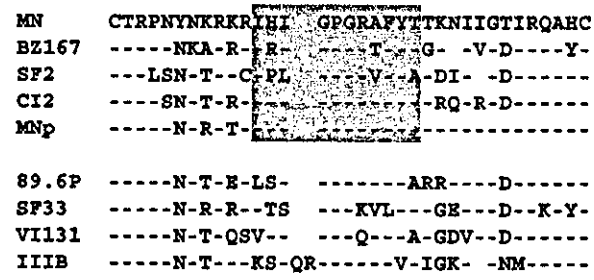


FIG. 5. Alignment of the amino acid sequences of HIV-1 Env V3 from primary and laboratory isolates. The spaces indicate amino acid deletions; dashes indicate homology. The V3 motif of a neutralization-sensitive HIV-1 strain is enclosed in the shaded rectangle (37).

crease in viral load following challenge, and the levels remained high until the animal was sacrificed. These results demonstrate that vaccination with rBCG Env V3 can induce protective immunity in rhesus macaques against a low-dose challenge with SHIV-MN.

**Challenge with high-dose SHIV-MN.** The second group of eight macaques (R-09 through R-16) was similarly challenged with a higher dose (200 $TCID_{50}$) of SHIV-MN by intravenous inoculation at 24 weeks p.i. (Fig. 6). Measurements of the viral loads in PBMC and plasma indicated that all the macaques were infected by the high-dose SHIV-MN challenge. However, the level of viremia during the acute phase of viral infection was reduced by 1 to 2 log units in macaques immunized with rBCG Env V3 compared with controls (from $10^6$ to $10^7$, to $<10^5$ to $10^4$ RNA copies/ml) (Fig. 6A). The control macaques developed a transient decrease in $CD4^+$-T-cell counts that rebounded to normal levels ~3 weeks postchallenge (Fig. 6B). In contrast, macaques vaccinated with rBCG Env V3 had little or no change in $CD4^+$-T-cell numbers.

Despite the low levels of V3 peptide-specific IFN-γ ELISPOT activities noted for animals R-09 and R-10 above (Table 2), these animals exhibited a plasma viral load and a rate of $CD4^+$-cell loss after SHIV challenge that was comparable to those seen in the immunized animals designated R-11, -12, and -13. Thus, immunization with rBCG Env V3 generated even low levels of T-cell responses in only 2 animals out of 5 in this group and out of a total of 15 immunized animals. No evidence of higher virus-specific IFN-γ ELISPOT activity was demonstrated in samples obtained 0, 4, or 6 and 24 weeks after vaccination (Table 2), suggesting that few significant cellular anti-SHIV responses were generated and that those few did not affect virus control in this macaque population.

**Challenge with pathogenic SHIV-89.6PD.** The third group of macaques (R-17 through R-24) was challenged with pathogenic SHIV-89.6PD (20 $TCID_{50}$) 24 weeks postinoculation. The effects of vaccination with rBCG Env V3 on immune induction against the pathogenic virus were followed for 32 weeks, and the macaques were then autopsied. As shown in

TABLE 2. SHIV-MN-specific serum IgG neutralization titers and Env V3-specific ELISPOT responses[a]

| Monkey no. | Immunogen | $IC_{50}$ of neutralization serum IgG (μg/ml)[b] | | | V3-specific IFN-γ SFCs/$10^6$ cells[c] | | |
|---|---|---|---|---|---|---|---|
| | | 0 week | 4 or 6 weeks[d] | 24 weeks[e] | 0 week | 4 or 6 weeks | 24 weeks |
| R-01 | rBCG Env V3 | >50 | 0.5 | 0.6 | <20 | 30 | 20 |
| R-02 | rBCG Env V3 | >50 | 0.3 | 0.4 | <20 | 40 | 40 |
| R-03 | rBCG Env V3 | >50 | 0.5 | 0.6 | <20 | 40 | 30 |
| R-04 | rBCG-Env V3 | >50 | 0.2 | 0.3 | <20 | 20 | 40 |
| R-05 | rBCG-Env V3 | >50 | 0.08 | 0.3 | <20 | 30 | 80 |
| R-06 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-07 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-08 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-09 | rBCG-Env V3 | >50 | 0.04 | 0.3 | <20 | 180 | 120 |
| R-10 | rBCG-Env V3 | >50 | 0.1 | 0.2 | <20 | 160 | 110 |
| R-11 | rBCG-Env V3 | >50 | 0.05 | 0.2 | <20 | 20 | 30 |
| R-12 | rBCG-Env V3 | >50 | 0.03 | 0.4 | <20 | 60 | 20 |
| R-13 | rBCG-Env V3 | >50 | 0.02 | 0.4 | <20 | 30 | 30 |
| R-14 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-15 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-16 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-17 | rBCG-Env V3 | >50 | 0.2 | 0.6 | <20 | 40 | 90 |
| R-18 | rBCG-Env V3 | >50 | 0.3 | 0.3 | <20 | 50 | 60 |
| R-19 | rBCG-Env V3 | >50 | 0.3 | 0.4 | <20 | 40 | 30 |
| R-20 | rBCG-Env V3 | >50 | 0.5 | 0.7 | <20 | 20 | 50 |
| R-21 | rBCG-Env V3 | >50 | 0.4 | 0.5 | <20 | 20 | 40 |
| R-22 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-23 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |
| R-24 | rBCG-α | >50 | >50 | >50 | <20 | <20 | <20 |

[a] Animals were inoculated with either rBCG Env V3 or the vector control. Blood samples were obtained at 0, 4, or 6 and 24 weeks p.i., and antibody inhibitory concentration and the V3-specific IFN-γ ELISPOT activity were compared.
[b] The $IC_{50}$ was derived from the data in Fig. 2 based on neutralization dose-response curves similarly obtained from Fig. 3.
[c] Freshly isolated PBMC were assessed for their ability to produce IFN-γ in response to HIV-$1_{MN}$ Env V3 peptide.
[d] Mean $IC_{50}$s: R-01 to R-05, 0.32; R-09 to R-13, 0.05; R-17 to R-21, 0.35.
[e] Mean $IC_{50}$s: R-01 to R-05, 0.44; R-09 to R-13, 0.30; R-17 to R-21, 0.50.

TABLE 3. Comparison of low-dose SHIV-MN infections in macaques vaccinated with either rBCG Env V3 or rBCG-α (control)

| Monkey | Immunogen (10 mg) | Efficacy analysis | Results[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0[b] | 2 | 4 | 6 | 8 | 10 | 12 | 16 |
| R-01 | rBCG Env V3 | Virus isolation | <1 | <1 | 2 | <1 | <1 | <1 | <1 | <1 |
| | | Provirus by PCR | <500 | <500 | >500 | <500 | <500 | <500 | <500 | <500 |
| | | Plasma viral load | <500 | <500 | 20,000 | <500 | <500 | <500 | <500 | <500 |
| R-02 | rBCG Env V3 | Virus isolation | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| | | Provirus by PCR | <500 | <500 | <500 | <500 | <500 | <500 | <500 | <500 |
| | | Plasma viral load | <500 | <500 | <500 | <500 | <500 | <500 | <500 | <500 |
| R-03 | rBCG Env V3 | Virus isolation | <1 | 32 | <1 | <1 | 2 | <1 | ND | ND |
| | | Provirus by PCR | <500 | >500 | <500 | <500 | >500 | <500 | ND | ND |
| | | Plasma viral load | <500 | 310,000 | <500 | <500 | 20,000 | <500 | ND | <500 |
| R-04 | rBCG Env V3 | Virus isolation | <1 | <1 | <1 | <1 | <1 | <1 | ND | ND |
| | | Provirus by PCR | <500 | <500 | <500 | <500 | <500 | <500 | ND | ND |
| | | Plasma viral load | <500 | <500 | <500 | <500 | <500 | <500 | <500 | <500 |
| R-05 | rBCG Env V3 | Virus isolation | <1 | <1 | <1 | <1 | <1 | <1 | <1 | ND |
| | | Provirus by PCR | <500 | <500 | <500 | <500 | <500 | <500 | <500 | ND |
| | | Plasma viral load | <500 | <500 | <500 | <500 | <500 | <500 | <500 | <500 |
| R-06 | rBCG-α | Virus isolation | <1 | 32 | 16 | <1 | 2 | 2 | <1 | 1< |
| | | Provirus by PCR | <500 | >500 | >500 | >500 | >500 | >500 | >500 | 500 |
| | | Plasma viral load | <500 | 300,000 | 50,000 | 20,000 | 20,000 | 20,000 | 20,000 | 20,000 |
| R-07 | rBCG-α | Virus isolation | <1 | 2 | 32 | <1 | <1 | <1 | <1 | ND |
| | | Provirus by PCR | <500 | >500 | >500 | >500 | >500 | <500 | <500 | ND |
| | | Plasma viral load | <500 | 27,000 | 310,000 | 350,000 | 25,000 | <500 | <500 | <500 |
| R-08 | rBCG-α | Virus isolation | <1 | 32 | 16 | <1 | 2 | 2 | ND | ND |
| | | Provirus by PCR | <500 | >500 | >500 | >500 | >500 | >500 | ND | ND |
| | | Plasma viral load | <500 | 300,000 | 50,000 | <500 | 20,000 | 20,000 | ND | <500 |

[a] Viral loads were determined by either limiting dilution of PBMC or competitive PCR for HIV-1 Env V3 genes, and the results are expressed as the number of infected cells per million PBMC and virus copies per milliliter of blood. Nested PCR for HIV-MN Env V3 was used in all macaques to detect the provirus genome. Naïve macaques were injected intravenously with 20 $TCID_{50}$ of SHIV-MN and used as controls for SHIV infection. The results are expressed as the mean of three different assays; <1, <500, and <500 were the detection limits of virus isolation, proviral copy number, and plasma viral load, respectively. ND, not determined.

[b] Weeks after challenge.

Fig. 7, high levels of plasma viremia were detected in the control macaques, with a viral set point of ~$10^6$ RNA copies/ml, accompanied by an abrupt decline in $CD4^+$-T-cell counts. Prior vaccination with rBCG Env V3 appeared to have no positive effect on the viral load and $CD4^+$-T-cell counts compared with the control animals.

**Association of in vitro neutralization antibody responses following rBCG Env V3 immunization with control of viremia after SHIV challenge.** Of the macaques challenged with low doses of homologous SHIV-MN (group 1), the three virus-controlled macaques R-02, -04, and -05 (Table 1) had higher $IC_{50}$s of SHIV-MN-specific neutralizing antibodies as measured in M8166 cells at 24 weeks p.i. or on the day of challenge, with serum IgG concentrations of 0.4, 0.3, and 0.3 μg/ml, respectively (Table 2). The $IC_{50}$s of the uncontrolled macaques R-01 and -03 (Table 1) were both 0.6 μg/ml (Table 2).

When the challenge dose was increased 10-fold (Fig. 1), all five animals in group 2 had high neutralizing antibody titers with a mean $IC_{50}$ of 0.30 μg/ml on the day of challenge (Table 2). These animals in group 2 showed partial protection against the same homologous virus challenge (Fig. 6). In contrast, no animals similarly immunized with rBCG elicited any in vivo protection against a low-dose, heterologous viral challenge with SHIV-89.6PD (Table 2 and Fig. 7).

In summary, the rBCG Env V3-elicited NAb response afforded some degree of protection against a homologous viral challenge. However, infection by the heterologous virus SHIV-89.6PD was not controlled by heterologous virus SHIV-MN- or $HIV\text{-}1_{MN}$-specific NAb generated by the recombinant $HIV\text{-}1_{MN}$ Env V3-expressed BCG immunization.

## DISCUSSION

First, our study demonstrates the potential of anti-Env V3 NAb induced by immunization of rhesus macaques with rBCG Env V3 to afford protection against homologous challenge with SHIV-MN but not against the heterologous SHIV-89.6PD. With the low-dose homologous SHIV-MN challenge (20 $TCID_{50}$), sterile protection was achieved in three of five immunized animals. These findings correlate well with our in vitro neutralization data for these animals. Protected animals showed higher levels of potent neutralization antibodies than did unprotected animals. Macaques serving as vector and naïve controls experienced high levels of replication of the SHIV-MN challenge virus. With a high-dose challenge, rBCG Env V3 vaccination was effective at reducing viremia during acute infection by ~100-fold. The vaccine consisted of an rBCG vector that expresses a chimeric HIV-1 Env V3 region peptide and the α-antigen of *M. bovis*. The kinetics and magnitude of the HIV-1 Env V3-specific antibody responses elicited in macaques were comparable to those observed in our previous studies using guinea pigs vaccinated with rBCG Env V3 (9, 16).

Secondly, the levels of neutralizing antibodies generated after injection with a recombinant BCG vector-based vaccine expressing a chimeric protein of HIV-1 Env V3 peptide and α-antigen protein were maintained for at least 24 weeks p.i. with no diminishment in titer. A plausible explanation for the longevity of the neutralizing antibody titers after rBCG immunization is that the carrier protein, α-antigen (also known as MPT59 or antigen 85B), is derived from mycobacteria and has

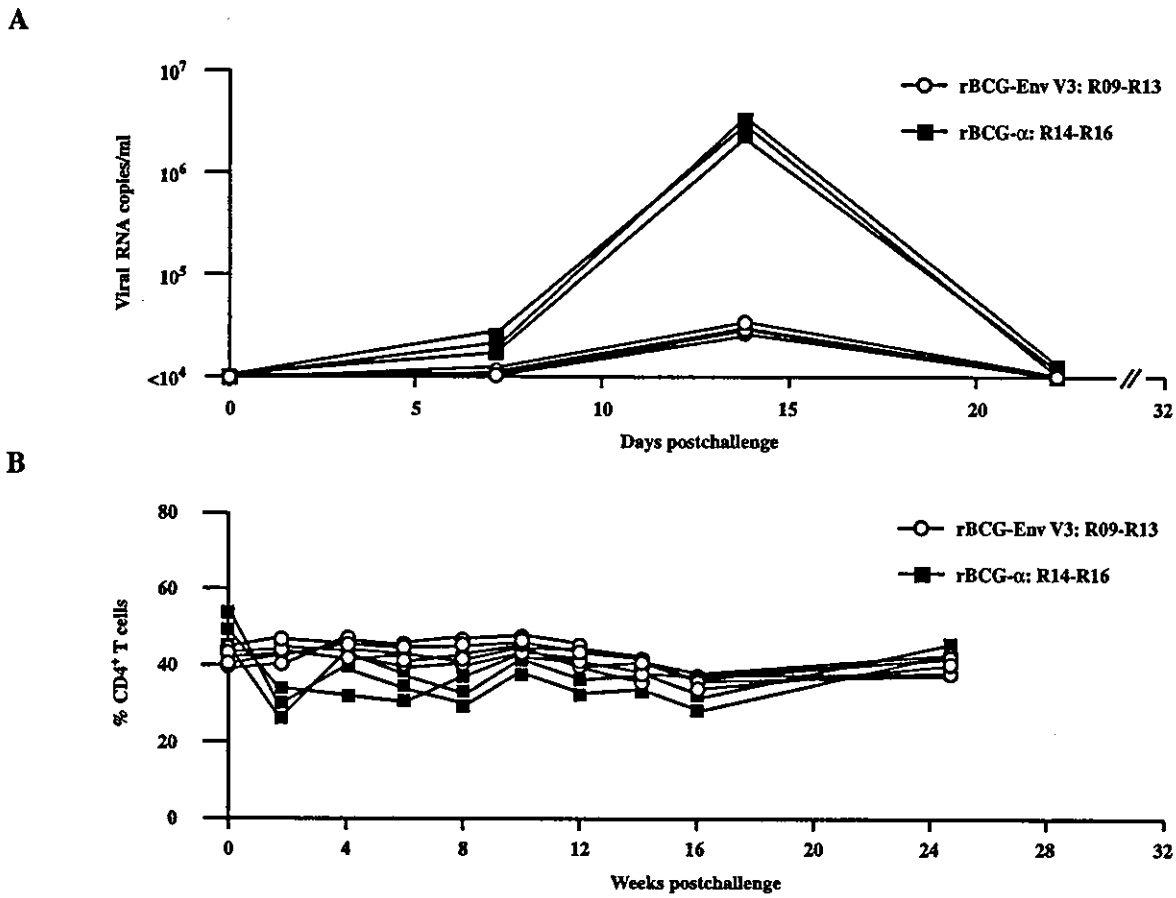


FIG. 6. Comparison of infection kinetics following high-dose (200 $TCID_{50}$) inoculation of SHIV-MN in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Viral RNA copy number per milliliter of serum. (B) $CD4^+$-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

the ability to elicit potent Th1-type immune responses (24, 43). Our result is consistent with those of other groups, which have shown that BCG immunity is maintained for at least a few years and that the BCG bacillus is effective at increasing NAb responses (40). These characteristics might help to explain the long-lasting enhanced levels of NAb elicited by vaccination with rBCG Env V3.

The concentration of purified macaque IgG in serum was determined to be ~10 mg/ml. By this estimation, 0.5 mg corresponds to a serum dilution of 1:1 in virus neutralization assays. The $IC_{50}$ and $IC_{90}$ values for neutralization of SHIV-MN were $10^3$ to $10^4$ and 166, respectively (similar values were obtained for neutralization of HIV-$1_{MN}$). These neutralization titers suggest that antibody responses generated de novo may contribute to a degree of protection against SHIV-MN. The observed relationship of the NAb titer and viral protection is consistent with results obtained by repeated immunization with SHIV-89.6 C4-V3 peptides in guinea pigs and rhesus macaques (6, 27). In this case, NAb titers to homologous SHIV-89.6 were ~$10^3$ greater than those against heterologous HIV-$1_{MN}$, while responses to HIV-1 R5 viruses were weak or absent. This suggests that the protection mediated by a C4-V3 peptide vaccine against SHIV-89.6 may be type (or strain) specific. Thus, we assume that the NAb generated by SHIV-89.6 C4-V3 peptide immunization (6) would not mediate protection against a heterologous SHIV-MN challenge.

The present study suggests that the vaccine-elicited antibodies directed against the HIV-1 Env V3 peptide can in some cases confer a degree of neutralization against primary isolates of HIV-1 (26). Following vaccination of rhesus macaques with rBCG Env V3, both binding and NAb responses against this novel construct were clearly evident. At the time of SHIV challenge, immune sera from the vaccinated macaques efficiently neutralized a homologous, type-specific TCLA HIV-1 strain (HIV-$1_{MN}$) and a related SHIV strain (SHIV-MN) with $IC_{90}$ values of <5 μg/ml. Controls, including preimmune sera and sera from macaques vaccinated with rBCG vector alone, had no neutralizing activity in assays using GHOST cells expressing either CCR5 or CXCR4 or in M8166 cells. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize several primary HIV-1 X4 isolates (HIV-$1_{BZ167}$, HIV-$1_{SF2}$, and HIV-$1_{CI2}$); however, neutralization of an X4-R5 dual-tropic strain (HIV-$1_{MNp}$) was weak. No neutralization of HIV-1 R5 isolates and primary HIV-1 isolates from different clades was observed. These findings were confirmed in an independent international neutralization trial (conducted by Simon Beddows and Jonathan Weber, Imperial College School of Medicine, Medical Research Council, London, En-

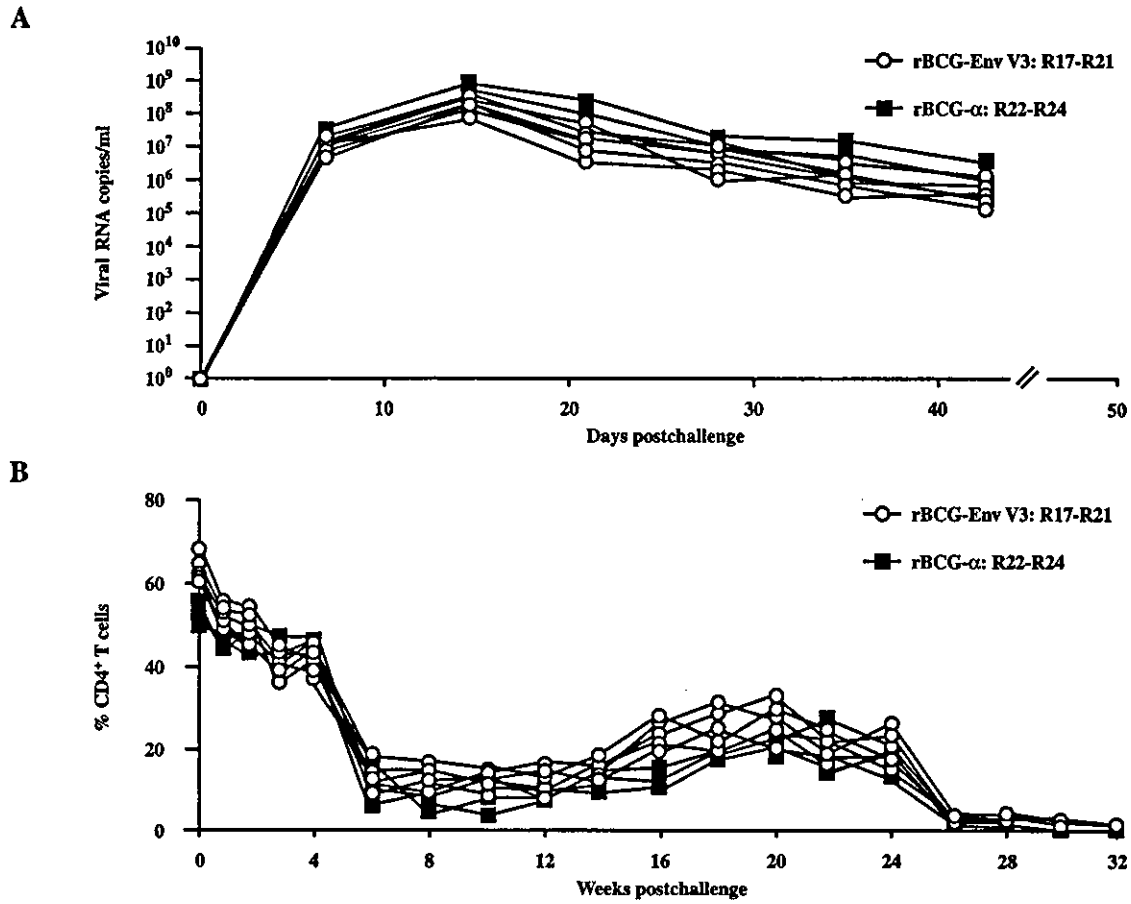


FIG. 7. Comparison of infection kinetics following challenge with pathogenic SHIV-89.6PD in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Plasma viral-RNA copy numbers per milliliter. (B) $CD4^+$-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

gland, and Pia Scott and Eva-Maria Fenyo at Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden). Preliminary results from this study have had been summarized and reported (11). Despite similarities in the V3 sequence motif, neutralization of the TCLA strain HIV-$1_{MN}$ was found to be 10- to 50-fold more sensitive than neutralization of primary HIV-1 isolates, such as HIV-$1_{CI2}$, HIV-$1_{MNp}$, or HIV-$1_{JR\text{-}CSF}$ (11). A reasonable explanation for the relative insensitivity of primary HIV-1 isolates—particularly primary HIV-1 R5 isolates—to neutralization is the presence of cryptic or occluded sites within the virus-associated V3 region (13, 53).

In the Japanese consensus HIV-1 Env V3 expressed in the rBCG construct, the core V3 motif of the neutralization epitope is IHIGPGRAF (39). Although the consensus sequence of the V3 loop differs from the MN-V3 sequence in five amino acid positions, the neutralization epitope of the tip V3 region in the Japanese consensus is identical to that of MN-V3. Some substitutions of amino acids at certain positions within this motif (for example, H to R and A to T in the core motif in BZ167) are tolerated, suggesting that NAbs generated by immunization with rBCG Env V3 are not strictly type specific. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize primary HIV-1 X4 and some HIV-1 X4-R5 dual-tropic isolates, suggesting that the antigenic structure of the chimeric V3 peptide mimics to some extent that of the virus-associated V3 region. Indeed, the chimeric V3–α-antigen protein is estimated to be 38 kDa and contains four cysteine residues, suggesting the possible formation of a new loop structure in the V3 portion of the protein. With regard to the heterologous SHIV-89.6PD challenge in macaques vaccinated with rBCG Env V3, NAbs specific for SHIV-89.6PD were not generated efficiently ($IC_{50}$, >50 μg of immune serum IgG/ml) and did not provide any protection against the SHIV-89.6PD challenge. The V3 neutralization site of SHIV-89.6PD may differ in sequence or structure or both from that of SHIV-MN or other viral strains, including some of the HIV-1 isolates, making it unrecognizable to antibodies. Such a difference could account for the poor cross-neutralization activity against SHIV-89.6PD.

Thus, our data from the SHIV-macaque models show that the in vitro neutralization titers generated in rBCG-immunized animals correlate with protection. Although a present goal of HIV-1 vaccine development is to reduce the viral set point by eliciting high levels of virus-specific cellular immune responses, induction of cross-reactive NAbs may also contribute to control virus replication in the course of HIV-1 infection and may therefore be useful in the context of a preventive vaccine. Furthermore, although the choice of HIV Env V3 and the

autologous challenge virus SHIV-MN are unlikely to provide information that predicts efficacy in humans, the results presented here demonstrate that recombinant BCG vectors have the potential to deliver a more appropriate immunogen for desirable immune elicitations.

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# Sequence Note

# Determination of HIV Type 1 CRF01_AE gag p17 and env-V3 Consensus Sequences for HIV/AIDS Vaccine Design

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## ABSTRACT

**A molecular epidemiological study of the gag p17 and env-V3 regions on HIV-infected drug users and blood donors was carried out in northern Thailand from 1998 through 2002 to determine the predominant subtype and consensus sequence (CS) for circulating HIV-1 strains. CRF01_AE was concluded to be a predominant strain and the nucleotide CSs in gag p17 and env-V3 showed only 1.26% and no difference from CS in the Los Alamos database, respectively. Our env-V3 CS was identical to the previously published CSs, suggesting that the CS was very conserved from 1990 through 2002 in Thailand. Gag p17 and env-V3 nucleotide sequences of seroconvertors in our subjects were quite similar to the CS and conserved for at least 9 and 6 years postinfection, respectively. These results suggest that the CS approach to the HIV-1 antigen design could overcome HIV diversity and help us develop an effective HIV/AIDS vaccine.**

THE ANTIGEN GENES IN THE FIRST-GENERATION HIV/AIDS CANDIDATE VACCINES were obtained from isolated viruses. However, the range of amino acid (AA) changes of each isolate demonstrated more than 30% difference from the CRF01_AE consensus envelope sequence.[1] Considering the diversity of each circulating virus, investigators find it very difficult to select an optimal antigen from numerous isolates to develop an efficacious preventive HIV/AIDS vaccine. Currently, three computational methods (consensus, ancestral, and center of the tree) are being considered as a strategy for a novel antigen design of an HIV/AIDS vaccine to overcome HIV diversity.[1,2] However, these novel types of antigen have never been used for vaccine construction to control the HIV-1 CRF01_AE epidemic in Thailand. From 1998 to 2002, to characterize currently circulating viruses in northern Thailand, we determined HIV-1 subtypes among HIV-1-seropositive drug users (DUs) and blood donors (BDs) in Chiang Rai in northern Thailand using provirus sequences of gag p17 and env-V3 regions. Furthermore, CSs of both regions were classified and compared with those in the database,[3] in 1990s isolated samples[4] and in seroconvertors,[5] respectively.

One hundred and nineteen HIV-1-infected DUs and 96 BDs were investigated. Their $CD4^+$ and $CD8^+$ T lymphocyte absolute count indicated a mean value of 308/$\mu$l (range: 8–1449/$\mu$l) and 747/$\mu$l (range: 98–3079$\mu$l) in DU, respectively. Phylogenetic tree analysis of the gag p17 and env-V3 regions revealed that the predominant HIV-1 subtype was CRF01_AE with 88% and 97% in the DU and BD groups, respectively (data not shown). Other minor subtypes were classified as B′ (Thailand variant of subtype B) and B in the both groups.

CSs of the CRF01_AE gag p17 (CSg) and env-V3 (CSe) regions were determined using proviral sequences derived from

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35 and 126 individuals, respectively. These CSs were compared to the available CRF01_AE CS (CSD) in the database (Table 1).[3] In the gag p17 region, nucleotide sequence and AA alignment difference showed means of 2.66 and 5.11% from our CSg, respectively. CSg showed a difference of 1.26% in nucleotides and 3.79% in AA, as compared with CSD.[3] The average magnitude ($n = 16$) of the gag p17 nucleotide and AA difference between CSg and each isolate in Table 1 showed no significant difference from circulating viruses in our specimens (Student's $t$ test). Furthermore, CSg and CSD of gag p17 were close to the isolates prior to 1993 than to those after 1994.

In the case of the env-V3 region, the mean differences of our nucleotide and AA sequences from CSe were 5.55 and 11.59%, respectively. The CSe of nucleotide and AA sequences were

TABLE 1. COMPARISON OF OUR CONSENSUS SEQUENCES WITH DATABASE OF HIV-1 CRF01_AE gag P17 AND env-V3[a]

| | *Compared to our CS of gag p17* | | *Compared to our CS of env-V3* | |
|---|---|---|---|---|
| *Sample name* | *Different nuc (%)* | *Different AA (%)* | *Different nuc (%)* | *Different AA(%)* |
| 93TH057 | 4.55 | 9.85 | 7.62 | 14.29 |
| 93TH065 | 2.27 | 5.30 | 0.95 | 2.86 |
| 95TH253 | 3.03 | 6.06 | 12.38 | 37.14 |
| 93TH902 | 4.29 | 8.33 | 7.62 | 8.57 |
| 94TH702 | 3.03 | 6.06 | No data | No data |
| 94TH7091 | 3.03 | 6.06 | No data | No data |
| 95TNIH022 | 3.79 | 6.82 | 20.95 | 42.86 |
| 95TNIH047 | 3.79 | 7.58 | 16.19 | 31.43 |
| 97TH6-107 | 3.54 | 6.06 | 0.95 | 0.00 |
| CM235 | 1.26 | 2.27 | 2.86 | 5.71 |
| CM238 | 1.77 | 4.55 | 0.95 | 2.86 |
| CM239 | No data | No data | 0.95 | 2.86 |
| CM240 | 2.27 | 5.30 | 2.86 | 5.71 |
| CM241 | No data | No data | 0.00 | 0.00 |
| CM242 | No data | No data | 0.95 | 2.86 |
| CM243 | 1.01 | 1.52 | 5.71 | 14.29 |
| CM244 | No data | No data | 0.00 | 0.00 |
| TN240 | 1.52 | 3.79 | No data | No data |
| TN245 | 2.02 | 3.03 | No data | No data |
| 92TH022 | 1.01 | 2.27 | 0.95 | 2.86 |
| CS from database[3] | 1.26 | 3.79 | 0.00 | 0.00 |
| CS from McCutchan *et al.*[4] | No data | No data | 0.00 | 0.00 |
| CS from Subbarao *et al.*[5] | No data | No data | 0.00 | 0.00 |
| Calculated by above samples | | | | |
| Mean ($n = 16$) | 2.64 | 5.30 | 5.12 | 10.89 |
| Range | 1.01–4.55 | 1.52–9.85 | 0–20.95 | 0–42.86 |
| Calculated by our samples | | | | |
| Mean ($n$ = gag p17, env-V3 = 35,126) | 2.66 | 5.11 | 5.55 | 11.59 |
| Range | 0.76–4.80 | 2.27–9.73 | 0–15.24 | 0–40.00 |
| Calculated by seroconvertors' samples[5] | | | | |
| Mean ($n = 102$) | No data | No data | 3.14[b] | 6.49[b] |
| Range | No data | No data | 0–11.43 | 0–22.86 |
| Years after seroconversion in our samples | | | | |
| <1: Mean ($n$ = gag p17, env-V3 = 9, 13) | 3.17 | 4.80 | 4.91 | 9.67 |
| Range | 1.52–5.05 | 1.52–6.82 | 0.95–7.62 | 2.83—17.14 |
| 1–3: Mean ($n$ = gag p17, env-V3 = 0, 12) | No sample | No sample | 5.16 | 9.29 |
| Range | | | 1.90–0.48 | 2.86–17.14 |
| 3–6: Mean ($n$ = gag p17, env-V3 = 9, 21) | 2.40 | 4.58 | 5.62 | 12.24 |
| Range | 0.76–4.13 | 2.27–7.03 | 0.95–15.24 | 2.86–31.43 |
| >6–9: Mean ($n$ = gag p17, env-V3 = 9, 12) | 2.81 | 6.05 | 7.70[c] | 17.38[d] |
| Range | 1.52–4.29 | 3.03–9.73 | 1.90–14.29 | 2.86–31.43 |

[a]CS, consensus sequence; nuc, nucleotide; AA, amino acid.
[b]Significantly different from our samples ($p < 0.0001$).
[c,d]Significantly different from <1 sample ($p = 0.038$ and 0.010).

identical to those of CSD. The average distance ($n$ = 16) of the env-V3 nucleotide and AA sequence of each isolate in the database[3] from CSe was 5.12 and 10.89%, respectively, and they showed no significant differences from circulating viruses in our specimens. CSs of nucleotide and AA of env-V3 in seroconvertors[5] who were infected during 1995–1998 were also identical to CSe. However, there was an average of 3.14% in nucleotide and 6.49% in AA differences that were significantly closer to CSe than to other available sequences. Furthermore, the CS of env-V3 that was determined by six specimens (CM235–CM244) isolated in 1990 in northern Thailand[4] was identical to CSe. 93TH065[6] and 92TH022[7] isolated from early seroconvertors indicated high similarity to CSs in both regions.

From our investigation, it was revealed that the consensus nucleotide and AA sequences of env-V3 in circulating CRF01_AE were very conserved from 1990 through 2002 in Thailand[4,5,7–9] and that the nucleotide sequence distance in the C2–V3 region from seroconvertors in DU was low[5] and each isolate was more similar to the env-V3 CS than our isolates. In addition, seven of 102 samples in DU seroconvertors in Bangkok[5] and two of six CM series samples[4] showed quite high homogeneity to CSe. In our subjects, 27 of 35 in gag p17 and 58 of 126 in env-V3 had definitive risk for HIV-1 infection from the data of Sawanpanyalert *et al.*[10–12] and were divided into four groups by year after seroconversion (less than 1 year, 1–3 years, 3–6 years, and >6–9 years). Using nucleotide and AA sequences in both regions, we compared seroconvertors (less than 1 year) with other year groups and with seroconvertors in a Bangkok DU cohort[5] as an env-V3 control. The sequence differences in gag p17 and env-V3 were not statistically significant among all year groups or the Bangkok DU group except for the >6–9 years group, which showed significant difference ($p$ = 0.038 in nucleotide and 0.010 in AA levels), suggesting that seroconvertors possessed a quite similar sequence and maintained the similarity for at least 6 years. These results suggest that the CRF01_AE sequence was conserved for quite a long time and a certain specific type of virus whose sequence is close to the CS might be the one that is mainly transmitted. Furthermore, taking account of the high homogeneity of our CSs to the early isolates during the pandemic in Thailand (93TH065, 92TH022, and CM series), CRF01_AE has not been evolving in a unique direction but at random and then consequently conserves its CSs, as compared to the sequence of isolates in the pandemic stage.

As for another subtype, the env and gag AA sequences in HIV-1 subtype C isolates shared identity 92–95% and 90.5–98.7% with the South African CS, respectively.[13] Subtype C gag p17 and p24 AAs were relatively conserved and showed less than 10% diversity to CS in Botzwana.[14] Abidjan AA CS of gag p24 was 99.32% identical to reference CRF02_AG.[15] The 4 incidence and 19 prevalence cases were 97.1 and 96.6% homologous to Abidjan CS, respectively, and the range in incidence cases was narrower than that in prevalence cases.[15] These results suggest that the CS could provide a less distant virus sequence in the population than each circulating isolate even in the different subtypes.

In summary, a consensus approach for antigen genes could minimize the sequence distance from each circulating isolate and may provide an effective strategy to construct an HIV/AIDS vaccine candidate that could induce broad protective efficacy against diverse HIV-1 isolates.

## ACCESSION NUMBERS

gag p17: AB115505–AB115540; env-V3: AB115782–AB115907.

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# *The Normalization of Guinea Pig Leukocyte Fractions and Lymphocyte Subsets in Blood and Lymphoid Tissues using a Flow Cytometric Procedure*

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***Abstract:*** *Many hematological and immunological parameters remain unclear in the study of the guinea pig. In this study, we established the mean values of blood counts, the percentage of leukocyte fractions and lymphocyte subsets in blood and various lymphoid tissues of the guinea pig with a flow cytometric procedure using MIL4/SSC. The mean counts of WBC and RBC in the blood were lower, and MCV and MCH were higher than those of other rodents, resembling those of humans. Furthermore, the mean percentages of blood lymphocytes were smaller and that of granulocyte was larger than those of other rodents, resembling those of humans. We further established a flow cytometric procedure for lymphocyte subsets and clarified the mean percentages of T- and B-cells, $CD4^+$-, $CD8^+$- and MHC Class $II^+$- T-cells, and $CD4^-CD8^-$ T-cells. The latter were morphologically larger in cell size and cytoplasm than $CD4^+$- plus $CD8^+$ T-cells, and this subset had a significantly higher percentage in newborn animals. Furthermore, the appearance of the MHC Class $II^+$ T-cell subset was suggested to be a marker of hyper-activation of T-cells in BCG-immunized animals. Thus, both the novel flow cytometric procedure for leukocyte fractions and lymphocyte subsets, and the established normal values will be useful tools in studying guinea pigs as models of various diseases and biological phenomena.*

***Key words:*** *flow cytometry, guinea pig, immunological values, leukocyte fraction, T-cell subpopulation*

## Introduction

The guinea pig has been a widely used experimental animal for studies on human tuberculosis and other mycobacterial infections because of the particular susceptibility of this species to *Mycobacterium tuberculosis* [15]. Furthermore, vaccinating guinea pigs with *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) has

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several important advantages over vaccinating mice such as delayed-type hypersensitivity skin reactions (DTH) to PPD and development of lesions that are similar to those induced in humans [1]. Moreover, the animal has been also used as an animal model for syphilis [30, 31], other genital diseases [6], tinnitus [7, 8, 17], shock [19], allergic diseases [10, 29] and various tumors [5, 22].

Monoclonal antibody and flow cytometric analysis have been widely used to study immunology, pathology and physiology including experimental disease in humans and mice [2, 4]. The number and composition of the blood counts and lymphocyte subsets in the blood and tissue are assumed to be influenced by many factors. However, the precise phenotype of cells and immune responses in the guinea pig have been difficult to analyze because of the limited availability of anti-guinea pig antibodies and animal strains, which remain to be established.

In this study, we devised a flow cytometric method using commercially and personally available antibodies to fractionate guinea pig leukocytes, i.e., lymphocytes, monocytes, granulocytes and Kurloff cell included fractions in the blood and various lymphoid tissues of guinea pigs. Furthermore, we clearly identified T-cells and B-cells, $CD8^+$ T-cells, $CD4^+$ T-cells and MHC Class $II^+$-activated T-cells, and $CD4^-CD8^-$ T-cell subsets, most of which were compared between adult and newborn guinea pigs. Further, we suggest that the appearance of MHC Class $II^+$ T-cell subpopulation in BCG-hyperinoculated animals may be a marker of lymphocyte hyperactivation.

## Materials and Methods

*Animals*: Hartley strain guinea pigs were obtained from Japan SLC, Inc., Japan. We used three groups of female guinea pigs. The first group comprised 3 days old guinea pigs (N=12), the second group, 8 weeks old guinea pigs (N=60), and the third group, 2 years old guinea pigs (N=12). The animals were kept in a specific-pathogen-free area according to the Institutional Animal Care and Use Guidelines of the National Institute of Infectious Diseases (NIID), Japan.

*Antibodies*: This study used FITC-labeled mouse monoclonal antibodies (mAbs) anti-guinea pig CD4 (T helper/inducer, CT7, Serotec Ltd., Kidlington, Oxford, UK), anti-guinea pig CD8 (CT6, Serotec), anti-guinea pig B-cells (Msgp9, Serotec) and anti-guinea pig T-cells (CT5, Serotec) [24, 28], anti-porcine granulocyte (MIL4, Serotec) [25] and anti-guinea pig MHC Class II antigen (27E7) [24], anti-asialo GM1 (Wako Pure Chem.Ldt., Tokyo, Japan) [14], PE-labeled Goat anti-rabbit IgG-$F(ab')_2$ (Caltag Lab., Burlingam, CA), streptavidin-cychrome (Pharmingen, Sam diego, CA ) and PE-labeled rabbit anti-mouse IgG-$F(ab')_2$ (Serotec).

*Isolation of blood and various tissue cells*: Blood cells were hemolized by adding ACK lysis buffer consisting of 0.15 M $NH_4Cl$, 0.1 mM EDTA-2Na, and 1.0 mM $KHCO_3$ at ratio of 13:1 and incubated for 5 min at RT. The cells were washed twice and were resuspended in a PBS staining buffer with 0.1% $NaN_3$ and 3% FCS. Splenocytes, lymph node cells and thymocytes were teased out using a mesh filter Cell Strainer (Becton Dickinson Labware, Franklin, NJ) and hemolized by adding ACK lysis buffer. After washing, the cells were resuspended in a PBS staining buffer.

*Flow cytometric assay*: Three color staining. The cells ($5 \times 10^5$ cells/50 µl) were incubated with purified antibody at 4°C for 30 min and washed twice with a cold staining buffer. PE-conjugated rabbit anti-mouse IgG $F(ab')_2$ was then added at 4°C for 30 min, and the cells were washed twice with the staining buffer. Finally, the cells were stained with FITC-conjugated antibodies at 4°C for 30 min followed by 2 more washes with the staining buffer.

*Four color staining*: The cells ($5 \times 10^5$ cells/50 µl) were incubated with a purified anti-asialo GM1 rabbit antibody at 4°C for 30 min and washed twice with a cold staining buffer. PE-conjugated goat anti-rabbit IgG $F(ab')_2$ was then added at 4°C for 30 min, and the cells were washed twice with the staining buffer. Then, the cells were stained with biotin-conjugated CT7 (CD4) and CT6 (CD8) at 4°C for 30 min followed by 2 washes with the staining buffer. Streptavidin-cychrome (Pharmingen) was added 4°C for 30 min, and the cells were washed twice with the staining buffer. Finally, the cells were stained with FITC-conjugated CT5 (T cell) at 4°C for 30 min followed by 2 more washes. Target cells were stained with respective Abs according to the manufacturer's instruction. To remove dead cells 200 µl of propidium iodide (Sigma Chem. Co., St Louis, USA) at a concentration of 5 µg/ml was added, and viable cells were analyzed on a FACScalibur

**Table 1.** Panel of anti-guinea pig mAb used in the flow cytometry[a]

| Clone name | Specificity | Sections[b] | Dye[c] | Ig class |
|---|---|---|---|---|
| Msgp9 | Pan B-cell | F, C | FITC | IgG |
| 31D2 | IgM | F, I | Purify | IgG |
| CT5 | Pan T-cell | F, C, P | FITC, Purify | IgG |
| MIL4 | Porcine neutrophil and eosinophil | F, C | FITC | IgG |
| CT7 | CD4 (T helper/inducer) | F, C | FITC, biotin | IgG |
| CT6 | CD8 (T suppressor/cytotoxic) | F, C | FITC, biotin | IgG |
| R27E | MHC Class II | F, I | Purify | IgG |
| Asialo GM1 | Mouse and rat NK cells | F, I | Purify | IgG |

[a] Anti-guinea pig mAb R27E was a kind gift from Dr. R. Burger, Robert Koch Institute, Berlin, Germany, and the others were obtained from Serotec. [b] Method identifying the specificity of mAb: F, flow cytometry; I, immunohistology; C, immunohistology-cryostat; and P, immunohistology-paraffin. [c] Labeled dye of mAb; FITC, FITC-labeled mAb; Purify, purified mAb; and biotin, biotinylated mAb.

(Becton-Dickinson, San Joes, CA, USA) equipped with an argon laser set at 488 nm using Cell Quest software (Becton-Dickinson). The emission wavelengths used for FITC, PE, PI and cychrome were 530 nm, 580 nm, 610 nm and 630 nm respectively [32, 33]. The viable cells were sorted with a FACS Vantage SE (Becton-Dickinson) equipped with argon, dye and UV lasers.

*Differentiation of leukocyte fractions of guinea pig and further study of its lymphocyte subpopulations*: Leukocyte of guinea pig was initially gated into 4 fractions by flow cytometry using MIL4/SSC parameter [26]. Then, the lymphocyte fraction of guinea pig gated with another SSC/FSC parameter was adjusted by the results, which were simultaneously obtained with the lymphocyte fraction gated by the MIL4/SSC parameter as described above. The lymphocytes subpopulations were further studied with the adjusted lymphocyte population by using various guinea pig antibodies as described in Table 1. Data were expressed as the mean percent positive cells ± SD.

*Blood count*: Blood counts were performed according to the manufacturer's instructions with an automated blood analyzer Celltac (Nihon Koden, Tokyo, Japan). Both white and red blood cells of guinea pigs were also counted with a hemacytometer chamber and the results obtained with the two methods described above were confirmed as identical.

*Morphological studies*: The sorted cells were cytocentrifuged using Cytospin 3 (Shandon, Life Sciences International, Runcorn, UK) and stained with the May-Giemsa solution (Merck KGaA, Darmstadt, Germany) as previously described [21, 33].

**Table 2.** Mean value of the hemograms of blood sample from normal guinea pigs[a]

| Item | Unit | Mean | SD |
|---|---|---|---|
| RBC | $10^4/\mu l$ | 499 | 47 |
| WBC | /μl | 5,438 | 1,689 |
| Hb | g/dl | 14.7 | 1.3 |
| Ht | % | 43.0 | 7.8 |
| MVC | fl | 87.7 | 4.9 |
| MCH | pg | 29.7 | 1.4 |
| MCHC | g/dl | 33.8 | 2.3 |
| PLT | $10^4/\mu l$ | 41.7 | 10.5 |

[a] Data are expressed as the mean ± SD. N=60.

*Statistical analysis*: Calculations were performed to determine the geometric mean ± SD. Statistical comparisons were performed using Student's *t*-test. Statistical significance was defined as $P<0.05$.

## Results

*Mean values of the blood counts of the guinea pigs*

We initially determined the mean values of the hemograms of heart-punctured blood samples from 60 Hartley guinea pigs with 8 weeks old weighing 400 g by adjusting the automated blood analyzer to study guinea pig blood samples (Table 2). The mean absolute counts of white blood cells (WBC), red blood cells (RBC) and platelets (PLT) were 5438 ± 1689, $499 \times 10^4 \pm 47$ and $41.7 \times 10^4 \pm 10.5/\mu l$, respectively. The

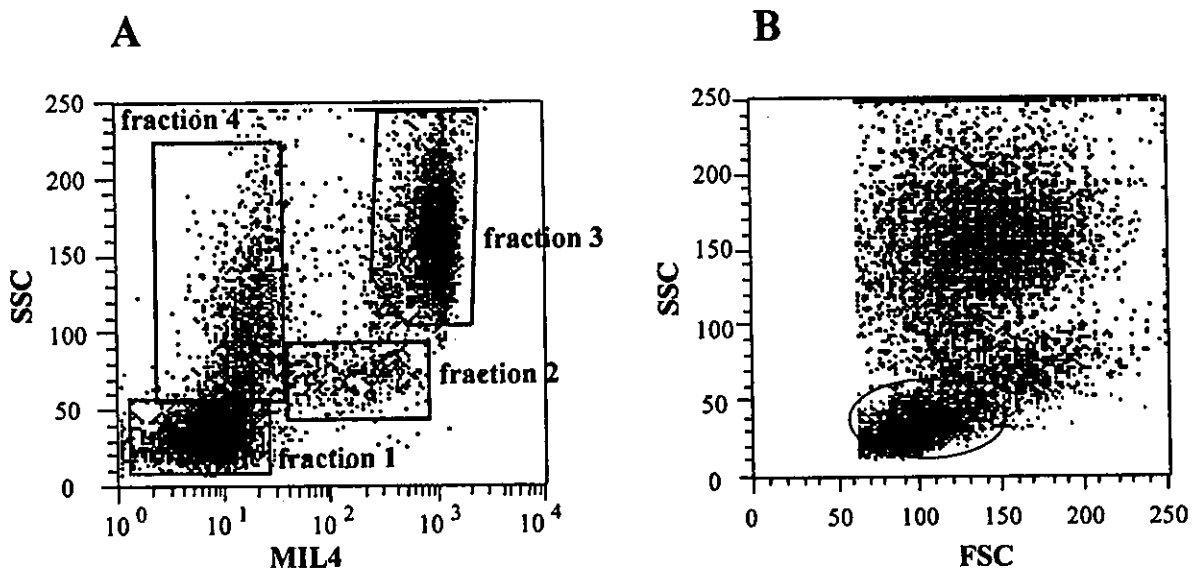


**Fig. 1.** The leukocyte fractions in various lymphoid tissues of guinea pigs using flow cytometry. (A) Two-color analysis was performed with the leukocytes of lymphoid tissues. Dead cells were removed by propidium iodide and viable cells were differentiated with MIL4/SSC. Fraction 1, lymphocyte population (Green dots); fraction 2, monocyte population; fraction 3, granulocyte population; and fraction 4, Kurloff cell-containing population. (B) Accurate gating of the lymphocyte population reflected by MIL4/SSC flow cytometry for the lymphocyte fraction described above. The green dots show the lymphocyte population by gating with FSC/SSC. Two color flow cytometry was applied to the gated lymphocyte population to study lymphocyte subsets and subpopulations.

mean values of hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were 14.7 ± 1.3 g/dl, 43.0 ± 7.8%, 87.7 ± 4.9 fl, 29.7 ± 1.4 pg, and 33.8 ± 2.3 g/dl, respectively.

*Gating of WBC fractions by flow cytometric analysis using MIL4/SSC*

One problem in studying immunological reactions in guinea pigs is due to difficulty in identifying each blood fraction by conventional gating of blood cells using light scatter (FSC/SSC). As shown in Fig. 1, the guinea pig leukocytes were successfully gated into 4 leukocyte fractions using a novel gating method with MIL4/SSC in the blood and lymphoid tissues of the 60 young guinea pigs at the age of 8 weeks old.

As summarized in Table 3, the lymphocyte fraction was the major fraction at a level of 95% of tissue cells in both the lymph node (LN) and the thymus. The predominance of lymphocyte fraction was less in the spleen and the blood, at 68.2 ± 7.2 and 48.7 ± 14.1%, respectively. This lymphocyte fraction dominance in the

**Table 3.** Mean percentage of leukocyte fraction in the blood sample and lymphoid tissues of normal guinea pigs by novel flow cytometry using the MIL4/SSC parameter[a)]

| | | Mean | SD |
|---|---|---|---|
| Blood | Lymphocyte | 48.7 | 14.1 |
| | Monocyte | 4.7 | 2.6 |
| | Granulocyte | 33.6 | 14.2 |
| | KC | 11.7 | 6.5 |
| Spleen | Lymphocyte | 68.2 | 7.2 |
| | Monocyte | 4.4 | 1.6 |
| | Granulocyte | 3.1 | 1.4 |
| | KC | 19.4 | 7.3 |
| Thymus | Lymphocyte | 96.5 | 0.6 |
| | Monocyte | 0.6 | 0.2 |
| | Granulocyte | 0.1 | 0.0 |
| | KC | 2.5 | 0.8 |
| LN | Lymphocyte | 94.8 | 1.4 |
| | Monocyte | 1.1 | 0.4 |
| | Granulocyte | 0.1 | 0.1 |
| | KC | 1.8 | 0.4 |

[a)] Leukocytes of guinea pigs were gated into 4 fractions by flow cytometry using the MIL4/SSC parameter shown in Fig. 1A. Data are expressed as the mean ± SD. N=60.

**Table 4.** Mean percentage value of the lymphocyte subset in the blood samples and various lymphoid tissues of normal guinea pigs by novel flow cytometry consisting of MIL4/SSC followed by FSC/SSC and specific antibody analysis[a)]

| | | Mean | SD |
|---|---|---|---|
| Blood | T | 54.2 | 10.7 |
| | $CD4^+$ | 26.6 | 9.9 |
| | $CD8^+$ | 16.1 | 12.1 |
| | $CD4^-CD8^-$T | 16.6 | 6.7 |
| | B | 45.7 | 11.8 |
| | R27E | 50.8 | 13.6 |
| Spleen | T | 56.1 | 4.3 |
| | $CD4^+$ | 26.4 | 4.1 |
| | $CD8^+$ | 22.8 | 6.6 |
| | $CD4^-CD8^-$T | 5.6 | 2.0 |
| | B | 43.1 | 4.4 |
| | R27E | 42.1 | 8.5 |
| Thymus | T | 94.0 | 2.6 |
| | $CD4^+$ | 6.5 | 3.1 |
| | $CD8^+$ | 11.2 | 6.2 |
| | $CD4^+CD8^+$ | 70.7 | 8.4 |
| | $CD4^-CD8^-$T | 8.8 | 3.7 |
| | IgM (31D2) | 1.5 | 0.6 |
| | R27E | 1.2 | 0.7 |
| L.N | T | 34.3 | 11.3 |
| | $CD4^+$ | 22.5 | 5.4 |
| | $CD8^+$ | 11.2 | 5.3 |
| | $CD4^-CD8^-$T | <0.1 | – |
| | B | 59.1 | 10.3 |
| | R27E | 61.1 | 13.1 |

[a)] Lymphocyte fraction was gated by the MIL4/SSC parameter, and each lymphocyte subset was studied by flow cytometry using the specific antibody shown in Table 1. The data are expressed as the mean ± SD. N=60.

lymphoid tissues and the blood has been similarly reported in humans and other rodents. The granulocyte fractions were 33.6 ± 14.2 and 3.1 ± 1.4% in blood and the spleen, respectively, and those in the thymus and LN were almost null. The MIL4-/$SSC^{high}$ fractions containing Kurloff cells were 11.7 ± 6.5, 19.4 ± 7.3, 2.5 ± 0.8 and 1.8 ± 0.4% in the blood, spleen, thymus and LN, respectively, indicating that MIL4-/$SSC^{high}$ Kurloff cells are mainly located in the spleen and blood of normal healthy guinea pigs (Table 3).

*Detection of guinea pig-lymphocyte subsets in blood and various lymphoid tissues*

The lymphocyte subsets of the guinea pigs were determined by flow cytometry with three color staining using a consecutive flow cytometric procedure. The lymphocyte gating procedure initially used gating with MIL4/SSC, followed by gating with FSC/SSC (Fig. 1). The available antibodies against guinea pig-cell surface markers that are described in Table 1 were used to detect specific lymphocyte subsets and subpopulations (Table 4). Initially, the MHC Class II antigen expression on B-cells was verified with the staining of B (Msgp9) and MHC Class II (R27E) (data not shown). The mean percentage of T- and B-cells was 54.2 ± 10.7 and 45.7 ± 11.8% in the total lymphocytes of the blood samples, respectively.

The lymphocyte subsets of the $CD4^+$ and $CD8^+$ cells were 26.6 ± 9.9 and 16.1 ± 12.1% in the blood, 26.4 ± 4.1 and 22.8 ± 6.6% in the spleen, and 22.5 ± 5.4 and 11.2 ± 5.3% in LN, respectively, demonstrating that $CD4^+$ lymphocytes were greater in number than the $CD8^+$ lymphocytes in blood samples, the spleen and the lymph nodes. The thymocytes mostly expressed T-cell marker CT5 at a rate of 94.0 ± 2.6%, and $CD4^+CD8^+$ double positive cells were 70.7 ± 8.4%. However, membrane $IgM^+$ thymocytes were negligible.

Since the accumulated percentage of $CD4^+$ plus $CD8^+$ lymphocytes was smaller than that of the pan-T-cells, we further analyzed the lymphocyte fraction by three color immunostaining for CD4 plus CD8 antigens vs. Pan-T-cell antigen (Fig. 2A). As expected, we identified the $CD4^-CD8^-$ T-cell population at 16.6 and 5.6% in the blood samples and the spleen, respectively. We then sorted the $CD4^-CD8^-$ T-cell population and a mixed population with the $CD4^+$ plus $CD8^+$ T-cell population. As shown in Figs. 2B and 2C, the $CT7^-CD8^-$ T-cells were bigger in size and richer in cytoplasm than those of $CD4^+$ plus $CD8^+$ T-cells as revealed by May-Giemsa staining. The mixed cell population with $CD4^+$ plus $CD8^+$ T-cells was homogeneous and was the so-called small lymphocyte that possesses a pyknotic nucleus and a narrow cytoplasm. Furthermore, approximately 40% of the $CD4^-CD8^-$ T-cell subpopulation was positive for asialo GM1 (Figs. 2D and 2E).

*Comparison of percentage positives of lymphocyte subsets between the adult and newborn guinea pigs*

The mean percentage for T, B, $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^-$ T-cells were compared between adult and newborn guinea pigs at the ages of 2 years old (N=12)

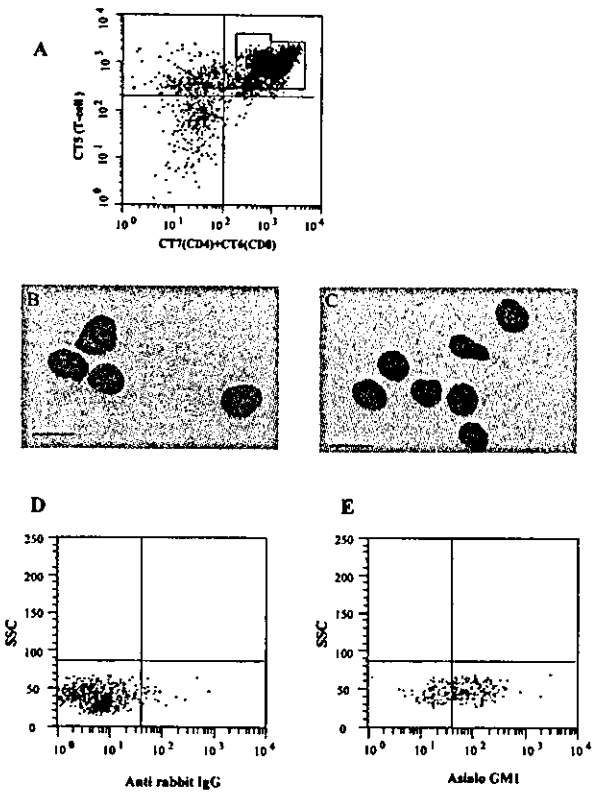


**Fig. 2.** Detection of a CD4⁻CD8⁻ T-cell subset and its morphological characterization. (A) The population of CD4⁻CD8⁻ T-cells was detected by flow cytometry using three color staining of the lymphocyte fraction with FITC-CT6, FITC-CT7 and PE-CT5. Dead cells were removed by propidium iodide, and viable cells were analyzed. The $CD4^{high}CD8^{high}$ and CD4⁺CD8⁺ subpopulations represent CD8⁺ and CD4⁺ T-cell subsets, respectively. B and C: Light-microscopic analysis of CD4⁻CD8⁻ (B) and CD4⁺CD8⁺ T-cells (C) by May-Giemsa staining. The lymphocyte subpopulation was purified by flow cytometric sorting from normal guinea pig blood. Detection of asialo GM1⁺ cells in the CD4⁻CD8⁻T-cell subpopulation. The asialo GM1⁺ cell subpopulation was detected by four color staining. Dead cells were removed by propidium iodide and remaining viable cells were analyzed. Lymphocyte population was initially gated with FSC/SSC followed by gating with CT5/CT7 and CT6 for detecting the CD4⁻CD8⁻ T-cell subpopulation. Then, gating with SSC/anti-rabbit IgG was used as reference for the gating (D), and asialo GM1⁺ cells in CD4⁻CD8⁻ T-cell subpopulation were detected by gating with SSC/anti-asialo GM1 rabbit antibody followed by PE-anti-rabbit IgG (E).

and 3 days old (N=12) (Fig. 3). The newborn animals were significantly lower in CD8⁺ lymphocytes at a percentage of 10.2 ± 2.7 than the adult animals (17.4 ± 5.6%, $P<0.01$). On the other hand, the newborn animals had a significantly higher percentage of CD4⁻CD8⁻ T-cells, at 25.4 ± 10.1, than the adults with 15.5 ± 4.3% ($P<0.05$). The other subsets of the T, B and CD4⁺ lymphocyte rates did not differ between the two groups.

*Appearance of MHC Class II⁺ T-cell subset in the blood after intravenous inoculation of a large amount of BCG*

We studied the effect of BCG inoculation on the percentage change in lymphocyte subsets using the procedure established above. By intravenous inoculation of 5 mg of BCG-Tokyo vaccine strain, the immunized animals expressed MHC Class II antigen on approximately 30% of the T-cells in the spleen at 6 weeks post-inoculation (Fig. 4A). In contrast, spleen cells from non-immunized animals or animals immunized with 0.1 mg of BCG intradermally showed no MHC Class II⁺ T-cells (Fig. 4B), suggesting that expression of MHC Class II antigen on T-cells may be a marker of hyper-activation of T-lymphocytes in animals inoculated with a large amount of BCG.

## Discussion

The guinea pig has been used as model animal to study various infectious diseases, allergies and tumors, and it has been especially used for the study of BCG and *Mycobacterium tuberculosis*. However, guinea pigs have limitations for immunological study because of a lack of immunological procedures and reagents compared with mice.

In this study, we established flow cytometric procedures enabling differentiation between guinea pig leukocyte fractions and lymphocyte subsets. Applying anti-MIL4 antibody, which recognizes porcine neutrophils, eosinophils and basophils [9], and was cross-reacted with guinea pig granulocyte and monocytes, and SSC to gate the leukocyte fractions, the mean percentages of the lymphocyte and granulocyte fractions in the blood were 48.7 ± 14.1 and 33.6 ± 14.2%. In humans, blood lymphocytes and granulocytes are 40 ± 13 and 53 ± 17% [20]. On the other hand the mean values of lymphocytes and granulocytes were reported as 81 ± 10 and 15 ± 11% in mice, 70 ± 13 and 24 ± 18% in rats, and 73 ± 19 and 22 ± 5% in hamsters [20]. Thus, other rodents show a higher percentage of lymphocytes and a lower percentage of granulocytes than

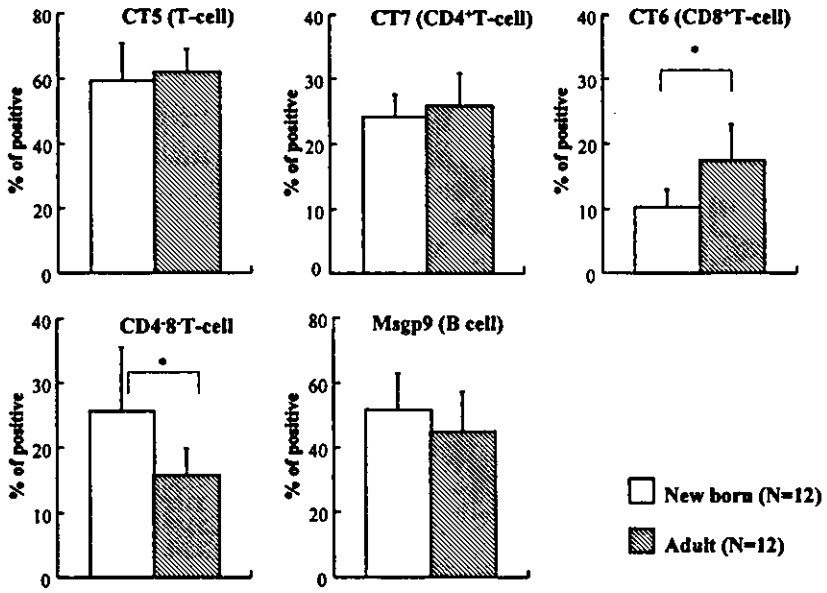


**Fig. 3.** Comparison of percentages of lymphocyte subsets between adult and newborn guinea pigs. The open column shows the mean results from newborn guinea pigs between 3 to 11 days, and the hatched column shows the mean results from adult guinea pigs aged 2 years. The data are expressed as the mean ± SD. *, $P<0.05$ significant difference between the two groups of guinea pigs.

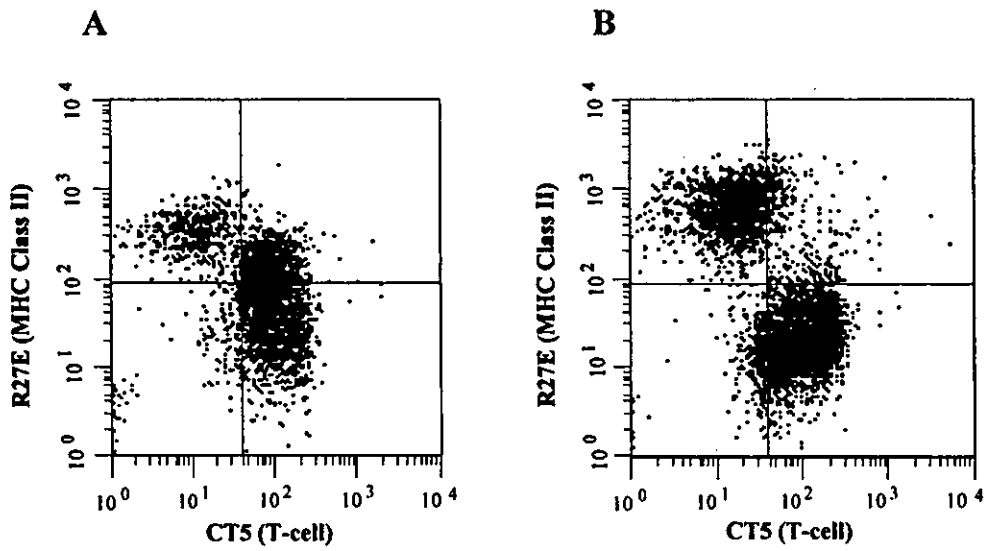


**Fig. 4.** An MHC Class II+ T-cell subpopulation appeared in the spleen at 6 weeks post-intravenous inoculation with large amounts of BCG. Dead cells were removed by propidium iodide, and viable cells of the lymphocyte gate were analyzed. (A) Appearance of the unique MHC Class II+ T-cell subpopulation in animals inoculated with a high dose of BCG intravenously. (B) MHC Class II+ T-cell subpopulation in normal control animals was not detected.

those of guinea pigs. Are there any differences in the leukocyte fractions obtained by the different methods of morphological analysis by other researchers and our flow cytometry using MIL4/SSC parameter? In the study of human leukocyte fractions, human CD45/SSC gating separated the leukocyte fractions consisting of

lymphocytes, monocytes and granulocytes. Moreover, the flow cytometric procedure obtained an identical count for each leukocyte [16]. These results suggest that the mean values of each leukocyte fraction of the guinea pigs are more similar to those of humans than those of other rodents.

Interestingly, DTH induction and hormone physiology in BCG-immunized guinea pigs are reported to be more similar to those of humans than other rodents [13, 18, 27]. Recently, molecular analysis has revealed that the CD1 gene family of the guinea pig is much more similar to humans than mice and other rodents [3, 11, 12]. Thus, the guinea pig should be a better model animal because of the resemblance of immunity, hormone physiology and genetic background against pathogens to those of humans.

Comparing lymphocyte subsets between newborn and adult animals, the $CD8^+$ T-cell subsets in newborn animals were significantly lower than those of adult animals, while the $CD4^-CD8^-$ T-cell subset was higher than that of the adult group. The T-cell fraction rate in the total leukocytes between both groups was similar. Although the increase in the $CD8^+$ T-cell rate in proportion to age seems reasonable, the presence of the $CD4^-CD8^-$ T-cell subset and the decrease in the rate in the elderly population is not well understood. The $CD4^-CD8^-$ T-cells were bigger in size than the $CD4^+$ or $CD8^+$ T-cells, and possessed abundant cytoplasm. More than half of the cell population was positive for asialo GM1, suggesting that this unique T-cell subset might function as NK or CTL. Further study on these functions is required to clarify this subpopulation.

The present flow cytometric procedure is a useful tool for studying T-cell subsets and subpopulations. Intravenous inoculation with a high dose of BCG specifically induced MHC Class $II^+$ T-cells in inoculated guinea pigs, whereas it was not detected in immunized animals intradermally inoculated with the common dose of BCG or in normal healthy control animals. Although these results suggest that the expression of MHC Class II antigen on T-cells may be related to T-cell activation due to BCG infection, the appearance of the MHC Class $II^+$ T-cells is probably related to the hyper-activation of T-cells, because the common dose of BCG vaccination did trigger the induction of the antigen specific immune-competent T-cells in the immunized animals. Our results of induction of MHC Class $II^+$ T-cells in guinea pigs inoculated with a high dose of BCG seem to be partly explained by a report by Zhou *et al.* [34] in which intravenous inoculation with a high dose BCG enhanced the pathogenicity of immunodeficiency virus infection.

Previously, other researchers reported that the mean value of the leukocyte count in guinea pigs was 10,830, 12,000, 8,280, 10,000 and 9,000/μl using the hemacytometer chamber [23], which is significantly higher than our result of 5,438/μl detected by the automated blood analyzer. We speculate that these differences are not due to the two different procedures of the cell counting using the hemacytometer chamber and automated blood analyzer, but are mainly due to differences in the environmental factors of raising and feeding animals, because blood cell counts of the guinea pigs by the two different procedures were identical as described in Materials and Methods. Thus, novel procedures for analysis of cell count, leukocyte fractions and lymphocyte subsets and subpopulations in the blood and lymphoid tissues, and their established normal values in this study, will provide an effective tool for studying the guinea pig as a model animal.

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# Potent Anti-R5 Human Immunodeficiency Virus Type 1 Effects of a CCR5 Antagonist, AK602/ONO4128/GW873140, in a Novel Human Peripheral Blood Mononuclear Cell Nonobese Diabetic-SCID, Interleukin-2 Receptor γ-Chain-Knocked-Out AIDS Mouse Model

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**We established human peripheral blood mononuclear cell (PBMC)-transplanted R5 human immunodeficiency virus type 1 isolate JR-FL (HIV-$1_{JR-FL}$)-infected, nonobese diabetic-SCID, interleukin 2 receptor γ-chain-knocked-out (NOG) mice, in which massive and systemic HIV-1 infection occurred. The susceptibility of the implanted PBMC to the infectivity and cytopathic effect of R5 HIV-1 appeared to stem from hyperactivation of the PBMC, which rapidly proliferated and expressed high levels of CCR5. When a novel spirodiketopiperazine-containing CCR5 inhibitor, AK602/ONO4128/GW873140 (molecular weight, 614), was administered to the NOG mice 1 day after R5 HIV-1 inoculation, the replication and cytopathic effects of R5 HIV-1 were significantly suppressed. In saline-treated mice ($n = 7$), the mean human $CD4^+/CD8^+$ cell ratio was 0.1 on day 16 after inoculation, while levels in mice ($n = 8$) administered AK602 had a mean value of 0.92, comparable to levels in uninfected mice ($n = 7$). The mean number of HIV-RNA copies in plasma in saline-treated mice were $\sim 10^6$/ml on day 16, while levels in AK602-treated mice were $1.27 \times 10^3$/ml ($P = 0.001$). AK602 also significantly suppressed the number of proviral DNA copies and serum p24 levels ($P = 0.001$). These data suggest that the present NOG mouse system should serve as a small-animal AIDS model and warrant that AK602 be further developed as a potential therapeutic for HIV-1 infection.**

Highly active antiretroviral therapy has brought about a major impact on the AIDS epidemics in the industrially advanced nations (5, 22). However, eradication of human immunodeficiency virus type 1 (HIV-1) is thought to be currently impossible, due in part to the viral reservoirs remaining in blood and infected tissues (6). The limitation of antiviral therapy of AIDS is exacerbated by complicated regimens, the development of drug-resistant HIV-1 variants (11), and a number of inherent adverse effects (2, 31). Hence, the identification of new antiretroviral drugs that have unique mechanisms of action and produce no or minimal adverse effects remains an important therapeutic objective. In regard to development of potential anti-HIV therapies or vaccines, experimental animal models for AIDS which allow the determination of the possible efficacy of antiviral agents or vaccines have been sought since severe combined immunodeficiency (SCID) mice engrafted with human fetal thymus, liver, or peripheral blood mononuclear cells (PBMC) were first exploited to examine antiretroviral agents (19, 25). However, a number of mouse models have suffered from false-positive and false-negative results in detecting or quantifying HIV-1 infection and replication and have required a large number of samples and mice for testing (25, 29).

In the present work, we established human PBMC-transplanted R5 HIV-$1_{JR-FL}$-infected, nonobese diabetic (NOD)-SCID, interleukin 2 receptor γ (IL-2Rγ)-chain-knocked-out (NOG) mice, in which massive and systemic HIV-1 infection occurs, human $CD4^+/CD8^+$ cell ratios significantly decrease, and high levels of R5 HIV-1 viremia reaching as high as $10^6$ copies/ml are achieved. Furthermore, we demonstrated that this unprecedented susceptibility of the implanted human PBMC to the infectivity and cytopathic effects of R5 HIV-1 infection stems from hyperactivation of the PBMC. Here, we also report a novel small nonpeptide CCR5 antagonist, AK602/ONO4128/GW873140, which exerts potent anti-HIV-1 activity in vitro against laboratory and clinical strains of HIV-1, including highly multidrug-resistant (MDR) variants.

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