vaccinia virus DIs generates both $CD4^+$ and $CD8^+$ T cell responses specific for SIV Gag, resulting in protection of the immunized macaques from pathogenic SHIV. However, it remains to be elucidated whether the *gag/pol*-encoding vaccine may elicit a protective effect against various viral challenges, such as CCR5-tropic viruses and other primary viruses. Nonetheless, this new regimen's twin merits of safety and efficacy position it as a promising vaccine candidate against HIV-1 infection as well as against HIV-induced disease progression.

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Disclosures

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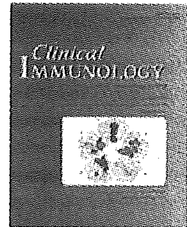

1

2 Intradermal and oral immunization with recombinant 3 *Mycobacterium bovis* BCG expressing the simian 4 immunodeficiency virus Gag protein induces 5 long-lasting, antigen-specific immune 6 responses in guinea pigs

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13 **KEYWORDS**
14 Recombinant BCG;
15 HIV-1;
16 SIV;
17 Gag p27;
18 Proliferation;
19 IFNγ;
20 Human dose;
21 Guinea pig

Abstract To develop a new recombinant BCG (rBCG) vaccine, we constructed rBCG that expresses the full-length Gag protein of simian immunodeficiency virus (rBCG-SIVGag) at a level of 0.5 ng/mg after 3 weeks of bacterial cell culture. Intradermal (i.d.) inoculation of guinea pigs with 0.1 mg of rBCG-SIVGag resulted in the induction of delayed-type hypersensitivity (DTH) responses to both purified protein derivative (PPD) of tuberculin and SIV Gag p27 protein; responses that were maintained for the duration of the 50-week study. In contrast, guinea pigs orally vaccinated with 160 mg of the same antigen exhibited a long-lasting DTH response to the SIV Gag p27 protein but mounted no response to PPD. Proliferative responses to SIV Gag p27 and PPD antigens were detected in both i.d. and orally immunized animals; however, the levels of PPD-specific responses were significantly higher in guinea pigs immunized by the i.d. than the oral route. A significant increase in the level of PPD- and SIV Gag p27-specific IFNγ mRNA expression was also detected in both immunization groups receiving rBCG-SIVGag. In addition, both i.d. and oral immunization with rBCG-SIVGag induced PPD- and SIV Gag p27-specific serum IgG responses. Insertion of the SIV *gag* gene into BCG did not appear to change the ability of rBCG-immunized animals to elicit PPD-specific immune responses. These results indicate that rBCG-SIVGag has the ability to effectively induce long-lasting, cell-mediated and humoral immunity against both viral and bacterial antigens in guinea pigs, suggesting that rBCG-Gag has the potential to elicit immunities specific not only for tuberculosis but also for HIV at human doses.
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Introduction

The epidemic of human immunodeficiency virus type 1 (HIV-1) infection and AIDS (HIV/AIDS) continues to spread worldwide, particularly in Asia and Africa. Globally, 40 million people are now living with HIV/AIDS [1]. In 2003, there were five million new HIV-1 infections, over 90% of them in developing countries [1] where rapid disease progression is more likely to occur due to co-infection with both HIV-1 and *Mycobacterium tuberculosis*. The best hope for individuals living in these countries is the development of a safe, effective and affordable vaccine to prevent HIV-1 infection. Despite recent advances in medical treatments for HIV-1, including highly active anti-retroviral therapy (HAART), most therapeutic drugs remain prohibitively expensive and inaccessible to people living in countries hardest hit by the epidemic [1].

With this dilemma in mind, our group has developed a recombinant BCG (rBCG) vector system designed to address both the problem of HIV-1 and *M. tuberculosis* co-infection and the issue of cost facing those in developing countries. The rBCG vector system has been shown to induce immune responses against both HIV-1 and *M. tuberculosis*, and its use is supported by a number of studies demonstrating efficacy in the induction of antigen-specific immunity. For example, it has been reported that BCG and its cell wall components possess adjuvant properties for enhancing the immunogenicity of an antigen when administered to animals [2–4]. Moreover, rBCG expressing HIV-1 antigens can act simultaneously as both an adjuvant and a vehicle to induce antigen-specific immunity [5]. Our own group has previously demonstrated that rBCG containing a 19-amino-acid insert from the HIV-1 Env V3 region (rBCG Env V3) expressed sufficient V3 antigen to induce HIV-1-specific cell-mediated and humoral immune responses in a small-animal model [6–9]. In addition, several groups have also shown the induction of cellular and/or humoral immune responses by inoculation with rBCG expressing HIV or simian immunodeficiency virus (SIV) proteins (10–14). However, 10- to 100-fold higher doses than that needed for a common BCG vaccination against tuberculosis in humans, or repeated inoculations, were needed to effectively elicit HIV- or SIV-specific immunity in animal models [6–14]. Moreover, previous studies often used intravenous or subcutaneous routes of inoculation; however, vaccination regimens such as these are not practical for use in humans in terms of safety. Furthermore, these BCG recombinants contained a single epitope from HIV or SIV; however, it was reported that rBCG expressing a SIV gag single epitope failed to protect macaques against intravenous challenge with SIV [15].

One of the strategies to practically use a rBCG-based HIV vaccine is to inoculate 0.1 mg of the vaccine into humans via intradermal (i.d.) route as a priming or boosting immunogen because the dose and route of immunization is commonly used for BCG vaccination in humans. For this purpose, we sought to construct a novel rBCG capable of effectively inducing long-lasting, virus-specific immunity by a single i.d. vaccination with 0.1 mg. To elicit antigen-specific immunity with a multi-epitope rBCG vaccine, we chose to target HIV-1 Gag based on evidence of several cytotoxic T lymphocyte (CTL) epitopes in this region [16], some of which are MHC linked and known to be immunodominant and relatively conserved among various HIV-1 clades [16–19]. Recently, it was shown that Gag-specific T helper cells and CTL correlate inversely with the level of plasma HIV-1 RNA [20–22]. These findings suggest that the HIV-1 Gag region is strongly immunogenic and may induce effective anti-viral responses.

In the present study, we inserted the full-length *gag* gene of SIV into BCG to create rBCG-SIVGag. We then investigated its ability to elicit antigen-specific immune responses in guinea pigs immunized either intradermally (i.d.) or orally with rBCG-SIVGag at human doses and assessed the possibility of the replacement of common BCG vaccination (0.1 mg by i.d. inoculation) by administration of a rBCG-based vaccine.

Subjects and methods

Animals

Female guinea pigs of the Hartley strain (Shizuoka Laboratory Center, Shizuoka, Japan), weighing 200 to 250 g each, were used in a P2-level animal facility at the National Institute of Infectious Diseases (NIID), Tokyo, Japan. The animals were fed in a specific pathogen-free level 2 facility according to NIID animal care guidelines. The study was conducted in the experimental animal area of a biosafety level 2 NIID facility under the guidance of an institutional committee for biosafety and animal experiments.

Construction of a plasmid containing the full-length SIV gag gene

A recombinant *Mycobacterium bovis* BCG substrain Tokyo was produced by transfection of BCG–Tokyo strain cells with either the plasmid pSO246 [23] or pSO246SIVGag. The SIVmac239 *gag* gene [24] was amplified by PCR from simian immunodeficiency virus DNA [25] using primers 5′-CCCGGATCCATGGGCGTGAGAAACTCC-3′ (forward) and 5′-CCGCCCGGGCTACTGGTCTCCTCCAAAGAG-3′ (reverse). The resulting PCR product was inserted into the multi-cloning site of pSO246 under control of the *hsp60* promoter of BCG [26]. BCG was transformed with the recombinant plasmid by electroporation and selected on Middlebrook 7H10 agar (BBL Microbiology Systems, Cockeyville, MD) containing 10% OADC enrichment (BBL Microbiology Systems) and 20 μg/ml kanamycin. The resulting recombinant clones containing either pSO246SIVGag or pSO246 were designated rBCG-SIVGag and rBCG-pSO246, respectively.

Western blot and ELISA detection of expressed SIV Gag

Expression of the SIV Gag protein by rBCG-SIVGag was determined by both Western blot and ELISA. rBCG-SIVGag was harvested from Middlebrook 7H9 broth containing ADC (BBL Microbiology Systems) 3 weeks after initiation of the culture, when the growth curve of the transformant had reached its peak. The harvested rBCG-SIVGag was sonicated completely and centrifuged, and the supernatant was heated at 95°C for 5 min in sample buffer (10% 2-mercaptoethanol, 20% glycerol, 123.9 mM Trizma base, 138.7 mM SDS, 3.0 mM bromphenol blue). SDS-polyacryl-

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amide gel electrophoresis (PAGE) was performed with a 4–20% gradient polyacrylamide gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The separated proteins were transferred to nitrocellulose membranes at 144 mA for 30 min, after which the membranes were probed with mouse anti-SIV p27 (kindly supplied by Dr. Sata of NIID) followed by anti-mouse IgG-alkaline phosphatase conjugate (New England BioLabs, Inc., Beverly, MA). Proteins were visualized by reactivity with NBT/BCIP (Roche Diagnostics Co., Indianapolis, IN). The concentration of SIV Gag p27 antigen in the cell extract was determined by a commercial antigen ELISA (SIV Core Antigen Assay kit, Coulter Corporation, Miami, Florida) as per the manufacturer's instructions.

Immunization of guinea pigs with rBCG-SIVGag

Guinea pigs were inoculated with either rBCG-SIVGag or BCG by the i.d. or oral route. For i.d. immunization, six guinea pigs were given a single inoculation of 0.1 mg of rBCG-SIVGag. Three separate control groups consisting of three animals each received i.d. inoculation with either (1) rBCG-pSO246 as a plasmid vector control, (2) BCG–Tokyo as a BCG vector control or (3) saline alone. Prior to oral immunization, fifteen guinea pigs were deprived of food and water overnight. The following day, 1 ml of 3% sodium bicarbonate was administered orally to each animal via a micropipette to neutralize stomach fluid, after which 500 μl of saline containing either 80 mg of rBCG-SIVGag ($n = 6$), rBCG-pSO246 ($n = 3$), BCG–Tokyo ($n = 3$) or saline alone ($n = 3$) was administrated by the same route. To flush the remaining antigens in their mouths and esophagi, saline (500 μl) was given orally to the animals after antigen ingestion. These procedures were performed on the animals under non-anesthetic conditions. Oral immunization was performed once a week for 2 consecutive weeks, providing a total dose of 160 mg of either rBCG-SIVGag, rBCG-pSO246 or BCG–Tokyo per animal.

Induction of a delayed type hypersensitivity (DTH) skin reaction

To investigate antigen-specific T cell immunity, DTH skin tests were performed at 8 and 50 weeks after immunization with either rBCG-SIVGag, rBCG-pSO246 or BCG–Tokyo. To evaluate SIV Gag-specific DTH responses, 10 μg of SIV Gag p27 protein (Advanced Biotechnologies Inc., Columbia, MD) per 100 μl of saline was injected i.d. into both immunized and non-immunized guinea pigs. To evaluate tuberculosis-specific DTH responses, 0.5 μg of PPD was administered by the same procedure. Saline (100 μl) was used as a negative control. After 24, 48 and 72 h, the diameter of each area of induration was measured.

Isolation of peripheral blood mononuclear cells (PBMC), spleen cells, intestinal intraepithelial lymphocytes (i-IEL) and lamina propria lymphocytes (LPL)

PBMC were separated from heparinized blood using Lymphosepar according to the manufacturer's instructions (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). To isolate spleen cells, guinea pigs were sacrificed while under anesthesia with ketamine hydrochloride (Sankyo Co., Ltd., Tokyo, Japan), and their spleens were harvested. Spleen cells were prepared by gentle dispersion through a 70-μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ). The preparations were treated with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) for 1 min at room temperature to remove red blood cells, and the remaining cells were washed three times in PBS. Preparation of i-IEL was performed as previously described [27–29]. Briefly, large or small intestines were opened longitudinally and washed with PBS containing 1 mM DTT. The tissues were then placed in 20 ml of RPMI 1640 containing 1 mM EDTA in a 50-ml centrifuge tube and incubated for 20 min at 37°C with shaking. After incubation, the tube was shaken vigorously for 15 s, and the cell-containing medium was removed and saved. This process was repeated three times. To isolate LPL, the remaining intestinal tissues were treated with 0.5 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) and 1.0 mg/ml hyaluronidase (Sigma) for 30 min at 37°C with shaking, and the cell-containing medium was removed and saved. This process was repeated twice, and the harvested cells were then purified through a discontinuous 40/75% percoll gradient (Pharmacia, Uppsala, Sweden).

Antigen-specific T cell proliferative responses

PBMC were re-suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 10 μg/ml gentamicin. The cells were cultured in triplicate wells at a density of 2×10^6 cells/ml in the presence or absence of antigen (20 μg/ml of either PPD or SIV Gag p27) in 96-well U-bottomed plates (Costar, Cambridge, MA) for 3 days at 37°C under 5% CO_2. The cells were then pulsed with 0.5 μCi [^{3}H] thymidine (Amersham, Arlington Height, IL) for the last 6 h of incubation and harvested onto filter paper disks. Uptake of [^{3}H] thymidine was determined by scintillation counting, and the results were expressed as the stimulation index (S.I.), which was calculated as a ratio of the counts per minute in the presence or absence of antigen.

RNA extraction from PBMC, spleen cells, i-IEL and LPL

Isolated PBMC, splenocytes, i-IEL and LPL were adjusted to a concentration of 0.5×10^7 to 1.0×10^7/ml in RPMI 1640 supplemented with 10% FCS, 50 μg of streptomycin, 50 U of penicillin and 10 μg of gentamicin/ml, and then cultured with either 20 μg/ml of PPD or SIV Gag p27 at 37°C for 4 days. Non-stimulated cells were used as controls. Following culture, total cellular RNA was extracted according to the instructions provided with the RNeasy Mini Kit (QIAGEN, Valencia, CA) and stored at −80°C.

To investigate Gag-specific IFNγ responses in T cell subsets, $CD4^+$ and $CD8^+$ T cell populations from the immunized guinea pigs were obtained from PBMC and spleen cells using magnetic cell sorting (autoMACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, after in vitro stimulation of total PBMC and spleen cells with 20 μg/ml of SIV Gag p27 at 37°C for 4 days, the cells were incubated with FITC-conjugated anti-guinea pig CD4 (Serotec Ltd., Oxford UK) or CD8 antibodies (Serotec Ltd.) followed by anti-FITC MicroBeads (Miltenyi Biotec). Non-stimulated cells were used as controls. $CD4^+$ T cell or $CD8^+$ T cell subpopulations

265 were negatively selected, and then total RNA was extracted
266 with the RNeasy Mini Kit (QIAGEN).

267 Amplification of guinea pig IFNγ by reverse 268 transcription (RT) and fluorogenic PCR

269 To examine antigen-specific IFNγ mRNA expression in PBMC, 270 spleen cells, i-IEL and LPL, RT-PCR was performed using a 271 TaqMan EZ RT-PCR kit according to the instructions provided 272 (Applied Biosystems, Foster City, California). The reaction 273 mixture consisted of 3 μl of appropriately diluted RNA 274 sample; 5 μl of 5 × TaqMan EZ buffer; 3 μl of 25 mM 275 manganese acetate; 0.75 μl each of dATP, dCTP, dGTP and 276 dUTP; 0.25 μl of primer for IFNγ at 100 μM; 1 μl of fluorogenic 277 probe; 2.5 U of recombinant Tth DNA polymerase; 0.25 U of 278 AmpErase uracil-*N*-glycosylase; and 8.25 μl of RNase-free 279 water in a final volume of 25 μl. Thermal cycling conditions 280 consisted of 2 min at 50°C, 30 min at 60°C and 5 min at 95°C, 281 followed by 50 cycles of 10 s at 95°C and 45 s at 62°C. The ABI 282 Prism 7700 sequence detection system (Perkin-Elmer, Applied 283 Biosystems, Inc.) was employed for PCR cycling, real-time 284 data collection and analysis.

285 Ribosomal RNA (rRNA) was used as an internal control, 286 and its expression level was quantitatively determined using 287 the TaqMan rRNA control reagent (Applied Biosystems) under 288 the same conditions as described above.

289 The level of cytokine expression was determined in three 290 independent samples for each animal. Quantification was 291 normalized by dividing the amount of IFNγ mRNA in the 292 target sample by the amount of rRNA in the same sample. 293 Data are shown as fold induction of mRNA and expressed as 294 the ratio of values obtained for antigen-stimulated cells to 295 non-stimulated cells; ratios <1 indicate down-regulation, 296 ratios >1 indicate up-regulation. Oligonucleotide primers 297 (5′-CATGAACACCATCAAGGAACAAAT-3′, 5′-TTTGAAT-298 CAGGTTTTTGAAAGCC-3′) and a fluorogenic-labeled probe 299 (5′-6-carboxyfluorescein-TTCAAAGACAACAGCAGCAA-300 CAAGGTGC-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine-301 3′) specific for guinea pig IFNγ mRNA were used for detection 302 and quantification [30]. The RNA standard template used for 303 quantitative determination of guinea pig-specific IFNγ mRNA 304 was prepared as described by us elsewhere [30].

305 Antigen-specific serum antibody titration by 306 ELISA

307 Sera were collected from each guinea pig at 50 weeks after 308 immunization and stored at −80°C. Antigen-specific antibody 309 titers were determined by ELISA. Maxisorp plates (Nunc A/S, 310 Roskilde, Denmark) were coated with either PPD (0.5 μg/ 311 well) or SIV Gag p27 (0.1 μg/well) and incubated overnight at 312 4°C. Serially diluted sera were added to the wells and 313 incubated for 2 h at 37°C. After three washes, rabbit anti-314 guinea pig IgG-horseradish peroxidase (HRP) conjugate 315 (Zymed Laboratories, Inc., San Francisco, CA) was added to 316 the wells and incubated for 2 h at 37°C, and the plates were 317 then washed and visualized by adding TMB substrate (Moss, 318 Inc., Pasadena, MD). After 30 min at room temperature, rates 319 of absorbance were measured at 450 nm with an ELISA reader. 320 Endpoint titers for antigen-specific IgG were calculated using 321 Microsoft Office Excel and expressed as the last dilution 322 giving an OD_{450} of 0.1 U above pre-immunization serum 323 samples.

Statistical analysis 324

Data analysis was carried out with the Statistica program 325 (StatSoft, Tulsa, OK). Data are expressed as the mean ± 326 standard deviation (SD), and *P* values <0.05 were considered 327 significant. DTH responses for the i.d. and orally immunized 328 groups were compared using the unpaired *t* test, and the 329 responses at week 8 and week 50 for each group were 330 compared using the Student's paired *t* test. Levels of 331 antigen-specific proliferative responses and IFNγ mRNA 332 expression for the two groups were compared using the 333 Mann–Whitney *U* test because of variability in values among 334 animals in each group. 335

Results 336

Construction of a rBCG-SIVGag vector 337 expressing full-length SIV Gag 338

To achieve expression of the complete SIV Gag protein, we 339 inserted the full-length *gag* DNA fragment of SIVmac239 into 340 the plasmid pSO246, yielding a rBCG clone. The resulting 341 recombinant clones (pSO246SIVGag and pSO246) were 342 designated as rBCG-SIVGag and rBCG-pSO246, respectively. 343 Transformation of cells with rBCG-SIVGag and analysis of the 344 cell lysates by Western blot revealed a single band 345 corresponding to 55 kDa consistent with the expected 346 molecular weight of the SIV Gag protein (Fig. 1). The 347 concentration of SIV Gag in the cell lysates was determined 348 by SIV Gag p27 antigen ELISA and found to be 0.5 ng/1 mg of 349 rBCG-SIVGag. 350

DTH skin responses to PPD and SIV Gag p27 351 antigen 352

For each vaccine, DTH skin tests for PPD and SIV Gag p27 353 antigens were performed at 8 and 50 weeks after immuni-354 zation. DTH responses to PPD and Gag p27 antigens peaked 355 24 h after antigen injection. At week 8, DTH responses to 356 PPD were detected in all six guinea pigs immunized i.d. with 357 rBCG-SIVGag with a mean area of induration of 15.0 mm 358 (Fig. 2A). The magnitude of induration in this group was 359 similar to that seen in both the rBCG-pSO246- and BCG–360 Tokyo inoculation groups (mean indurations = 15.5 and 15.0 361 mm, respectively). In contrast, only three of six animals 362 orally immunized with rBCG-SIVGag exhibited a PPD-specific 363 DTH response, and that response of six animals had a mean 364 induration of 4.1 mm (Fig. 2A). Thus, it appeared that 365 immunization with rBCG-SIVGag induced stronger DTH 366 responses via the i.d. than the oral route (Fig. 2A). The 367 three guinea pigs that showed no PPD-specific DTH 368 responses were also included in further analyses of prolif-369 eration, levels of IFNγ mRNA expression and antibody 370 production. Evaluation of animals receiving rBCG-pSO246 371 and BCG–Tokyo by the oral route also showed similar levels 372 of PPD-specific DTH reactions (4.6 and 4.3 mm, respective-373 ly). At week 50, PPD-specific DTH responses were again 374 detected in all six animals immunized i.d. with rBCG-375 SIVGag. The mean area of induration of these responses 376 was 12.4 mm (Fig. 2A), equivalent to that seen in animals 377 inoculated i.d. with either rBCG-pSO246 (11.0 mm) or BCG–378 Tokyo (13.5 mm). However, no DTH responses were seen in 379

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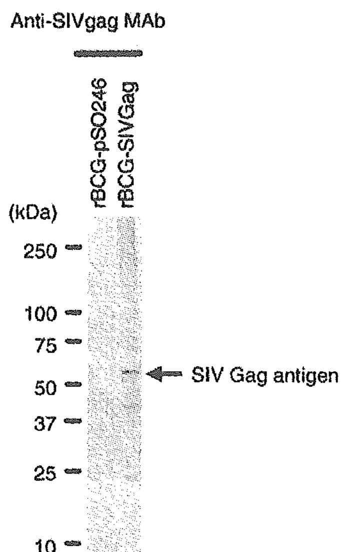

Figure 1 Western blot detection of SIV Gag in rBCG cell lysates. A 55-kDa molecule was identified, corresponding in size to the expected molecular weight of SIV Gag. Insertion of the empty plasmid 246 into rBCG (rBCG-pSO246) was used as a negative control.

380 any of the animals immunized orally with either rBCG-
381 SIVGag, rBCG-pSO246 or BCG—Tokyo (Fig. 2A). With respect
382 to DTH responses against SIV Gag p27, similar responses
383 were seen at 8 weeks in groups i.d. and orally immunized
384 with rBCG-SIVGag, with mean indurations of 15.8 and 16.1
385 mm, respectively (Fig. 2B).

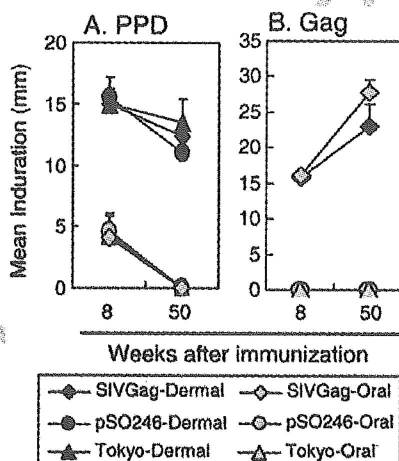

Figure 2 Induction of DTH skin reactions against (A) PPD and (B) SIV Gag p27 in guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG—Tokyo or rBCG-pSO246. DTH induction was performed at 8 and 50 weeks post-immunization. PPD (0.5 μg) and SIV Gag p27 (10 μg) were intradermally injected into the immunized animals, and the diameter of each area of induration was measured 24 h later. Only 3 of 6 animals orally immunized with rBCG-SIVGag exhibited PPD-specific DTH responses. Shown are the mean values ± standard deviation of all animals per group.

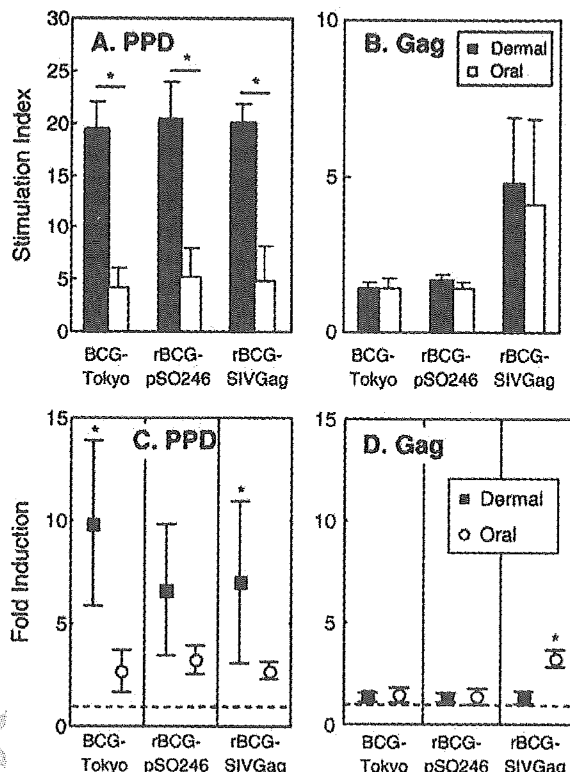

Figure 3 Induction of PPD- or SIV Gag-specific T cell proliferative responses (A, B) and IFNγ mRNA expression (C, D) in PBMC from guinea pigs intradermally or orally immunized with either rBCG-SIVGag, BCG—Tokyo or rBCG-pSO246. PBMC were isolated at week 20 after immunization and were cultured with or without antigen (20 μg/ml of either PPD or SIV Gag p27) for 3 days. During the final 6 h of incubation, 0.5 μCi [^{3}H] thymidine was added to each well. The cells were harvested, and the levels of [^{3}H] thymidine incorporation were determined by scintillation counting. Data are expressed as the stimulation index, as described in Materials and methods. Shown are the means (solid bars, intradermal immunization; open bars, oral immunization) ± standard deviations. To investigate antigen-specific IFNγ responses, PBMC harvested 20 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of either PPD or SIV Gag p27) for 4 days. Total RNA was extracted, and IFNγ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction, as described in Materials and methods. Shown are the mean values (symbols; ■, intradermal immunization; ○, oral immunization) ± standard deviations. *$P < 0.05$ (i.d. vOersus oral groups for each vaccine strain).

Interestingly, animals immunized with rBCG-SIVGag via 437
either the i.d. or oral route showed strong, statistically 438
identical SIV Gag p27-specific DTH responses (23.1 and 27.8, 439
respectively) at week 50 (Fig. 2B). The magnitude of the 440
DTH to Gag at week 50 was significantly higher than that at 441
week 8 in the group orally immunized with rBCG-SIVGag (P = 442
0.004), while it did not reach statistical significance in the 443
i.d. immunized group. No significant levels of Gag p27 444
antigen-specific DTH responses were detected in animals 445
inoculated with either rBCG-pSO246 or BCG—Tokyo via 446
either the i.d. or oral routes (Fig. 2B). In addition, no 447

significant DTH responses to PPD and Gag p27 antigens were found in control animals inoculated with saline alone (data not shown).

Antigen-specific proliferative responses and IFNγ mRNA expression in PBMC

To investigate the T cell responses specific for SIV Gag p27 and PPD antigens, proliferation assays were performed at week 20 after immunization (Figs. 3A and B). In the groups immunized with rBCG-SIVGag, PPD-specific proliferative responses were evaluated in PBMC from the immunized animals. The levels of these responses were found to be significantly higher in the i.d. immunized group than in the orally immunized group (19.9 ± 2.0 and 4.9 ± 3.2, respectively, $P = 0.02$) (Fig. 3A). Conversely, similar levels of proliferative responses to SIV Gag p27 were elicited by either i.d. or oral immunization with rBCG-SIVGag (4.8 ± 2.1 and 4.1 ± 2.8, respectively) (Fig. 3B). PPD-specific proliferative responses to BCG—Tokyo or rBCG-pSO246 were 19.3 ± 2.8 and 20.2 ± 3.9, respectively, in the i.d. group, while those inoculated by the oral route had proliferative responses of 4.2 ± 1.9 and 5.3 ± 2.6, respectively.

To assess Th1-type helper T cell functions specific for PPD and SIV Gag, IFNγ mRNA expression in PBMC was measured quantitatively at week 20 using real-time, fluorogenic RT-PCR (Figs. 3C and D). In animals receiving rBCG-SIVGag i.d. or orally, the level of IFNγ mRNA expression was elevated following stimulation with PPD over baseline values obtained from non-stimulated PBMC. Mean values specific for PPD in the i.d. and orally immunized groups were 7.0 ± 3.9 and 2.8 ± 0.4, respectively ($P = 0.04$, Fig. 3C). With respect to IFNγ mRNA expression specific for SIV Gag p27, the level was enhanced in animals immunized orally with rBCG-SIVGag, whereas not activated in animals vaccinated i.d. with the vaccine (3.3 ± 0.4 and 1.3 ± 0.3, respectively, $P = 0.02$, Fig. 3D). By comparison, the levels of PPD-specific IFNγ mRNA expression were 9.9 ± 4.0 and 6.8 ± 3.2, respectively, in animals receiving either BCG—Tokyo or rBCG-pSO246 by the i.d. route, and 2.7 ± 1.0 and 3.2 ± 0.7, respectively, for animals inoculated by the oral route.

Long-term antigen-specific IFNγ mRNA expression in PBMC, spleen cells, i-IEL and LPL

To assess whether Th1-type T cells persist in systemic and mucosal compartments, the level of IFNγ mRNA expression was determined at week 50 using PBMC, splenocytes, i-IEL and LPL from guinea pigs immunized either i.d. or orally with rBCG-SIVGag (Fig. 4). Higher levels of IFNγ mRNA induced by PPD were clearly detected in PBMC from guinea pigs immunized with rBCG-SIVGag (Fig. 4A). However, the mean levels of PPD-specific IFNγ mRNA expression were significantly higher in animals immunized by the i.d. route compared to the oral route (205.0 ± 51.2 and 15.5 ± 19.4, respectively, $P = 0.02$) (Fig. 4A). Similarly, the levels of Gag p27-specific IFNγ mRNA expression in the rBCG-SIVGag immunized animals were higher in the i.d. group compared with those in the orally immunized group, but these differences did not reach significance (53.3 ± 50.9 and 6.4 ± 3.2, respectively, $P = 0.15$, Fig. 4B). PBMC from control animals inoculated with

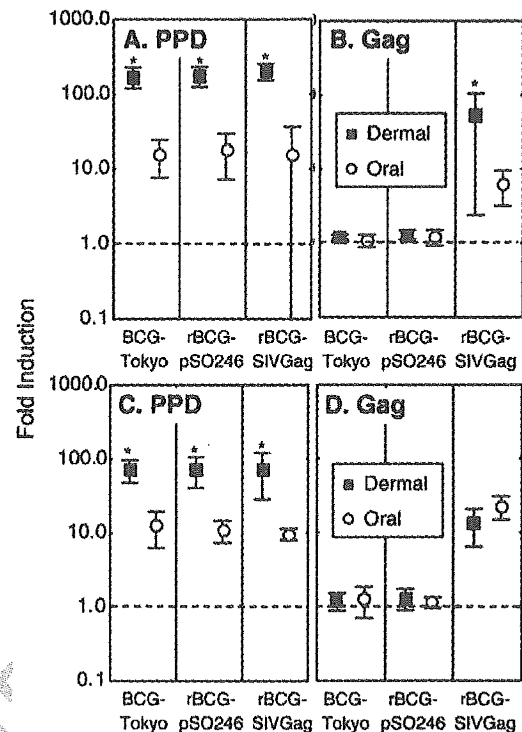

Figure 4 Profile of IFNγ responses at 50 weeks against PPD and SIV Gag p27 antigens in PBMC (A, B) and spleen cells (C, D) from guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG—Tokyo or rBCG-pSO246. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of either PPD or SIV Gag p27) for 4 days. Total RNA was extracted, and IFNγ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction. Shown are the mean values (symbols; ■, intradermal immunization; ○, oral immunization) ± standard deviations. *$P < 0.05$ (i.d. versus oral groups for each vaccine strain).

either BCG—Tokyo or rBCG-pSO246 had levels of PPD-specific IFNγ mRNA comparable to animals vaccinated with rBCG-SIVGag via same immunization route (Fig. 4A). As expected, no Gag-specific IFNγ mRNA responses were found in animals inoculated with either BCG—Tokyo or rBCG-pSO246 (Fig. 4B).

Splenocytes from guinea pigs receiving rBCG-SIVGag expressed considerably higher levels of IFNγ mRNA in response to PPD and SIV Gag p27 compared to baseline values obtained from non-stimulated splenocytes (Figs. 4C and D). PPD-specific IFNγ responses were significantly higher in animals immunized with rBCG-SIVGag by the i.d. route (75.0 ± 46.5) compared with those immunized by the oral route (9.7 ± 1.7, $P = 0.02$, Fig. 4C). Marked increases in the response to SIV Gag p27 were found in both i.d. and orally immunized animals receiving rBCG-SIVGag (13.6 ± 7.2 and 22.8 ± 7.8, respectively, Fig. 4D). Splenocytes from animals inoculated with either BCG—Tokyo or rBCG-pSO246 had levels of PPD-specific IFNγ responses similar to those from animals vaccinated with rBCG-SIVGag via the same immunization route (Fig. 4C), while no Gag-specific IFNγ responses were observed in these animals (Fig. 4D).

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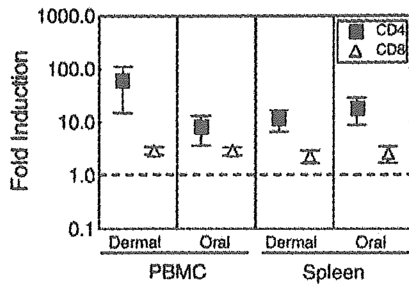

Figure 5 Profile of IFNγ responses at 50 weeks against SIV Gag p27 antigen in PBMC and spleen cells from guinea pigs intradermally or orally immunized with rBCG-SIVGag. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of SIV Gag p27) for 4 days. After separation of $CD4^+$ and $CD8^+$ T cell subsets, total RNA was extracted, and IFNγ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction. Shown are the mean values (symbols; ■, $CD4^+$ T cell subsets; △, $CD8^+$ T cell subsets) ± standard deviations.

To further investigate Gag-specific IFNγ responses in T cells, PBMC and splenocytes from guinea pigs vaccinated with rBCG-SIVGag were separated at week 50 into $CD4^+$ and $CD8^+$ T cell subsets. Higher levels of IFNγ mRNA expression were detected in $CD4^+$ T cells in comparison to $CD8^+$ T cells from PBMC and splenocytes from guinea pigs in both the i.d. and oral immunization groups (Fig. 5). The mean values of Gag-specific IFNγ responses were 63.5 ± 48.4 and 8.4 ± 4.8 for PBMC $CD4^+$ T cells, and 11.7 ± 5.2 and 18.8 ± 10.0 for spleen-derived $CD4^+$ T cells, in the i.d. and orally immunized groups, respectively. Comparatively, the magnitude of Gag-specific IFNγ responses in $CD8^+$ T cell subsets from PBMC and splenocytes ranged from 2.3 to 3.0 in the i.d. and orally immunized animals (Fig. 5). These results indicate that long-lasting, Gag-specific IFNγ responses are induced by immunization with rBCG-SIVGag, and these responses are mediated to a large extent by $CD4^+$ T cells at 50 weeks after vaccination.

PPD- and Gag-specific IFNγ responses were also determined for i-IEL from the large intestines of guinea pigs receiving rBCG-SIVGag. The mean values of PPD-specific IFNγ responses were 1.1 ± 0.4 and 0.7 ± 0.1 for the i.d. and oral groups respectively, while those for Gag-specific IFNγ responses were 1.7 ± 0.7 and 0.8 ± 0.1, respectively. Similarly, LPL from the small intestines of the i.d. and orally immunized animals had mean values for PPD-specific IFNγ responses of 1.0 ± 0.2 and 1.1 ± 1.0, respectively, while the means for Gag-specific IFNγ responses were 1.4 ± 0.2 and 1.3 ± 0.7, respectively. LPL from the large intestines exhibited no significant increase in antigen-specific IFNγ mRNA expression. i-IEL from the small intestines were not isolated in sufficient quantity for analysis due to the enormous quantity of mucus, which is copious in the small intestine. Thus, our results indicate that no significant increases occurred in levels of IFNγ mRNA expression upon in vitro stimulation with PPD or SIV Gag p27 in i-IEL and LPL from guinea pigs immunized with rBCG-SIVGag by either route. Previous studies have suggested that i-IEL and LPL have different activation requirements than do PBMC [31,32]. Further study is needed on the antigen-specific mucosal immunity induced by rBCG-SIVGag.

Induction of antigen-specific serum antibody responses

To investigate the induction of humoral immune responses to PPD and SIV Gag p27 in guinea pigs immunized with rBCG-SIVGag, antigen-specific serum IgG titers were determined by ELISA (Fig. 6). Even at 50 weeks after immunization,

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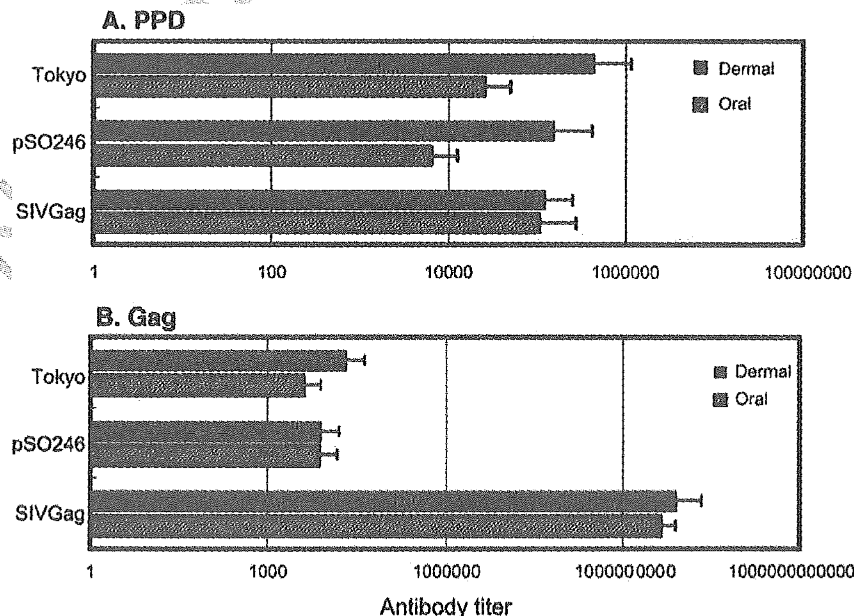

Figure 6 Induction of serum IgG responses against (A) PPD and (B) SIV Gag p27 antigens in guinea pigs intradermally (solid bars) or orally (hatched bars) immunized with either BCG—Tokyo, rBCG-pSO246 or rBCG-SIVGag 50 weeks after immunization. Shown are the mean values ± standard deviations.

575 significant levels of serum IgG specific for PPD and SIV Gag
576 p27 were detected in all animals from both immunization
577 groups. In comparison to animals inoculated with control
578 preparations of BCG–Tokyo or rBCG-pSO246, the levels of
579 Gag-specific serum IgG in the animals receiving rBCG-SIVGag
580 were 10^6-fold higher, although low but detectable levels of
581 non-specific IgG against Gag p27 antigen could be found in
582 the animals receiving control inoculations compared to non-
583 immunized healthy animals (Fig. 6B). As expected, animals
584 inoculated with BCG–Tokyo and rBCG-pSO246 had serum IgG
585 titers against PPD similar to those seen in the rBCG-SIVGag-
586 immunized group (Fig. 6A).

587 Discussion

588 We report here that i.d. or oral inoculation with rBCG-
589 SIVGag induces SIV- and tuberculosis-specific immune
590 responses in guinea pigs, and these responses persist for at
591 least 1 year (the duration of the study period). These
592 findings suggest that rBCG-Gag might be used as an
593 immunogen to elicit responses specific for HIV as well as
594 tuberculosis at human doses.

595 In this study, we used BCG–Tokyo as a parental strain to
596 develop a rBCG vector and confirmed that both the parental
597 BCG and the rBCG had the same advantage of providing long-
598 lasting, cell-mediated, PPD-specific immunity after a single
599 i.d. inoculation. Furthermore, two oral inoculations of rBCG
600 also produced levels of immunity against PPD identical to
601 those seen with the parental BCG. We have evidence that
602 both i.d. and oral inoculation of rBCG can elicit positive
603 immunity against challenge by inhaled *M. tuberculosis* with
604 similar efficacy to that conferred by vaccination with BCG–
605 Tokyo [S. Haga et al., personal communication]. Thus, the
606 ability to induce antigen-specific immunity and provide
607 protective efficacy against *M. tuberculosis* may apply to
608 rBCG as well as the BCG–Tokyo vaccine strain.

609 In studying the rBCG, we sought to determine how
610 effectively BCG might express an inserted gene from a
611 foreign immune deficiency virus. We found that rBCG
612 produced intracellular SIV Gag protein at a level of 0.5 ng/
613 mg. Even though i.d. inoculation dosages were small, 0.1 mg
614 of rBCG-SIVGag and 0.05 ng of SIV Gag protein per animal, we
615 achieved the induction of a strong immune response specific
616 for SIV Gag that was maintained over a 50-week period of
617 observation. Several possible attributes may contribute to
618 the ability of rBCG to elicit potent immune responses: (i) the
619 BCG bacillus is known to increase immune responses by
620 acting as an adjuvant; (ii) the persistence of live rBCG in host
621 cells may account for its ability to induce long-lasting,
622 specific immunity; and (iii) secretory proteins derived from
623 the mycobacteria, such as α-antigen (also known as MPB59 or
624 antigen 85B), can elicit potent Th1 immune responses [33–
625 35], which have been shown to be beneficial for controlling
626 pathogenic infectious agents [36–39].

627 The rBCG-SIVGag used here was able to effectively elicit
628 long-term, SIV Gag-specific DTH, proliferative and IFNγ
629 responses in PBMC and splenocytes from either i.d. or
630 orally immunized guinea pigs. Previous reports have shown
631 that the degree of HIV-specific DTH responsiveness, which
632 generally depends on the intensity of helper T cell
633 function, correlates with clinical stability in infected
individuals [40,41]. Furthermore, several lines of evidence 634
support the importance of maintaining helper T cell 635
function in controlling viral infection and replication 636
[42,43]. A related study has suggested that the mainte- 637
nance of HIV-1 Gag-specific proliferative responses helps 638
preserve Gag-specific CTL activity [44]. In addition, IFNγ 639
has been shown to play an important role in controlling 640
HIV-1 and SIV replication [45–48] and *M. tuberculosis* 641
infection [49–51]. In a recent study of HIV-1-infected 642
subjects, the production of IFNγ in response to Gag was 643
associated with a lower viral load set point [52]. Based on 644
these observations, it is conceivable that immunization 645
with rBCG-SIVGag might help control viral load and curb 646
disease progression, although this has yet to be tested in 647
the appropriate animal models. 648

In the present study, the levels of IFNγ mRNA induced in 649
response to both PPD and Gag were elevated at week 50 650
compared to week 20. The cells were re-stimulated in vitro 651
with the respective antigens; therefore, our results reflect 652
the IFNγ reaction of effector cells that were differentiated 653
from memory T cells and activated by in vitro restimulation. 654
This finding might be attributed to the number of memory T 655
cells or the ability of the memory cells to differentiate into 656
effector cells. The amount of rBCG-produced Gag antigen is 657
very low, perhaps resulting in low levels of Gag-specific IFNγ 658
responses at week 20. However, once the Gag-specific IFNγ 659
response was evoked by rBCG-Gag, the response was 660
maintained for more than 1 year after immunization. As 661
shown in Fig. 6, serum IgG against PPD and Gag was detected 662
at high levels even at week 50 in rBCG-Gag-immunized 663
guinea pigs, suggesting persistent antigenic stimulation by 664
chronic rBCG infection. Such chronic infection might grad- 665
ually lead to an increase in memory T cell counts and/or 666
enhancement of differentiation into effector cells. 667

In guinea pigs, IFNγ-specific tetramers, ELISPOT assays 668
and flow cytometric analyses remain to be developed. 669
Hence, we relied on a method for quantitative determina- 670
tion of antigen-specific IFNγ mRNA expression in $CD4^+$ and 671
$CD8^+$ T cell subpopulations using real-time RT-PCR. Our 672
results indicate that long-lasting IFNγ responses against SIV 673
Gag p27 induced by rBCG-SIVGag inoculation occur mainly in 674
the $CD4^+$ T cell population and not the $CD8^+$ T cell population 675
at the 50-week time point. However, it is unclear whether a 676
$CD8^+$ T cell IFNγ response to Gag p27 may have occurred 677
because the level of Gag-specific IFNγ expression was 678
normalized by using the amount of rRNA in the sample. 679
Namely, in case of a low frequency of Gag-specific memory 680
$CD8^+$ T cells in the sample, it may be difficult to accurately 681
detect enhancement of the Gag-specific IFNγ expression 682
even if such memory $CD8^+$ T cells are activated by 683
restimulation with Gag antigen. 684

The current study demonstrates that i.d. immunization 685
with rBCG-SIVGag or BCG–Tokyo induces significantly higher 686
DTH responses to PPD than does oral immunization with the 687
same vaccines. Interestingly, guinea pigs lacking a PPD- 688
specific DTH response 8 weeks after oral inoculation with 689
rBCG-SIVGag still exhibited significant proliferative and IFNγ 690
responses to the PPD antigen. Moreover, a significant IFNγ 691
response to PPD was generated in guinea pigs in which a PPD- 692
specific DTH reaction was no longer detected at week 50. 693
These results clearly indicate that a DTH reaction to PPD does 694
not necessarily reflect proliferative and IFNγ responses [30]. 695

It is possible that a T cell subset mediating a DTH response to PPD [53] was transiently localized at a cutaneous area by oral inoculation with rBCG-SIVGag. However, oral immunization with rBCG-SIVGag effectively induced Gag-specific DTH responses over a 50-week period. These findings indicate that oral inoculation of guinea pigs with rBCG-SIVGag engenders distinct DTH kinetics between PPD and Gag antigens, suggesting that different T cell subsets may be responsible. It has been shown that T cells activated by oral vaccination of humans with common BCG preferentially express a mucosal homing $\alpha 4\beta 7$ molecule associated with T cell trafficking to mucosa, resulting in a failure of the immunization regimen to induce PPD-specific DTH responses [54]. However, in this study of guinea pigs, it is unclear why oral rBCG-SIVGag inoculation persistently elicited vigorous Gag-specific DTH responses. It is possible that intracellular expression of SIV Gag within BCG inoculated orally may not be efficiently recognized by antigen-presenting cells in gut-associated lymphoid tissue (GALT). Namely, SIV Gag antigens might be processed and presented after migration of rBCG-SIVGag-infected macrophages to systemic compartments (e.g., the spleen). In the oral immunization group, a Gag-specific T cell subset may then home to systemic compartments including a cutaneous area, rather than to the mucosa. It will be important in future studies to investigate whether mucosal and cutaneous homing molecules are expressed on PPD- and Gag-specific T cell subsets that are induced by oral inoculation with rBCG-SIVGag.

Intradermal or oral vaccination of guinea pigs with rBCG-SIVGag resulted in the production of serum IgG directed to SIV Gag p27 and PPD. Although it is unclear how Gag-specific IgG affects HIV-1 infection or replication in vivo, a reduction in anti-Gag antibody levels has been shown to correlate with the onset of disease progression [55,56]. More recently, it has been reported that IgG2 directed against Gag is associated with a low viral load and high levels of antigen-specific IFNγ production [57]. We were unable to determine the IgG subclass of the serum antibodies in the immunized guinea pigs because of the lack of species-specific reagents. However, it is possible that the Gag-specific IgG observed here might be classified into a subclass corresponding to IgG2 of humans, since a significant Gag-specific IFNγ response was detected. In addition, we previously found that nasal immunization of mice with rBCG Env V3 induced not only antigen-specific IFNγ but higher levels of V3 antigen-specific serum IgG2 than IgG1 [58].

Thus, we have shown that a rBCG vaccine can induce antigen-specific immunity to viral as well as bacterial antigens. It is especially interesting to note that significant levels of Gag-specific immunity were induced by inoculation with rBCG-SIVGag at the dose and route commonly used for BCG vaccination in humans (0.1 mg by i.d. inoculation). These findings suggest that a rBCG-based vaccine targeting the HIV-1 Gag region might be an effective immunogen. Currently, many candidate HIV-1 vaccines are multivalent, utilizing several viral proteins for the induction of broadly reactive virus-specific immune responses. However, recent studies have shown the effectiveness of SIV vaccines expressing a single viral Gag protein, including Mamu-A*01 macaques immunized with either SIV Gag DNA [59] or adenovirus type 5 vectors expressing SIV Gag proteins [60]. Results using these vaccines indicate that expression of Gag alone is sufficient to induce significant efficacy in the macaque model.

However, there are certain drawbacks to using rBCG as a live, vector-based vaccine. One of the most serious concerns is that it might interfere with immunity induced by other tuberculosis vaccines that are based on the same BCG vector. Pre-existing immunity in BCG-vaccinated individuals may lead to rapid neutralization of a rBCG vaccine. However, the anamnestic effect of BCG vaccination would be irrelevant if our ultimate goal is reached—that is, the replacement of common BCG vaccination by administration of a rBCG vaccine to newborns who have no pre-existing immunity to BCG. While it is clear that the safety of an rBCG vaccine must be established for use in humans, BCG—Tokyo may be one of the most suitable BCG substrains to use as an HIV vaccine vector because it is less virulent than other substrains, and its inoculation does not cause severe systemic infection in immune deficient animals [61—64]. Based on these findings, rBCG based on BCG—Tokyo may have promise as a suitable vector for an HIV/AIDS vaccine.

Although the current study did not directly address the efficacy of the rBCG-SIVGag vaccine against viral challenge due to the failure of guinea pigs to support infection with HIV or SIV, our results open up the possibility of i.d. immunization with a single, human dose of rBCG-HIVGag against both HIV and tuberculosis, an immunization regimen that might 1 day replace the common BCG vaccine without requiring any variation in the current dose or protocol.

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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 ($HIV\text{-}1_{JR\text{-}FL}$)-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 1.27×10^3/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 $HIV\text{-}1_{JR\text{-}FL}$-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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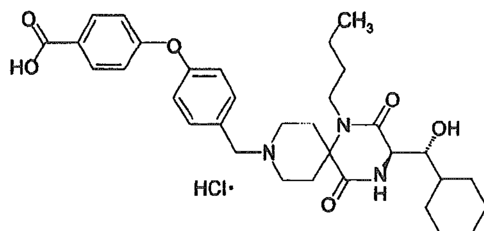

FIG. 1. Structure of AK602.

MATERIALS AND METHODS

Transplantation of human PBMC in NOG mice. NOD-SCID (NOG) mice (10, 33) were maintained in the Central Institute for Experimental Animals (Kawasaki, Japan). Mice were 4 to 6 weeks old at the time of transfer of human PBMC. The human PBMC-transplanted NOG (hu-PBMC-NOG) mice were generated by methods previously described (23, 24). Briefly, PBMC (10^7) were freshly prepared from heparinized blood of a single healthy HIV-1-seronegative donor by Ficoll-Hypaque density gradient centrifugation, resuspended in RPMI 1640-based culture medium (0.5 ml), and infused intraperitoneally to each mouse. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of the participating institutions.

Assay for proliferation and CCR5 expression of transplanted human PBMC recovered from hu-PBMC-NOG mice. Freshly isolated human PBMC (2×10^7 cells/ml) were incubated in phosphate-buffered saline (PBS) containing 10 μM 5-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oreg.) for 15 min at 37°C for CFSE labeling as previously described by Lyons (16), washed, and resuspended in RPMI 1640. One part of the labeled PBMC preparation was intraperitoneally injected (10^7 PBMC) to each NOG mouse, and human PBMC were recovered from peritoneal lavages and spleen. The other part of the preparation was immediately stimulated with 10 μg of phytohemagglutinin (PHA)/ml, cultured, and harvested. PBMC samples thus obtained were labeled with phycoerythrin (PE)-conjugated anti-CCR5 monoclonal antibody 3A9 or peridinin chlorophyll protein-conjugated anti-HLA-DR antibody (BD Pharmingen, San Diego, Calif.) and subjected to flow cytometric analysis with a Becton Dickinson FACscan cytometer; the data were analyzed by Cell Quest software (Becton Dickinson, Franklin Lakes, N.J.). A quantitative fluorescence-activated cell sorting (FACS) assay that relies on a series of precalibrated beads that bind to a fixed number of mouse immunoglobulin G molecules (Quantum Simply Cellular Kit; Sigma, Saint Louis, Mo.) to determine the absolute number of CCR5s on the cell surface was also conducted according to the manufacturer's instructions (15).

Cells and viruses. The HeLa-CD4-LTR-β-gal indicator cell line expressing human CCR5 ($CCR5^+$ MAGI) (18), a kind gift from Yosuke Maeda, was used for the present study. 293T cells (a human embryonic kidney cell line) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) and antibiotics and used for transfection of DNA plasmid containing the R5 $HIV-1_{JR-FL}$ genome (13). PBMC isolated from HIV-1-seronegative individuals were cultured with 10% FCS and antibiotics with 10 μg of PHA/ml for 3 days prior to anti-HIV-1 activity assay in vitro (PHA-PBMC). A panel of HIV-1 strains was employed for the drug susceptibility attempt: $HIV-1_{Ba-L}$ (7), $HIV-1_{JR-FL}$ (13), $HIV-1_{NL4-3}$ (32), a wild-type $HIV-1_{MOKW}$ isolated from a drug-naive AIDS patient (17), and MDR primary HIV-1 ($HIV-1_{MDR}$) strain ($HIV-1_{JSL}$ and $HIV-1_{MM}$) (35). All primary HIV-1 strains were passaged once or twice in PHA-PBMC cultures and the culture supernatants were stored at −80°C until use. Antiviral assays using PHA-PBMC were conducted as previously reported (12, 17, 35).

Antiviral agents and assay for inhibition of R5 HIV-1 infectivity and replication. A series of different spirodiketopiperazine (SDP) derivatives were newly designed, synthesized, and tested for their activity against in vitro infectivity and replication of R5 HIV-1 as previously described (17). AK602 was chosen for this study based on its CCR5-specific, potent activity against R5 HIV-1. A method for the synthesis of AK602 will be published elsewhere. The structure of AK602 is illustrated in Fig. 1. An approved drug for therapy for HIV-1 infection, 2′,3′-dideoxyinosine (ddI) (20, 21), was kindly provided by Ajinomoto Co., Inc, Tokyo, Japan. TAK779 and SCH-C were synthesized according to previously published data (1, 30). The MAGI assay using $CCR5^+$ MAGI cells was conducted as previously described (17) with minor modifications. Briefly, $CCR5^+$ MAGI cells were seeded in 96-well, flat-bottomed microculture plates (10^4 cells/well) for 24 h, exposed to 0.1 or 1 μM AK602 for 30 min, washed three times, exposed to R5 HIV-1 (100 50% tissue culture infectious doses) at various time points after AK602 removal, and cultured in Dulbecco's modified Eagle medium containing 15% FCS for 48 h. Following the removal of supernatants and lysis of the cells with PBS (100 μl) containing 1% Triton X-100, a solution (100 μl) containing 10 mM chlorophenol red-β-D-galactopyranoside, 2 mM $MgCl_2$, and 0.1 M KH_2PO_4 was added to each well; the mixture was incubated at room temperature in the dark for 30 min; and the optical density (wavelength, 570 nm) was measured with a microplate reader (*V*max, Molecular Devices, Sunnyvale, Calif). All assays were performed in triplicate.

Pharmacokinetic analysis of AK602 in hu-PBMC-NOG mice. Pharmacokinetic analysis of AK602 in hu-PBMC-NOG mice was performed as previously described (28). In brief, plasma samples were collected periodically over 12 h, following a single AK602 administration at a dose of 60 mg/kg of body weight dissolved in 400 μl of 4% hydroxypropyl-β cyclodextrin (HPBC). Each plasma sample (150 μl) was centrifuged at 3,000 rpm for 10 min, and the supernatant was vacuum concentrated and injected into the high-performance liquid chromatography (HPLC) system. The eluent was monitored at 255 nm of UV, and the AK602 concentration in plasma was determined.

Determination of amounts of AK602 persistently bound to CCR5 in hu-PBMC-NOG mice. Blood samples were collected from the tail vein of each hu-PBMC-NOG mouse at various time points following a single intraperitoneal administration of AK602 at a dose of 60 mg/kg. PBMC were isolated by density gradient centrifugation and stained with fluorescein isothiocyanate-conjugated monoclonal antibody 45531 (R&D Systems, Minneapolis, Minn.) specific for the C-terminal half of the second extracellular loop (ECL2B) of CCR5 (15) known to be competitively replaced by SDP derivatives (17) or with PE-conjugated monoclonal antibody 3A9, which binds to the N-terminus extracellular domain of CCR5 (17). PBMC were then subjected to FACS analysis.

Treatment of R5 HIV-1-infected hu-PBMC-NOG mice with anti-HIV-1 agents. Sixteen days after PBMC infusion, the mice were bled from the tail vein, and three-color flow cytometric analysis was performed to confirm positive engraftment of human HLA, CD4, and CD8 antigens on the cells recovered. $HIV-1_{JR-FL}$ (2,000 50% tissue culture infectious doses) was intraperitoneally inoculated to each mouse in which PBMC engraftment was confirmed. Twenty-four hours after the R5 HIV-1 inoculation, administration of AK602 (120 mg in 4% HPBC/kg/day, twice a day), ddI (50 mg in 4% HPBC/kg/day, twice a day), or saline was implemented and continued by day 16. On days 5 and 9 after the R5 HIV-1 inoculation, blood samples were collected from mouse tail veins for immunologic and virological monitoring (see below). On day 16, blood samples were collected by cardiocentesis, and the mice were sacrificed. The experimental protocol for the treatment is illustrated in Fig. 2.

Immunologic and virological monitoring. Human PBMC recovered from mice were subjected to immunologic and virological monitoring as previously described (23, 24). The $CD4^+$/$CD8^+$ cell ratios were determined by FACS analysis with PE-conjugated mouse anti-CD4 and peridinin chlorophyll protein-conjugated mouse anti-CD8 (BD Pharmingen) monoclonal antibodies. Determination of HIV-1 DNA copy numbers in recovered human PBMC was performed by real-time PCR assay with Taqman Master mixture (PE Biosystems) and HIV long terminal repeat-specific primers M667 (5′-GGC TAA CTA GGG AAC CCA CTG-3′) and AA55 (5′-CTG CTA GAG ATT TTC CAC ACT GAC-3′). HIV-1-specific products were quantified with the ABI 7700 detection system (Applied Biosystems, Foster City, Calif.), and cell numbers were determined with the RAG-1 gene. The numbers of $CD4^+$ cells were calculated based on the percentage of $CD4^+$ values obtained from the FACS analysis of each test PBMC sample, and R5 HIV-1 proviral DNA copy numbers were expressed as copy numbers per 10^5 $CD4^+$ cells. In some experiments, $CD4^+$ and $CD4^-$ cells were separated before real-time PCR assay with the rapid immunomagnetic CD4-positive cell isolation kit (Dynabeads M-450 CD4; Dynal Biotech, Inc., Lake

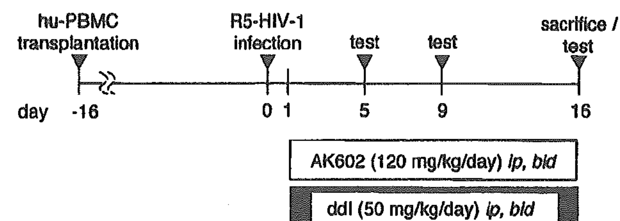

FIG. 2. Protocol for drug administration and immunological and virologic monitoring.

Success, N.Y.). The amounts of p24 antigen in murine sera were determined using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio, Inc., Tokyo, Japan) as previously described (12). Plasma viral load was quantified with the AMPLICOR HIV-1 monitor test kit, version 1.5 (Roche Diagnostics, Branchburg, N.J.).

Statistical analyses. Nonparametric statistical analyses were performed by using the Mann-Whitney U test (Statview, version 5.0; Abacus Concepts, Berkeley, Calif.). The difference between viremia levels in two groups of mice was determined by the Wilcoxon rank sum test. For each mouse, the value of $\log_{10}$ RNA copies was calculated, and the slope corresponding to the rate of increase per day was determined by simple linear regression for the days (5, 9, and 16) of blood collection. The resulting slopes for all mice in the untreated groups were compared to the slopes of mice in each of the other two groups.

RESULTS

Transplanted PBMC in hu-PBMC-NOG mice are intensely activated and express high levels of CCR5. When we examined the proliferation profile of PBMC stimulated with PHA in vitro by treatment with the vital dye CFSE, which allows the analysis of cell proliferation as the CFSE's fluorescence intensity is halved per each cell division, there was only a slight shift to the left in the flow cytometric profile on days 1 and 2 of culture (Fig. 3A). On day 4 of culture, a discrete shift to the left was identified, suggesting that the PHA-PBMC underwent up to four cycles of proliferation in vitro by day 4. In contrast, PBMC transplanted and recovered on day 2 had apparently undergone ~4 cycles of proliferation; by day 4, a majority of cells had undergone up to 10 cycles and beyond in proliferation (Fig. 3B). It was possible that the CFSE-negative and weakly CFSE-positive cells which accumulated on days 2 and 4 (Fig. 3B) were murine cells that engulfed and degraded CFSE. We therefore conducted experiments in which the cells with CFSE dilution were directly confirmed to be human CCR5-positive cells. As can be seen in Fig. 3C, when cells were recovered from the spleen of an NOG mouse into which CFSE-labeled PBMC had been transplanted and stained with monoclonal antibody 45531, which is specific for the C-terminal half of the second extracellular loop (ECL2B) of CCR5 (15), the majority of such human $CCR5^+$ cells proved to be CFSE negative. We also examined the levels of cellular activation by the expression of HLA-DR on cell surface. The levels of HLA-DR expression in PBMC recovered from uninfected NOG mice 3 days after transplantation were much greater than those in 3-day-cultured PBMC following PHA stimulation (Fig. 3D). The fluorescence intensity in the same donor's PHA-PBMC examined on three different occasions was 21 ± 4, while that of the PBMC recovered from mice was 91 ± 25 (Fig. 3D). When we further assessed the levels of CCR5 expression, the PBMC recovered from the mice on day 3 proved to be strongly positive for CCR5 (Fig. 3E). The CCR5-positive fraction in the PBMC recovered was 49.7%, while that in PHA-PBMC was 27.3%. The mean fluorescence intensity of the $CCR5^+$ cell population was 141, compared to the $CCR5^+$ cell population in PHA-PBMC with a mean fluorescence intensity of 51. The estimated number of CCR5 expressed on the PBMC recovered on day 3 was 25,348 (as antibody binding sites per cell) while that on PHA-PBMC on day 3 in culture was 8,981 antibody binding sites as examined by quantitative FACS assay. These data indicate that the transplanted human PBMC were intensely activated and rapidly proliferating and expressed high levels of CCR5 on their cell surfaces.

Potent activity of AK602 against R5 HIV-1 in vitro. Among SDP derivatives we designed and synthesized, AK602 was identified to be highly potent against a broad spectrum of R5 HIV-1 strains, including MDR clinical R5 HIV-1 isolates in vitro with 50% inhibitory concentration (IC_{50}) values of 0.3 to 0.6 nM, although two previously published CCR5 antagonists (TAK779 and SCH-C) were substantially less potent than AK602 (Table 1). AK602 and other CCR5 antagonists failed to inhibit the replication of an X4 HIV-1 strain, HIV-1_{NL4-3}.

Pharmacokinetics of AK602 in hu-PBMC-NOG mice. We examined the pharmacokinetics of AK602 in hu-PBMC-NOG mice by intraperitoneally administering the compound at a dose of 60 mg/kg. Plasma samples were collected periodically up to 12 h and subjected to HPLC analysis. As shown in Fig. 4A, the concentration of AK602 reached the maximal concentration immediately after intraperitoneal administration and decreased rapidly. The calculated plasma half-life in the α-phase of the concentration curve was as short as 29 min.

AK602 persists on cell surface CCR5. As shown above, the plasma half-life of AK602 turned out to be short; however, considering that AK602 possesses such a high affinity to CCR5 and potent activity against R5 HIV-1 in vitro, it was thought possible that AK602 would remain attached on cellular CCR5 for an extensive period of time and exert anti-R5 HIV-1 activity even when the compound was depleted from circulation. To examine this possibility, we used two monoclonal antibodies, 45531 and 3A9. When human PBMC were recovered from a hu-PBMC-NOG mouse 2 and 6 h after AK602 administration (60 mg/kg) and stained with 45531, AK602 proved to block the binding of 45531 to CCR5 (Fig. 4B), while AK602 failed to block 3A9 binding to CCR5 (Fig. 4C), suggesting that AK602 did not elicit CCR5 internalization or shedding at all at least for 6 h. We subsequently examined whether AK602 remained on cellular CCR5 with the 45531 monoclonal antibody. When the cells were recovered from mice 2, 6, and 14 h after the AK602 administration, the mean values of the percentage of AK602 occupancy were 85 (four mice), 54 (three mice), and 16 (three mice), respectively. It was calculated that it took about 9 h for AK602 occupancy to be reduced by 50% (Fig. 4D).

Anti-R5 HIV-1 activity of AK602 persistently seen after its removal from culture medium. In another depletion experiment, we exposed $CCR5^+$ MAGI cells to AK602 for 30 min, depleted the compound from the culture by thorough washing, incubated the cells for various lengths of time, exposed the cells to HIV-1_{Ba-L}, further cultured the cells for 48 h, and determined whether HIV-1_{Ba-L} infection was blocked by AK602 exposure (Fig. 4E). When the $CCR5^+$ MAGI cells were exposed to 0.1 and 1 μM AK602 and exposed to HIV-1_{Ba-L} immediately afterward, the values for protection were 68 and 85%, respectively. When the cells were exposed to HIV-1_{Ba-L} 4 h after depletion, 49 and 72% of the cells were protected by 0.1 and 1 μM AK602. When the cells were exposed to HIV-1_{Ba-L} 12 and 24 h after depletion, 57 and 45% of the cells were seen protected by 1 μM, respectively (Fig. 4E).

Effects of AK602 on $CD4^+$ and $CD8^+$ cell counts in R5 HIV-1-infected hu-PBMC-NOG mice. PBMC were recovered from murine blood samples collected on days 5, 9, and 16 after R5 HIV-1 inoculation and subjected to flow cytometric analysis for determination of $CD4^+$/$CD8^+$ cell ratios. As shown in Fig. 5A, in PBMC recovered on day 16 from a representative

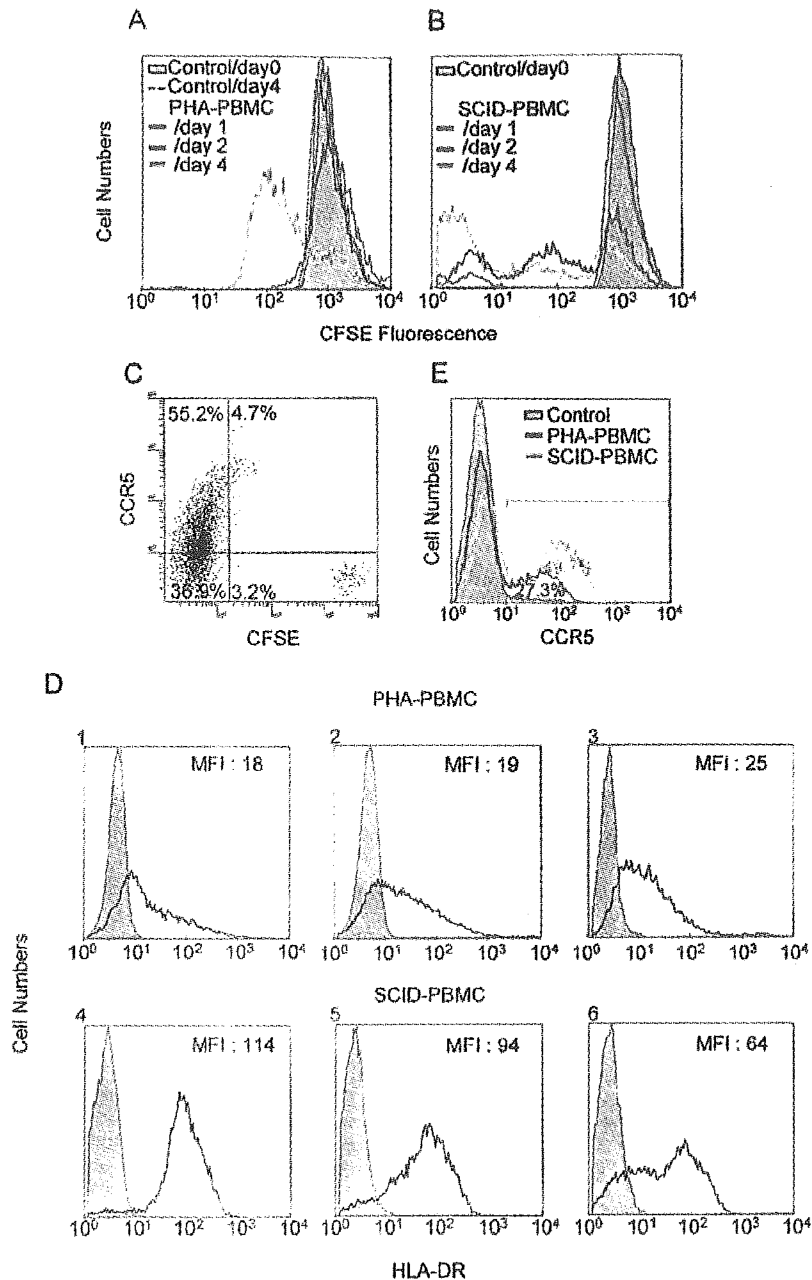

FIG. 3. Transplanted PBMC are intensely activated and express high levels of CCR5. (A and B) Proliferation profiles of PHA-PBMC and transplanted and recovered PBMC. Freshly prepared PBMC were incubated with the vital dye CFSE, and one part of such PBMC preparation was stimulated with PHA, while the other part was intraperitoneally transplanted to mice. On days 1, 2, and 4, the cells were harvested and the fluorescence intensity of CFSE was determined. Note that transplanted PBMC recovered on day 2 had undergone ~4 cycles of proliferation; by day 4, a majority of cells had undergone ~10 cycles and more of proliferation. (C) CCR5 expression level and CFSE intensity in human PBMC harvested from a spleen of hu-PBMC-NOG mouse on day 4. (D) Intense activation of PBMC after transplantation. PBMC stimulated with PHA and cultured for 4 days (panels 1 to 3) and transplanted PBMC recovered from the uninfected mice on day 4 (panels 4 to 6) were stained with an anti-HLA-DR monoclonal antibody. Note that HLA-DR expression levels in transplanted PBMC were much higher than those in PHA-PBMC. (E) CCR5 expression profiles of PHA-PBMC and transplanted PBMC. PBMC stimulated with PHA and cultured for 3 days and transplanted PBMC recovered from the uninfected mice on day 3 were stained with PE-conjugated anti-CCR5 monoclonal antibody 3A9 and subjected to flow cytometric analysis. SCID-PBMC, PBMC transplanted and recovered.

R5 HIV-1-infected, saline-treated mouse, there were only few $CD4^+$ cells (3.9% [1.4% + 2.5%]) resulting in a $CD4^+/CD8^+$ cell ratio of 0.05. However, a distinct $CD4^+$ cell population (55.1% [4.4% + 50.7%]) resulting in a $CD4^+/CD8^+$ ratio of 1.84 (Fig. 5B) was seen in PBMC recovered from an AK602-treated mouse, and the size of this $CD4^+$ cell population was comparable to that seen in a ddI-treated mouse (53.2% [3.8% + 49.4%]) and that in an uninfected mouse (48.9% [3.8% +

TABLE 1. Anti HIV-1 activity of novel SDP derivatives in PBMC[a]

Compound	IC_{50} value in p24 assay (nM)					
	HIV-$1_{Ba\text{-}L}$ (R5)	HIV-1_{JRFL} (R5)	HIV-1_{MOKW} (R5)	HIV-1_{MM} ($R5_{MDR}$)	HIV-1_{JSL} ($R5_{MDR}$)	HIV-$1_{NL4\text{-}3}$ (X4)
AK602	0.5 ± 0.3	0.2 ± 0.1	0.3 ± 0.2	0.7 ± 0.3	0.4 ± 0.2	>1,000
TAK779	14 ± 5	6 ± 2	9 ± 3	12 ± 4	10 ± 3	>1,000
SCH-C	3 ± 2	2 ± 1	2 ± 1.5	2.5 ± 1	2 ± 1	>1,000
ZDV	13 ± 5	7 ± 3	10 ± 6	520 ± 75	64 ± 13	9 ± 5
SQV	8 ± 3	6 ± 2	6 ± 3	212 ± 56	276 ± 44	10 ± 4

[a] IC_{50}s were determined by using PHA-PBMC isolated from three different donors, and the inhibition of p24 Gag protein production was used as an endpoint. All assays were conducted in triplicate. The results shown represent arithmetic means (±1 standard deviation) of three independently conducted assays. HIV-1_{MOKW} was isolated from a drug-naive AIDS patient, and HIV-1_{JSL} and HIV-1_{MM} were isolated from patients who received antiretroviral therapy for a long period of time and whose virus loads showed a number of RT and PR mutations. Two previously published CCR5 inhibitors, TAK779 and SCH-C, and zidovudine (ZDV) and saquinavar (SQV) were used as reference compounds.

45.1%]), resulting in the ratios of 1.43 and 1.40 (Fig. 5C and D), respectively. Figure 6A illustrates the overall profiles of $CD4^+/CD8^+$ cells ratios on day 16 in the four groups. The mean $CD4^+/CD8^+$ cell ratio in mice (n = 7) given saline was 0.1 (range, 0.06 to 0.20). In contrast, the ratios in AK602-treated mice (n = 8) were significantly higher with a mean value of 0.92 (range, 0.23 to 1.89; P = 0.001), which was comparable to that in ddI-treated mice (n = 9; mean, 1.29; range, 0.38 to 2.68; P = 0.001) and uninfected mice (n = 7; mean, 1.0; range, 0.50 to 1.49). The numbers of $CD4^+$ cells/μl

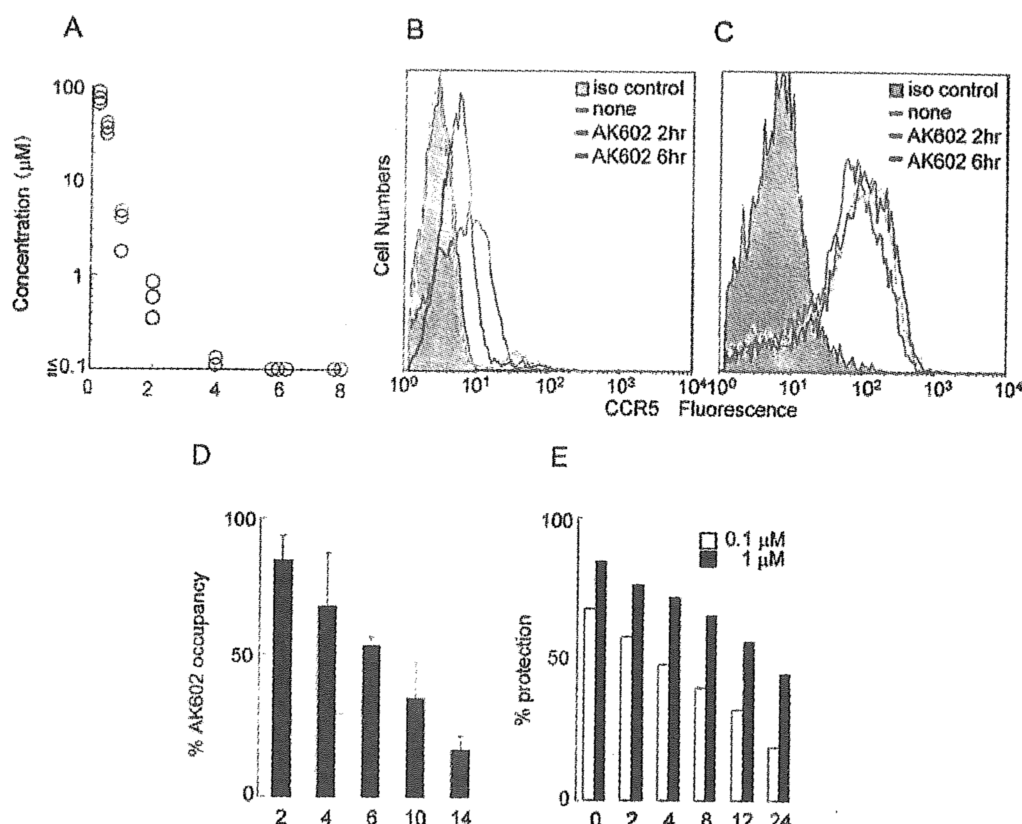

FIG. 4. Pharmacokinetics and persistence of anti-HIV-1 activity of AK602. (A) Pharmacokinetics of AK602. Each mouse was administered AK602 at a dose of 60 mg/kg, and blood samples were taken at 15, 30, 60, 120, 240, 480, and 720 min. Plasma concentrations of AK602 determined by HPLC analysis at 15, 30, 60, 120, and 240 min were 76.2, 36.1, 3.5, 0.6, and 0.13 μM, respectively. AK602 was not detected at later time points. (B and C) No CCR5 internalization or shedding was caused by AK602. Human PBMC were recovered 2 and 6 h after AK602 administration and stained with 45531 (B) or 3A9 (C). (D) Sustained AK602 occupancy on cell surfaces. At indicated periods of time after a bolus of AK-602 (60 mg/kg) was administered to hu-PBMC-NOG mice, PBMC were recovered and the percentages of AK602 occupancy on cellular CCR5 were determined with fluorescein isothiocyanate-conjugated monoclonal antibody 45531. (E) Persistence of in vitro activity of AK602 against R5 HIV-1 after AK602 depletion. $CCR5^+$ MAGI cells were exposed to 0.1 or 1 μM AK602 for 30 min and thoroughly washed to deplete AK602 from the medium. The cells were subsequently cultured for the indicated periods of time, exposed to HIV-$1_{Ba\text{-}L}$, and further cultured for 48 h, when the cells were harvested and lysed with Triton X-100-containing PBS. A solution containing chlorophenol red-β-D-galactopyranoside was added, the optical density was measured, and the percentage of protection was determined.

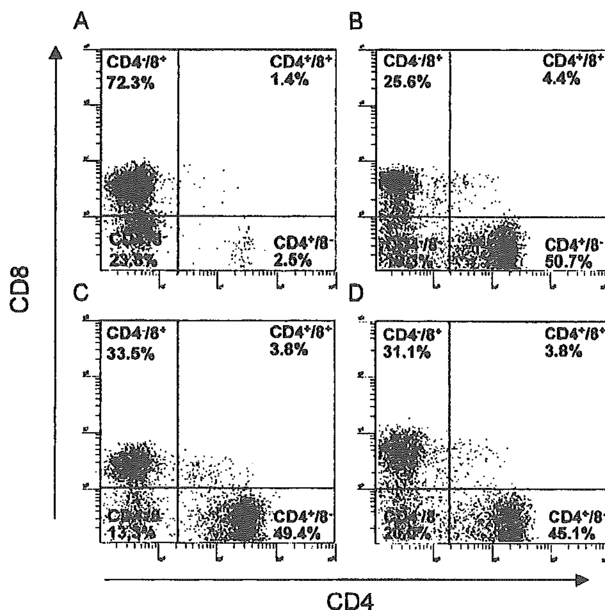

FIG. 5. Effects of AK602 on $CD4^+$ and $CD8^+$ cell counts in infected hu-PBMC-NOG mice. PBMC recovered on day 16 after R5 HIV-1 inoculation were subjected to flow cytometry. Shown are representative flow cytometric analysis profiles. Note that only 3.9% of $CD4^+$ cells were seen (A), resulting in a $CD4^+/CD8^+$ cell ratio of 0.05 in a mouse given saline, while distinct numbers of $CD4^+$ cells (55.1 and 53.2%) (B and C) were seen in AK602- and ddI-administered infected mice, resulting in $CD4^+/CD8^+$ cell ratios of 1.84 and 1.43, respectively. In an uninfected mouse (D), 48.9% of cells were positive for CD4, with a $CD4^+/CD8^+$ cell ratio of 1.40.

in saline-treated mice were significantly less than those of AK602-treated, ddI-treated, or uninfected mice (Fig. 6B).

Effects of AK602 on R5 HIV-1 proviral DNA copy numbers and serum p24 levels in R5 HIV-1-infected hu-PBMC-NOG mice. We next asked which population harbored proviral DNA in the cells recovered from R5 HIV-1-infected hu-PBMC-NOG mice, by purifying $CD4^+$ and $CD4^-$ cell populations and determining proviral DNA copy numbers in each population. As shown in Table 2, more than 99% of proviral DNA was found in $CD4^+$ cells and <0.3% of proviral DNA was detected in $CD4^-$ cells derived from saline-treated mice, indicating that R5 HIV-1 infection occurred in $CD4^+$ cells in the hu-PBMC-transplanted NOG environment. As illustrated in Fig. 6C, the mean number of R5 HIV-1 proviral DNA copies was 2.0×10^5 (range, 2.6×10^4 to 1.7×10^6) per 10^5 $CD4^+$ cells in R5 HIV-1-infected mice ($n = 7$) given saline. However, values for mice in groups given AK602 and ddI were 1.3×10^3 (range, 2.3×10^2 to 7.9×10^3; $P = 0.001$) and 1.8×10^2 (range, $<10^2$ to 7.9×10^2; $P = 0.001$), respectively.

The amounts of R5 HIV-1 p24 in serum were also found to be very high in saline-treated mice, with a mean amount of 1.1×10^5 pg/ml (range, 3.1×10^4 to 2.8×10^5 pg/ml). AK602 and ddI were found to significantly suppress the serum p24 amounts as examined on day 16 with a mean amount of 5.6×10^3 pg/ml (range, 8.1×10^2 to 2.1×10^4 pg/ml; $P = 0.001$) and 7.1×10^2 pg/ml (range, 1.3×10^2 to 1.1×10^4 pg/ml; $P = 0.001$), respectively (Fig. 6D).

AK602 suppressed R5 HIV-1 viremia in hu-PBMC-NOG mice. As described above, the PBMC transplanted to NOG mice were intensely activated in the xenogeneic environment and had undergone ~4 cycles of proliferation by day 2; a majority of the cells had undergone ≥10 cycles of proliferation by day 4 (Fig. 3B). These data suggested that R5 HIV-1 might extensively replicate in the hu-PBMC-NOG mice immediately after R5 HIV-1 inoculation. When we collected blood samples on days 5, 9, and 16 following the inoculation and determined R5 HIV-1 RNA copy numbers in infected, saline-treated mice ($n = 7$), the geometric mean copy number was 8.6×10^3/ml (range, 1.7×10^3 to 1.0×10^5) on day 5 and rapidly increased to 1.9×10^5/ml (range, 2.2×10^4 to 3.0×10^6) on day 9; by day 16, the mean copy number had reached 7.7×10^5/ml (range, 2.6×10^5 to 3.0×10^6/ml). However, AK602 significantly suppressed viremia by ~1.1 log, as examined on day 5; the mean numbers of R5 HIV-1 RNA copies in AK602-administered mice were 1.6 and 1.8 logs lower than those in saline-treated mice examined on days 9 and 16, respectively (Fig. 7). Comparable viremia suppression was seen in the mice receiving ddI (Fig. 7). It was noted that although AK602 did not completely prevent the viremia from further increasing after day 5, there was a clear reduction in the viremia increase rates. The mean slopes (change in RNA copies per day over the range of data from 5 to 16 days) for the group receiving saline was 0.167 ± 0.042, whereas those for the AK602 and ddI groups were 0.102 ± 0.041 and 0.091 ± 0.037, respectively. Thus, the rates of increase in the AK602 ($P = 0.0057$) and ddI ($P = 0.0023$) mice were significantly lower than that for the mice given saline, indicating that both of the agents significantly inhibited R5 HIV-1 replication in this mouse model over the range of days evaluated. No apparent AK602- or ddI-associated adverse effects were seen throughout the study period.

DISCUSSION

In the present hu-PBMC-NOG mouse model, human $CD4^+/CD8^+$ cell ratios went down to 0.1 by 16 days after R5 HIV-1 inoculation, the amounts of proviral DNA and p24 gag antigen reached 10^5 to 10^6 copies/10^5 $CD4^+$ cells and 10^5 pg/ml, respectively (Fig. 6), and no mice failed to be infected with R5 HIV-1. It is noteworthy that the use of NOG mice provides a higher engraftment rate than with other SCID mice such as NOD/Shi-SCID mice treated with anti-NK cell antibody or the β_2-microglobulin-deficient NOD-SCID mice (10). With NOG mice, the chimeric rate of 30 to 40% is achieved, and cord blood $CD34^+$ cells have been shown to "take" with as few as 100 cells (10). Moreover, all infected mice developed high levels of R5 HIV-1 viremia by day 16, reaching as high as 10^6 copies/ml (Fig. 7). It is worth noting that the notably high levels of HIV-1 viremia seen in the present mouse model by 16 days after R5 HIV-1 exposure can be seen only on acute infection or up to 10 years after HIV infection in humans (3, 4).

In the present study, we found that the conspicuous susceptibility to the infectivity and replication of R5 HIV-1 in these mice appeared to stem from the hyperactivation of the implanted human PBMC. The implanted PBMC were highly activated in the xenogeneic environment, expressed quite high