinant interleukin 2 as described previously (Shibata et al., 1991).

**Transfection and infection.** Samples of 10 μg of plasmid DNA were introduced into 2 x 105–3 x 106 cells of M8166 or CEMx174 by the DEAE–dextran method (Naidu et al., 1988). The culture medium was changed every 24 h and the supernatant was filtered (0.45 μm pore size) and stored at −80 °C. Then virion-associated reverse transcriptase (RT) activity was measured as previously described (Willy et al., 1988). The supernatant with the highest RT level was used as the virus stock. The TCID50 was determined using M8166 cells (Igarashi et al., 1994). The virus inocula used for infection were adjusted to contain equal RT units or TCID50 titres by adding an appropriate volume of the medium.
Fig. 1. Genetic structures of parental and HIV-1/SIVmac chimeric viruses. (a) Full-genome structures of NL432 (HIV-1), MA239 (SIVmac) and chimeric viruses. Solid and open boxes represent sequences derived from NL432 and MA239, respectively. Not-intact vpr and nef (due to chimeric recombinations) were previously described (Shibata et al., 1991) and are represented by dotted boxes. (b) Schematic representations of NL432/MA239 junctions of various chimeric viruses. (i) The MA239 vpx/NL432 vpr junction of the chimeric viruses NM-3rn, NM-3rN and NM-3r contains sequences of MA239 to the termination codon of vpx (to nt 6150) and those of NL432 from the initiation codon of vpr (from nt 5559). The nucleotide numbers given are from GenBank data base (accession numbers M19921 and M33262). The initiation codon of HIV-1 vpr was positioned at that of SIVmac vpr to maintain as similar a way of gene expression as possible. (ii) The MA239 vpr/NL432 tat junction of the chimeric viruses NM-3rn, NM-3rN and NM-3r contains sequences of MA239 to the termination codon of vpr (to nt 6456) and those of NL432 from 14 nt upstream of the initiation codon of tat (from nt 5816). (iii) The NL432 nef/MAM239 LTR junction of the chimeric viruses NM-3rn, NM-3rN and NM-3N was constructed by blunt-end ligation using a TaqI site (nt 9410) of NL432 and a Stul site (nt 9829) of MA239 as previously described (Igarashi et al., 1994). (iv) The NL432 env/MA239 nef junction of the chimeric viruses NM-3rn, NM-3rN and NM-3N contains sequences of NL432 to 1 nt downstream of the termination codon of env (to nt 8786) and those of MA239 from the initiation codon of nef (from nt 9077). The initiation codon of SIVmac nef was positioned at the same site as that of HIV-1 nef for the same reason as mentioned above.
to the virus stocks. Culture supernatants were collected every 3 days and their RT activities were monitored.

Western immunoblotting. Lysates of transfected cells were prepared as previously described (Willy et al., 1988) and proteins were resolved in SDS-polyacrylamide gels (10-18%) and transferred electrophoretically to nitrocellulose membranes (Immobilon; Millipore). The membranes were incubated with sera overnight at 4 °C and with horseradish peroxidase (HRP) — anti-human immunoglobulin (Caltag) or HRP-protein A (Amersham) for 1 h at 37 °C, washed and examined with an ECL western blotting detection system (Amersham).

Inoculation of monkeys and their follow-ups. Three male adult cynomolgus monkeys (M. fascicularis), three male adult rhesus monkeys (M. mulatta) and two female adult pig-tailed monkeys (M. nemestrina) were inoculated intravenously with NM-3RN. The virus stock of NM-3Rn was prepared 10 days after transfection into CEMx174 cells and 1 x 10^5 TCID_{50} per monkey were used for inoculation. Blood samples were collected periodically from the infected monkeys and their PBMCs were co-cultured with M8166 cells to test for virus re-isolation. Virus production was monitored by determination of the RT activity of the culture supernatants. Virus load was determined according to Kestler et al. (1991). Briefly, serial threefold dilutions of periodically collected CD8^+ cell-depleted PBMCs were performed and co-cultured with 1 x 10^6 M8166 cells for 6 weeks. The endpoint was determined by CPE observation and expression of viral antigens judged by indirect immunofluorescence assay. The antibody responses of the sera collected were examined by western immunoblotting, as described above, using HIV-1- and SIVmac-infected cells as antigens. Plasma antigenaemia was examined using an SIV core antigen assay kit (Coulter).

Results

Construction of chimeric viruses and their production of viral proteins

The genome organizations of the parental molecular clones of HIV-1 (NL432) and SIVmac (MA239) are very similar except for vpu and vpx. Since a prototype chimeric virus, NM-3, has defective vpr and nef genes, we designed new chimeric viruses with intact vpr and/or nef from either NL432 or MA239. As shown in Fig. 1 (a) seven new chimeras were constructed, each with a different combination of vpr and nef with respect to their genetic derivation. To examine whether viral proteins were expressed as intended for each DNA construct, the M8166 cell lysates obtained 3 or 4 days post-transfection were subjected to immunoblotting analysis. The serum from a cynomolgus monkey infected with NM-3 is known to recognize SIVmac Gag (p26) and HIV-1 Env (gp120) proteins and also to cross-react with HIV-1 Gag (p24) but not with SIVmac Env (gp130) (Sakuragi et al., 1992). By using this serum, it was confirmed that all the chimeras produced SIVmac Gag and HIV-1 Env proteins (data not shown), indicating that all the chimeras maintained their basic genetic structures. Similarly, Vpr proteins of HIV-1 (NM-3rn, NM-3Rn and NM-3r) and SIVmac (NM-3Rn, NM-3RN and NM-3r) were detected by using the anti-Vpr serum which reacts with Vpr of HIV-1 and SIVmac (Sato et al., 1990). HIV-1 Nef proteins were detected in NM-3rn, NM-3Rn and NM-3n by the anti-Nef serum which specifically reacts with Nef of HIV-1 (data not shown). However, the expression of Nef proteins of SIVmac was not examined because antisera to these proteins were not available.

The presence of nef accelerated replication

To compare the growth kinetics of all chimeras, infection experiments were performed in human CD4^+ cell line CEMx174 and monkey PBMCs (Fig. 2). Each graph shows the growth of a set of three chimeras which carried vpr of the same origin. Of the viruses with vpr from the same source, the chimeras carrying nef, irrespective of its origin, replicated more rapidly than the nef-defective chimera. The source of nef did not seem to have any significant effect. Similar results were obtained in M8166 cells and PBMCs from three different cynomolgus monkeys (data not shown).

Comparison of four chimeras having combinations of intact vpr and nef of different origins

To compare chimeras with vpr and nef of different origin, the following four viruses, NM-3rn, NM-3Rn, NM-3Rn and NM-3RN, were used for infection of H9, A3.01 and human or monkey PBMCs. All the virus stocks used for inoculation were found to be 1-1 x 10^6 TCID_{50}/ml using M8166 cells. In monkey PBMCs, chimeras with SIVmac nef replicated slightly faster than those with HIV-1 nef [Fig. 3 a (i)]. On the other hand, the chimeras with HIV-1 nef replicated to higher titres in human PBMC than those with SIVmac nef [Fig. 3 b (i)]. The parental viruses grew well in the original virus-host combinations [Fig. 3 a (ii), b (ii)]. Though these differences in replication were slight, it would seem that the growth potential of each chimera reflected that of the parental virus from which nef was derived. In comparison with parental viruses, the chimeras with SIVmac nef replicated faster, but to lower titres than parental SIVmac in monkey PBMCs, and the chimeras with HIV-1 nef replicated slower than parental HIV-1, but to the same titres in human PBMCs. Unlike nef, no effect of vpr was seen in human and monkey PBMCs since no significant difference was observed between two combinations of chimeras with vpr of different origin. Similar results were obtained with PBMCs from two cynomolgus monkeys and two human subjects. Moreover, essentially similar results to those with cynomolgus monkeys were obtained with two rhesus monkeys (data not shown).

The effect of nef origin was more clearly observed in H9 and A3.01 cells. In H9 cells, as in human PBMCs, parental NL432 replicated faster and to higher titre than
SIV Having vpr and nef of different origins

Fig. 2. Replication of chimeric viruses in (a) the human CD4+ cell line CEMx174 and (b) PBMC from a cynomolgus monkey. Cell-free virus stocks from M8166 cells transfected with the respective chimeric DNA clones were inoculated and RT production in the infected cells was monitored every 3 days. (i) Kinetics of three chimeric viruses carrying HIV-1 vpr. Symbols: ○, NM-3rn; ●, NM-3rN; □, NM-3r. (ii) Kinetics of three chimeric viruses carrying SIVmac vpr. Symbols: ○, NM-3Rn; ●, NM-3RN; □, NM-3R. (iii) Kinetics of three chimeric viruses carrying defective vpr. Symbols: ○, NM-3n; ●, NM-3N; □, NM-3.

MA239 [Fig. 4 a (ii)]. For the pair of chimeras with vpr of the same origin, those with SIVmac nef replicated slightly faster, but the titres of virus production of chimeras with HIV-1 nef were twice as high as those with SIVmac nef in H9 cells [Fig. 4 a (i)]. In A3.01 cells, the parental NL432 was replication competent whereas MA239 was not [Fig. 4 b (ii)]. The chimera with HIV-1 nef replicated faster and to higher titre (Fig. 4 b). These results support the notion that the growth of viruses was enhanced by HIV-1 nef in human cells. These experiments were performed three times, with similar results.

Though the growth of chimeras was not influenced by the vpr origin in PBMCs of both humans and monkeys, it was drastically different in H9 and A3.01 cells. In H9 cells, for the pair of the chimeras with nef of the same origin, the growth peaks almost reached the same height, but the onset of replication of chimeras with HIV-1 vpr was earlier than those with SIVmac vpr [Fig. 4 a (i)]. In A3.01 cells, virus production was observed only for chimeras with HIV-1 vpr (NM-3rn and NM-3rN) and the parental virus NL432, and not with chimeras with SIVmac vpr (NM-3Rn and NM-3RN) or another parental virus MA239 (Fig. 4 b). In attempts to rescue viruses from cultures infected with NM-3Rn, NM-3RN and MA239 in which no significant virus production was observed, the cultures 40 days after infection were cocultured with M8166 cells. Viruses were detected in cultures of NM-3Rn and NM-3RN, but not in those of MA239. These results indicate that all four chimeras (NM-3rn, NM-3rN, NM-3Rn and NM-3RN) replicated in A3.01, and that HIV-1 vpr was required for highly productive infection. The results suggest that the effect of
Fig. 3. Replication of four chimeric viruses having intact vpr and nef and parental viruses in (a) cynomolgus PBMCs and (b) human PBMCs. (i) Chimeric viruses: O, NM-3rn; ●, NM-3rN; □, NM-3Rn; ■, NM-3RN. (ii) Parental viruses: ▲, NL432; ▼, MA239; +, mock-infected. Infection of cynomolgus PBMCs was performed at an m.o.i. of 1:1 and that of human PBMCs at an m.o.i. of 0:1. All experiments were performed in duplicate and average RT values are shown.

vpr might be cell type dependent and that the origin of vpr reflected the growth of the parental virus in these cells.

Inoculation of macaque monkeys with NM-3rN

One of the newly constructed chimeras, NM-3rN, was examined for its ability to infect macaque monkeys in vivo. The virus stock of NM-3rN was prepared 10 days after transfection into CEMx174 cells. The virus stock of NM-3rN containing 1×10⁵ TCID₅₀ was inoculated intravenously into three rhesus monkeys, three cynomolgus monkeys and two pig-tailed monkeys, respectively. Viruses were consistently re-isolated from all monkeys 2 to 14 weeks post-inoculation (p.i.) (Table 1). Virus loads were very similar between the three species, though pig-tailed monkeys showed slightly higher virus loads. Virus loads reached the highest levels at 2 weeks p.i. (2.1×10⁴–1.9×10⁵) and decreased to 3.2×10⁴–1.2×10⁶ at 10–14 weeks p.i. An antigenaemia in cell-free plasma was detected at 2 weeks p.i. in two of three cynomolgus and two of three rhesus, but none of two
SIV Having vpr and nef of different origins

Fig. 4. Replication of four chimeric viruses having intact vpr and nef and parental viruses in (a) H9 cells and (b) A3.01 cells. (i) Chimeric viruses: ○, NM-3rn; ●, NM-3rN; □, NM-3Rn; ■, NM-3RN. (ii) Parental viruses: △, NL432; ▲, MA239; +, mock-infected. H9 cells were infected at an m.o.i. of 0.11, and A3.01 cells at an m.o.i. of 1.1. All experiments were performed in duplicate and average RT values are shown.

Table 1. Summary of virus recovery* and virus loads† of monkeys infected with NM-3rN

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<tr>
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<td>+</td>
<td>+</td>
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<tr>
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<td>-</td>
<td>+</td>
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<td>ND</td>
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<tr>
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<td>ND</td>
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<tr>
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<td>ND</td>
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<td>MN2150</td>
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* Re-isolation of virus was judged by RT production in co-cultures of M8166 cells and PBMCs from infected monkeys.
† Virus loads are show by the minimum numbers of PBMCs needed to yeild virus. ND, Not determined.
infected with NM-3rN, but only in one of two monkeys in the case of NM-3. Judging by virus re-isolation and NM-3rN inoculated monkeys and virus loads were present study, viruses were easily isolated from all the immune response of infected monkeys, the efficiency of infection with NM-3rN is higher than with our previous chimeric viruses, NM-3 and NM-3n. Li et al., constructed and infected cynomolgus monkeys with the rather high (to the level of about 10^4-10^6 PBMCs). Also, an antibody response was observed in all the monkeys and the western immunoblot analysis of sera from two rhesus, two cynomolgus and two pig-tailed monkeys is shown in Fig. 5. Antibodies to HIV-1 Env (gp120) and SIVmac Gag (p26) appeared about 6 weeks p.i. and increased thereafter in cynomolgus and rhesus monkeys. In pig-tailed monkeys, antibodies levels to HIV-1 Env (gp120) were very low, though antibodies to SIVmac Gag (p26) appeared in similar amounts to those found in the other two species. In the previous study with NM-3 or NM-3n, persistent viraemia was not observed in this period and virus recovery was successful from only one of two infected cynomolgus monkeys (Sakuragi et al., 1992; Igarashi et al., 1994). These results indicate that the infectivity of NM-3rN was higher than those of our former chimeras, NM-3 and NM-3n.

### Discussion

A series of chimeric viruses constructed in this study replicated in vitro, and the present in vivo experiments demonstrated that one of these chimeras, NM-3rN, infected macaque monkeys with high efficiency. In the case of NM-3 or NM-3n, virus isolation (for which 2 x 10^6-4 x 10^6 PBMCs were required) was positive at some time points from one of two inoculated monkeys (Sakuragi et al., 1992; Igarashi et al., 1994). In the present study, viruses were easily isolated from all the NM-3rN inoculated monkeys and virus loads were rather high (to the level of about 10^4-10^6 PBMCs). Also, an antibody response was observed in all the monkeys infected with NM-3rN, but only in one of two monkeys in the case of NM-3. Judging by virus re-isolation and immune response of infected monkeys, the efficiency of infection with NM-3rN is higher than with our previous chimeric viruses, NM-3 and NM-3n. Li et al. (1992) constructed and infected cynomolgus monkeys with the chimera designated SHIV-4 that has basically the same genome structure as NM-3RN but lacks the vpu gene carried by NM-3RN. However, they did not determine virus loads for SHIV-4. Therefore, we could not compare SHIV-4 with NM-3rN in terms of infection efficiency. In the case of SIVmac infection, 1 x 10^8 to 2 x 10^9 PBMCs were reported to be required for virus recovery in the early period (Daniel et al., 1992). Comparison with the results obtained with SIVmac may provide useful information on the efficiency of infection, although the experimental conditions, such as the cells used as an indicator, were not the same. In the very early period (2 and 4 weeks p.i.) the virus load of NM-3rN was comparable to that of parental SIVmac, though the virus load of NM-3rN thereafter decreased to a 10-100-fold lower level. In comparison with a nef deletion mutant of SIVmac, the virus load of NM-3rN was apparently higher. SIVmac/nef deletion, which did not induce disease, required > 10^6 PBMCs for virus recovery (Kestler et al., 1991). Plasma antigenaemia of NM-3rN-infected monkeys was detected in four of eight monkeys. Levels of p27 protein ranged from 0.04 to 0.37 ng/ml. These values are about 10-fold lower level than in SIVmac239 infection. In the case of SIVmac/nef deletion, however, no plasma antigenaemia was detected (Kestler et al., 1991). The results of virus loads and levels of plasma p27 in NM-3rN infection indicate that the in vivo replication ability of NM-3rN does not equal that of SIVmac239, but is apparently greater than that of SIVmac/nef deletion.

### Table 2. Plasma antigenaemia of monkeys infected with NM-3rN*

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<th>Species</th>
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<td>†</td>
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<td>—</td>
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<tr>
<td></td>
<td>MM64</td>
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<td>MF46</td>
<td>—</td>
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<tr>
<td></td>
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<tr>
<td>M. nemestrina</td>
<td>MN2150</td>
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<td></td>
<td>MN2170</td>
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* Plasma antigenaemia (ng/ml) was measured by a Coulter SIV core antigen assay kit.
† Values below 0.03 ng/ml were judged negative.

pig-tailed monkeys (Table 2). An antibody response to the virus was also observed in all monkeys and the western immunoblot analysis of sera from two rhesus, two cynomolgus and two pig-tailed monkeys is shown in Fig. 5. Antibodies to HIV-1 Env (gp120) and SIVmac Gag (p26) appeared about 6 weeks p.i. and increased thereafter in cynomolgus and rhesus monkeys. In pig-tailed monkeys, antibodies levels to HIV-1 Env (gp120) were very low, though antibodies to SIVmac Gag (p26) appeared in similar amounts to those found in the other two species. In the previous study with NM-3 or NM-3n, persistent viraemia was not observed in this period and virus recovery was successful from only one of two infected cynomolgus monkeys (Sakuragi et al., 1992; Igarashi et al., 1994). These results indicate that the infectivity of NM-3rN was higher than those of our former chimeras, NM-3 and NM-3n.

### Discussion

A series of chimeric viruses constructed in this study replicated in vitro, and the present in vivo experiments demonstrated that one of these chimeras, NM-3rN, infected macaque monkeys with high efficiency. In the case of NM-3 or NM-3n, virus isolation (for which 2 x 10^6-4 x 10^6 PBMCs were required) was positive at some time points from one of two inoculated monkeys (Sakuragi et al., 1992; Igarashi et al., 1994). In the present study, viruses were easily isolated from all the NM-3rN inoculated monkeys and virus loads were rather high (to the level of about 10^4-10^6 PBMCs). Also, an antibody response was observed in all the monkeys infected with NM-3rN, but only in one of two monkeys in the case of NM-3. Judging by virus re-isolation and immune response of infected monkeys, the efficiency of infection with NM-3rN is higher than with our previous chimeric viruses, NM-3 and NM-3n. Li et al. (1992) constructed and infected cynomolgus monkeys with the chimera designated SHIV-4 that has basically the same genome structure as NM-3RN but lacks the vpu gene carried by NM-3RN. However, they did not determine virus loads for SHIV-4. Therefore, we could not compare SHIV-4 with NM-3rN in terms of infection efficiency. In the case of SIVmac infection, 1 x 10^8 to 2 x 10^9 PBMCs were reported to be required for virus recovery in the early period (Daniel et al., 1992). Comparison with the results obtained with SIVmac may provide useful information on the efficiency of infection, although the experimental conditions, such as the cells used as an indicator, were not the same. In the very early period (2 and 4 weeks p.i.) the virus load of NM-3rN was comparable to that of parental SIVmac, though the virus load of NM-3rN thereafter decreased to a 10-100-fold lower level. In comparison with a nef deletion mutant of SIVmac, the virus load of NM-3rN was apparently higher. SIVmac/nef deletion, which did not induce disease, required > 10^6 PBMCs for virus recovery (Kestler et al., 1991). Plasma antigenaemia of NM-3rN-infected monkeys was detected in four of eight monkeys. Levels of p27 protein ranged from 0.04 to 0.37 ng/ml. These values are about 10-fold lower level than in SIVmac239 infection. In the case of SIVmac/nef deletion, however, no plasma antigenaemia was detected (Kestler et al., 1991). The results of virus loads and levels of plasma p27 in NM-3rN infection indicate that the in vivo replication ability of NM-3rN does not equal that of SIVmac239, but is apparently greater than that of SIVmac/nef deletion.

NM-3- or NM-3n-infected monkeys produced neutralizing antibodies to the chimera as well as the parental HIV-1, and cytotoxic T-lymphocytes (CTL) to HIV-1 Env (Igarashi et al., 1994). As NM-3rN has basically the same genome structure as NM-3 and NM-3n, the immune response of the NM-3rN-infected monkeys was probably similar, though neutralizing antibodies and CTL were not assessed in this study. At the time of submission, no clear clinical symptoms have been observed in monkeys infected with NM-3rN. But, even if NM-3rN does not induce any AIDS-like disease, its highly efficient infectivity shown in this study indicates that it may be suitable as a challenge virus for evaluating AIDS vaccine candidates based on HIV-1 Env, and allows the use of macaque monkeys instead of chimpanzees.

The chimeras constructed in this study have intact vpr and nef genes from either HIV-1 or SIVmac, or defective genes in some cases. The growth potential of these chimeras differed and this seemed to be due to the effects of vpr and nef. The effects of parameters other than vpr and nef, such as LTR and splice donors and acceptors, were not addressed in this study, since the principal aim was to select a chimera which displayed efficient macaque
Fig. 5. Antibody response of monkeys infected with NM-3rN determined by western immunoblotting. Plasma samples collected at intervals were treated with SDS-PAGE-resolved antigens of (a) parental HIV-1 (NL432) and (b) SIVmac (MA239) after transfer to membranes. The numbers of monkeys and weeks after inoculation are shown above. The serum from a cynomolgus monkey infected with NM-3 was tested as a positive control (P). Size markers are indicated on the left.
infection. We observed some effects of vpr and nef, though only the in vitro replication of chimeric viruses was compared. Acceleration of replication by nef was observed in all the cell types used. This effect of nef was shown not only with the chimeras, but also with a parental virus, MA239. MA239/nef-open (in which the stop codon TAA of nef was changed to GAA) replicated faster than MA239/nef-stop (original clone MA239) in monkey PBMCs (data not shown). These results are consistent with previous reports that nef has a positive effect on replication in PBMCs (De Ronde et al., 1992; Zazopoulos & Haseltine, 1993). Furthermore, results suggested that nef from both HIV-1 and SIVmac is functional in cells of both human beings and macaque monkeys and that the growth potential of each chimera reflects that of the parental virus from which nef was derived. This effect of nef may be related to the interaction between Nef and host cell protein(s).

The effect of vpr was observed in H9 and more clearly in A3.01 cells. Chimeras with HIV-1 vpr replicated faster than those with SIVmac vpr in H9 cells [Fig. 4 a (i)]. Furthermore, chimeras with HIV-1 vpr replicated in A3.01, but those with SIVmac vpr did not, although virus rescue was successful [Fig. 4 b (i)]. SIVmac (SIVmac251) is reported not to replicate in A3.01 cells (Koenig et al., 1989). We also observed that one parental virus HIV-1 (NL432) was competent for replication, whereas another parental virus SIVmac (MA239) showed no detectable virus production and even rescue of the virus was not successful [Fig. 4 b (ii)]. In addition to the region including tat, rev, vpu and env of HIV-1, which all four chimeras share, HIV-1 vpr appears to confer highly productive infection in A3.01 cells. Moreover, the growth potential of each chimera in these cells reflected that of the parental virus from which vpr was derived.

For in vivo study, only one of the chimeras could be used due to the limited availability of monkeys. We chose to test the chimera that grew best in macaque PBMC. Chimeras with SIVmac nef replicated faster in macaque PBMC. Moreover, infection with NM-3n, which carried HIV-1 nef, was no more efficient than with NM-3 which lacks an intact nef gene (Igarashi et al., 1994, Sakuragi et al., 1992). Thus, we considered that the nef gene from SIVmac would be more suitable for in vivo replication. With regard to choice of vpr genes, no clear results were observed in macaque PBMC with respect to its origin, but chimeras carrying HIV-1 vpr replicated more efficiently in established cell lines. In addition, the genetic structure of the vpr region is much closer (in terms of the sites of ORFs, splicing acceptors and donors) to parental viruses in chimeras carrying HIV-1 vpr than in those carrying SIVmac vpr. For these reasons, NM-3rN carrying HIV-1 vpr and SIVmac nef was inoculated into the monkeys. We can not exclude the possibility that other chimeras which were not tested in vivo in this study may present with higher in vivo infectivity. Comparative infection experiments using the other chimeras will be useful for clarifying the in vivo effects of both vpr and nef.

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References


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