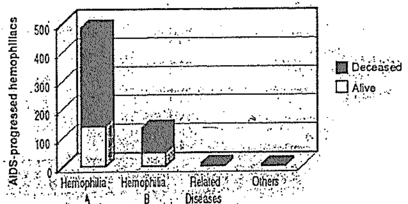

FIGURE 2. Total numbers of AIDS-progressed hemophiliacs treated with coagulant factor products prior to May 1996 in Japan. Deaths are shown in black.

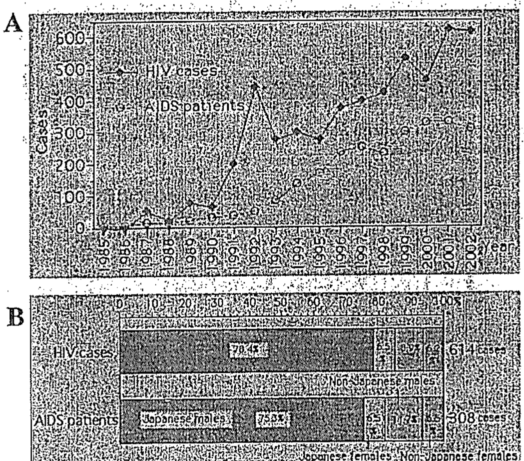

FIGURE 3. Increase of HIV/AIDS incidence in Japan. Increase of annual incidence of HIV/AIDS from 1985 to 2002 (A). HIV and AIDS cases in 2002 by gender and Japanese nationality (B). Japanese males predominated among both HIV-positive individuals and people with AIDS in 2002. Non-Japanese represented 15.1% of HIV-positive non-hemophiliac individuals and 18.7% of people with AIDS in 2002 (MHLW, 2002; NIID, 2003).

both of these groups, 78% and 75%, respectively (Figure 3B).

HIV infections among Japanese males have been continuously increasing; cases among Japanese females are also increasing but the total number has been less than 50 per year (Figure 4). It is important to note that HIV infections among Japanese men who have sex with men (MSM) are rapidly increasing (Figures 3 and 4). Cases among non-Japanese males are gradually increasing, and cases among non-Japanese females peaked

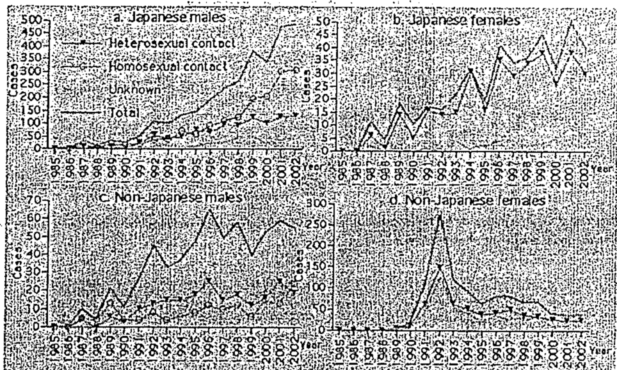

FIGURE 4. Study of the increase of HIV incidence by nationality, gender, and mode of infection from 1985 to 2002 in Japan (MHLW, 2002; NIID, 2003).

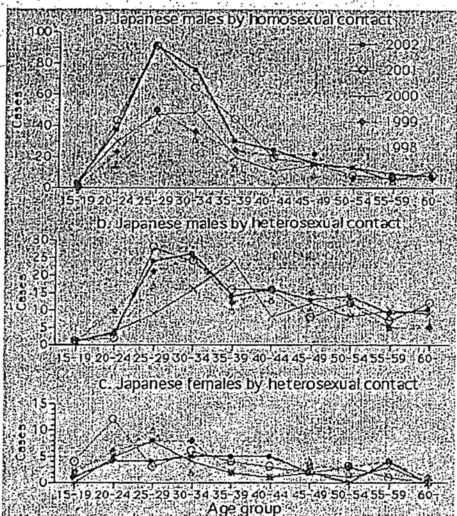

FIGURE 5. Age distribution analysis of HIV incidence by gender and mode of infection from 1998 to 2002 in Japan (MHLW, 2002; NIID, 2003).

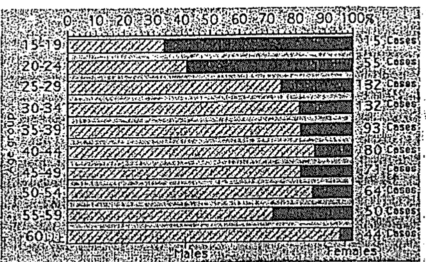

FIGURE 6. Distribution of heterosexual HIV infections by gender and age group, 1998–2002. Japanese females predominated among young HIV-positive Japanese contracting HIV by heterosexual contact from 1998 to 2002. Ten age categories were created to cover ages 15 to over 60 (MHLW, 2002; NIID, 2003).

in 1992, but the percentage of non-Japanese in the total of HIV cases is more than 18%, higher than that of other countries.

HIV infections are considered to be mainly due to sexual transmission among Japanese males in Japan (Figures 5 and 6). Although the prevalence is low as described above, heterosexually acquired HIV has been rapidly increasing since the early 1990s in Japan (Umeda, 1999).

Interestingly, the first HIV-antibody-positive case among blood donors was detected in 1987, and the positive rate per 100,000 donations increased to 1.368 (2.259 in males and 0.043 in females) in 2001, a rate approximately 10–100 times higher than that of European and American countries (Figure 7). Contaminated blood was found in 67, 79, and 82 donors in 2000, 2001, and 2002, respectively; six infections were detected by nucleic acid test with PCR detection. These data suggest the need for careful evaluation of whether HIV-positive blood donors may be more likely to donate blood frequently for the purpose of HIV testing, as speculated, or whether HIV may truly be spreading vigorously in Japan.

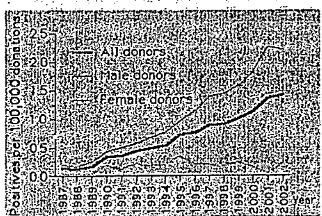

FIGURE 7. Rate of HIV-antibody positives per 100,000 blood donors, 1987–2002. HIV infections among blood donors were rapidly increasing from 1987 to 2002, at a rate much higher than that of European countries. Numbers of HIV-positive blood donors detected in 2000, 2001 and 2002 were 67, 79, and 82, respectively, and only 6 of 228 positives were detected by the nucleic acid amplification test (MHLW, 2002; NIID, 2003).

PHENOTYPIC CHARACTERIZATION OF HIV IN JAPAN

The molecular epidemiology of HIV in Southeast Asian countries has been well characterized by studying this unique geographical hot spot, where extensive recombination events appear to be ongoing. These events suggests the presence of highly exposed individuals and social networks of HIV transmission (Motomura *et al.*, 2003; Takebe *et al.*, 2003; Weniger *et al.*, 1994; Yang *et al.*,

2003). In Japan, hemophiliacs who received frequent transfusions of HIV-contaminated blood products comprised the largest population of HIV-positive cases until 1998 (Kamakura *et al.*, 1998). The HIV-1s detected in the hemophiliacs were all HIV-1 clade B; the major HIV clade circulating in Japan has also been identified as clade B by genotypic analysis of HIV-1 *env* C2-V3 region (Hattori *et al.*, 1991; Yamanaka *et al.* 1997). However, the National Survey of HIV/AIDS in 1999 found sexual transmission to be a major risk factor in the population of HIV-positive individuals in Japan. During the rapid increase in sexually transmitted HIV infections in Japan, which took place from 1996 to 1998, 40% of the HIV-positive individuals infected via MSM contact were grouped into subtype E. Others were subtype B, including Japanese males infected by heterosexual contact with multiple partners both abroad and in Japan (Kitsutani *et al.*, 1998). Furthermore, a replication-competent molecular clone of HIV-1 was isolated from an HIV-1 NH1 isolate in Japan as an CRF01_AE strain, and the infectious molecular clone was designated p93JP-NH1 (Kusagawa *et al.*, 2002). Further epidemiological research of HIV in Japan has detected a gradual shift toward an increase with predominantly heterosexual and MSM contact (Infectious Disease Surveillance Center, 2003). Apart from subtypes B and E, other HIV-1 subtypes A, C, D, and G, and HIV-2 have also been sporadically found in Japan (Hara *et al.*, 2001; Kusagawa *et al.*, 2003; Nakasone *et al.*, 1998). Efforts to isolate infectious clones of various HIV subtypes are continuing to define the HIV phenotypes spreading in the field (Mochizuki *et al.*, 1999).

ANTI-HIV CHEMOTHERAPY IN JAPAN

Between 1988 and 1999, HIV was isolated from 614 HIV-positive individuals out of a total 2785 cases. During those 11 years, annual HIV isolation rates were found to decrease, reflecting the success of anti-HIV chemotherapy with single, double, and triple combinations of antiretroviral drugs (Figure 8) (Nakamura *et al.*, 1987).

In 1997, three protease inhibitors, Saquinavir, Ritonavir, and Indinavir became available for clinical use in Japan, and highly active antiretroviral therapy (HAART) began being used for treatment of people with HIV/AIDS (PLWHA) in Japan. Among 446 cases, 87 (60.0%) out of 145 naïve cases and 150 (49.8%) out of 301 drug-treatment-experienced cases showed a reduction of plasma viral load to undetectable levels. Successful therapy with HAART was maintained for more than twelve months among 50% of the medicated patients (Oishi *et al.*, 1999). The prevalence of emerging drug resistance-related mutations has been systematically monitored to evaluate the efficacy of HAART in Japan (Sugiura, 2001; Sugiura *et al.*, 1999).

VERTICAL TRANSMISSION OF HIV IN JAPAN

A national collaborative group for studying vertical transmission of HIV in pregnant women and their infants was established by Dr. Ryozo Totani in Japan in 1989. Through their research, 42 infants, including 13 HIV-positive, 25 uninfected, four of undetermined status, and 15 control children born to HIV-positive mothers were diagnosed and followed from birth to 1.5 years (Yoshino *et al.*, 1998). All strains from HIV-positive infants were either clade E (eight infants, 61.5%) or B (five infants, 38.5%) according to DNA sequencing specific for the HIV-1 C2-V3 region. The 42 HIV-positive mothers were women with sexual risk behaviors from all regions, but were concentrated in the Kanto District. In this group of HIV-positive children, there was no significant difference between the transmissibility of their mother's clade E and B viruses. Eight (61.5%) of the 13 HIV-positive babies were Japanese and five (62.5%) of the

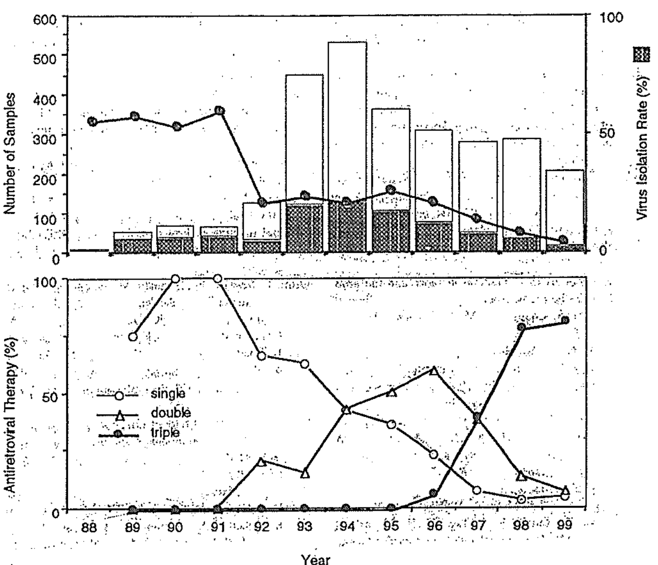

FIGURE 8. Viral isolation rate and antiretroviral therapy coverage, 1988–1999. Decline in the HIV-1 isolation rate in Japan may correspond to anti-HIV chemotherapy. Upper panel: light and dark dotted bars indicate the total number of samples, and sample numbers positive for HIV-1 isolation, respectively. Bold line indicates the kinetics of the rate of HIV-1 isolation. Lower panel: open circle, open triangle and closed circle lines indicate percentage of patients treated with single, double, and triple anti-HIV chemotherapies, respectively.

eight were positive for HIV-1 clade E. The V3 loop region of the clade E virus of the babies was conserved, but approximately 60% of the sequences showed a substitution of aspartic acid by asparagine at position 29. These results suggest that HIV clade E may be predominant in cases of vertical transmission and are phenotypically different from HIV in persons with various other risk behaviors in Japan. Prevention efforts against vertical transmission have been quite successfully undertaken through a combination of Caesarian-section deliveries and chemotherapy during the delivery stage.

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Japan's Collaboration with Thailand in the Development of an HIV/AIDS Vaccine

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INTRODUCTION

On March 26, 1998, the Japan Science and Technology Corporation, Japan's Ministry of Education, Science and Culture, Ministry of Health, Labor and Welfare, and Thailand's Department of Medical Science and Ministry of Public Health jointly launched a bilateral collaborative HIV vaccine project with equal partnership. This international collaboration primarily focused on in-country capacity building for HIV vaccine research and development, and preclinical development of the Thailand-Japan AIDS vaccine candidates that targeted the prevalent HIV-1 subtype in Thailand—HIV CRF01_AE—with potential for work with other types of vaccines as well in Thailand.

The Thailand-Japan Cooperative AIDS Vaccine Research Project (T-J CAVRP) initially targeted development of a recombinant Bacillus Calmette-Guérin (rBCG)-based HIV CRF01_AE *env* vaccine (Chujoh *et al.*, 2002). However, a more realistic goal of a preventive HIV vaccine may be to limit HIV infection by producing a cellular immune response to control viral load and prevent disease progression (Nabel, 2001; Schultz and Bradac, 2001). Therefore, the vaccine strategy was changed to apply the experimental results of a prime-boost regimen consisting of rBCG-SIV priming followed by recombinant

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collected and virus was isolated from HIV-1-infected individuals throughout Thailand by various local hospitals and institutions, including the Siriraj Hospital, Bamurasnaradura Hospital, Rajavithi Hospital, the Queen Sirikit National Institute of Child Health, and others. Sequence analysis of HIV-1 genes *env*, *env*-V3 (the variable loop 3 of the *env* gene), *pol, gag, tat,* and *nef* were performed. These studies demonstrated that HIV CRF01_AE is spreading all over Thailand. Most of these viruses had the amino acid sequence GPGQ at the tip of V3 of their *env* genes (Naganawa *et al.*, 1997; Sutthent *et al.*, 2001; Balachandra *et al.*, 2002). It was also shown that the HIV CRF01_AE isolates from genital fluid samples appeared to have less genetic divergence than those from the blood samples of the same individuals (Sutthent *et al.*, 2001). Moreover, the sero-reactivity of an HIV-infected individual appeared to decrease when the GPGQ sequence at the tip of V3 was substituted, and its reactivity in AIDS patients was inversely correlated with their tip sequences. The sequence substitutions at the V3 also seemed to correlate with the appearance of HIV-1 mutants that become unrecognizable by the neutralizing antibody in the hosts (Balachandra *et al.*, 2002). HIV CRF01_AE in cerebrospinal fluid was defined to be non-syncytium-inducing even when the patients progressed to the disease state, suggesting that central nervous tissue cells may be one of the reservoirs of CCR5-tropic virus in individuals with advanced disease (Albright *et al.*, 1999). The Gag sequence of HIV CRF01_AE also had a characteristic deletion of six amino acids in the p15 peptide, which was approximately 90% homologous to that of HIV-1 subtype B primary isolates. Another recent study of HIV-1 CRF01_AE showed that the consensus sequence of the *gag* and *env*-V3 region was very close to the consensus sequence of sero-converters in the early epidemic period in Thailand. These data suggest that CRF01_AE might have evolved randomly, and the consensus sequence strategy should be applicable to selecting appropriate vaccine antigens (Hamano *et al.*, 2004).

Various HIV-1 subtypes spread predominately in different human populations in the world. Our studies, together with studies from many other investigators, showed that the predominant HIV-1 subtype in Thailand, CRF01_AE, has significant genetic differences from the predominant HIV-1 subtype B in Japan (Weniger *et al.*, 1994; Kitsutani *et al.*, 1998; Kusagawa *et al.*, 2002). In order to make the candidate Thailand-Japan HIV-1 vaccines beneficial for the people in Thailand where future vaccine efficacy trials will be conducted, we devoted our efforts to match the origins of HIV-1 antigens in our vaccine designs with the HIV CRF01_AE that is prevalent in Thailand. In our opinion, in the absence of convincing evidence showing that the subtype of HIV-1 antigens in a vaccine design has nothing to do with potential vaccine efficacy, developing the Thailand-Japan HIV-1 vaccines that include CRF01_AE was the only ethically correct way to proceed.

HUMAN GENETICS, CELLULAR IMMUNE RESPONSES, AND HIV VACCINE DESIGN

Numerous factors and cofactors are believed to affect the infectivity of HIV and the disease progression of AIDS, some of which warrant special consideration in the HIV/AIDS vaccine design for Thailand. The design was focused on specifically mapping the HIV viral epitopes that are responsible for eliciting HLA restricted anti-HIV cytotoxic T lymphocytes (CTL) responses in HIV-infected people in Thailand. Using the classical chromium release CTL assay, intracellular cytokine staining, and ELISPOT assays, new CTL epitopes were identified in HIV-infected people in Thailand that differed from the dominant CTL epitopes in HIV-infected people in the United States (Ruxrungtham and Phanuphak, 2001; Ariyoshi *et al.*, 2002; Ananworanich *et al.*, 2003). Significant cross-clade CTL responses were also found in Thai HIV-infected people, characterized by reactions to peptide epitopes

derived from different subtypes, including Gag, Env, Pol and Tat antigens (Ruxrungtham *et al.*, 2003). HLA typing in Thai people revealed that the frequencies of HLA-A2, -A11, -B46, -B57 and DQB1*0303 were significantly increased as compared to controls from other populations ($p < 0.05$) (Ward *et al.*, 1995; Vejbaesya *et al.*, 1998; Fukada *et al.*, 2002; Pimtanothai *et al.*, 2003).

These studies provided additional support to the designing principle of the T-J CAVRP that the proposed HIV vaccine candidates for Thailand should be based on CRF01_AE rather than the HIV-1 subtype B that is prevalent in the United States and Japan. By characterizing the immune responses of Thai infected people to CRF01_AE, we also obtained useful information about potential CTL responses in Thailand where the proposed HIV vaccine would be evaluated. Moreover, by collaboratively conducting these state-of-art immunology and molecular virology studies in Thailand with necessary laboratory and clinical capacity and infrastructure building, new vaccine research capability was introduced into Thailand, to benefit future vaccine studies in the country.

HIV VACCINE DEVELOPMENT STRATEGY SPECIFICALLY DESIGNED FOR THAILAND

Increasing evidence suggests that cell-mediated immune responses play critical roles in controlling HIV-1 replication and disease progression. It is widely believed that an effective HIV vaccine should elicit both humoral and cell-mediated immunity in order to provide protection against HIV. Our approach to develop an HIV vaccine for Thailand is mainly focused on the induction of protective cellular immune responses to HIV. In the past five years, T-J CAVRP focused primarily on two types of recombinant vectors: *Mycobacterium bovis* BCG, which has been used in Thailand and Japan for many years as the vaccine against tuberculosis (TB), and vaccinia virus DIs (Tagaya *et al.*, 1961; Kitamura *et al.*, 1967), which had been tested in Japan as the vaccine against smallpox. These two vaccines not only have had excellent safety records for human use, they also have a number of features that are highly desirable for practical use in Thailand. BCG is known to induce long-lasting T helper type 1 responses, which are preferable for induction and maintenance of antigen-specific CTL responses. In addition, BCG can be produced at low cost and can be given by oral, nasal, and rectal administration for targeting mucosal delivery of recombinant antigens to induce effective mucosal immunity (Lagranderie *et al.*, 1997).

Based on the reports that previously used vaccinia strains posed potential safety hazards in immune-compromised individuals, associated with replication of the viruses, we sought to select and evaluate highly attenuated vaccinia virus (DIs) as another vaccine vector for T-J CAVRP. One desirable feature of this particular virus vector is its lack of replication capacity in mammalian cells.

The strategy of using recombinant BCG-Tokyo 172 and attenuated vaccinia virus DIs as the vectors for the proposed Thailand-Japan HIV vaccine also resulted from considerations beyond the proposed clinical testing. If these vaccine candidates turn out to be effective in future large-scale vaccine efficacy trials in Thailand, they should be made available immediately for the Thai government to use. Based on this principle, the transfers of these two recombinant vaccine technologies from Japan to Thailand, including molecular construction, pilot production, and quality control and assurances, has been a major goal of the T-J CAVRP. Moreover, the organizing committee of the T-J CAVRP has also initiated discussions about the intellectual property issues that will affect the use of these vaccines in Thailand. The intellectual property covering any new vaccine of the prime-boost regimen targeting HIV CRF01 AE Gag, achieved by the Thailand-Japan Collaborative

Project, will be equally shared between both countries.

The experimental part of T-J CAVRP has produced encouraging results. First, it was established that whole HIV-1 *gag* genes from subtype B, CRF01_AE, and SIV could be productively expressed by the recombinant BCG-vector. After optimizing the codon usage for viral antigen expression in mycobacteria, we demonstrated that the expression of the HIV-1 p24 antigen by the recombinant BCG increased more than 20-fold (Kanekiyo *et al.*, 2003). We obtained similar results using recombinant non-replicative vaccinia vector DIs, which productively expressed the Gag proteins from HIV subtype B, CRF01_AE, and SIV (Ishii *et al.*, 2002; Izumi *et al.*, 2003). In small animal experiments, these potential HIV vaccine candidates were shown to effectively induce strong anti-HIV specific CTL responses in mice (Ishii *et al.*, 2002).

The same prime-boost regimen, in which the recombinant BCG bacteria were used first and followed by a boost immunization with the recombinant vaccinia DIs that expressed the same HIV antigens, also showed a strong ability to induce CTL in immunized macaque monkeys (Matsuo *et al.*, 2002). This result was critically important to advance the T-J CAVRP into the preclinical testing stage of the HIV vaccine candidates.

PRECLINICAL EVALUATION OF THE THAILAND-JAPAN AIDS VACCINE CANDIDATES

As part of the research capacity building efforts of the T-J CAVRP, facilities and expertise were collaboratively established in the Sasakawa Memorial Animal Facility Building, Nonthaburi, Thailand, where the HIV vaccines would be evaluated in a hu-PBL-NOD-SCID mouse model. Such a complicated but useful animal model enabled us to demonstrate the capability of our prime-boost regimen to induce the desirable neutralizing antibodies that would be required in order to protect human vaccinees from HIV/AIDS. In one particular experiment, the IgG fraction collected from HIV-1-positive individuals, which has neutralizing activity *in vitro* checked by the MAGIC5 cell line, inhibited virus growth in hu-PBL-NOD-SCID mice more than 100 times compared to negative controls (Ogura *et al.*, 1996; Okamoto *et al.*, 1997; Okamoto *et al.*, 1998).

Next, we established the macaque monkey HIV/AIDS models using chimeric human-simian immunodeficiency virus (SHIV) that was specifically developed for this project. A panel of challenging SHIV stocks was prepared for the proposed vaccine efficacy studies, including the nonpathogenic SHIV-MN, SHIV-HXB, the pathogenic SHIV-C2/1, and its molecular clone, SHIV-C2/1 KS661 (Shinohara *et al.*, 1999; Sakai *et al.*, 2001). In order to demonstrate the potential vaccine efficacy for candidates with the SHIV model, we constructed new recombinant BCG and non-replicative vaccinia DIs that productively express the SIV *gag* gene to match the SIV *gag* gene in the challenging SHIV viruses. Results showed that the recombinant BCG-vectored and recombinant non-replicative vaccinia virus DIs-vectored SIV Gag vaccines induced strong CTL responses in immunized monkeys, respectively. However, the best immune responses in monkeys were obtained when the two vaccines were used in combination as a prime-boost regimen. When the immunized monkeys were challenged with the pathogenic SHIV-C2/1, the vaccination provided full protection for the monkey hosts from developing simian AIDS (Matsuo *et al.*, 2002). The CD4 T-cell decline in the vaccinated monkeys after the lethal challenge of SHIV, which is the hallmark of simian AIDS as well as AIDS in humans, was prevented. The plasma virus load in the vaccinated monkeys after the SHIV challenge also showed approximately 100- to 1000-fold reductions in comparison with unvaccinated monkeys (Matsuo *et al.*, 2002). Moreover, it was demonstrated that oral administration of the recombinant

BCG-based vaccines with two doses of 80 mg in monkeys was sufficient to induce the desired virus-specific cell-mediated immune responses, thus presenting the possibility of using the vaccine candidates orally in humans (Kawahara *et al.*, 2002).

These studies also demonstrated that the proposed prime-boost regimen was safe to use in the non-human primate hosts: no detectable adverse effects resulted from vaccination in any of the immunized monkeys. One of the concerns regarding the use of recombinant BCG in Thailand was possible side effects or reduced efficiency in people who were previously immunized with BCG. A series of animal experiments were conducted to address these issues. We showed that prior immunization with BCG did not affect the induction of HIV p24 antigen-specific DTH (delayed-type hypersensitivity) in guinea pigs that were immunized with the recombinant BCG-based HIV vaccines. However, previous exposure to BCG vaccination in monkeys did seem to reduce Gag-specific cellular immune responses in the immunized monkeys by about 50%.

We believe that the recombinant BCG-based HIV vaccines would be safe to use in Thailand. To date, more than two billion people worldwide have been vaccinated with BCG against TB with a proven safety record. With regard to the use of the recombinant vaccinia virus-based HIV vaccines, we believe that the non-replicative DIs strain presents a safer alternative to the MVA (modified vaccinia Ankara) that is being widely used in HIV-1 vaccine development elsewhere. Like the MVA-based HIV-1 vaccines, the recombinant non-replicative vaccinia virus DIs is capable of efficiently expressing the HIV-1 antigens in mammalian host cells. However, the DIs-based viruses lack the replicative ability in these cells, thus producing no infectious viruses. In contrast, MVA is able to replicate in mammalian cells like BHK and CV-1 following infection (Carroll and Moss, 1997). The structural basis for this distinction between the DIs virus and MVA is not yet known. It was shown that the DIs virus as a smallpox vaccine failed to induce a skin reaction in inoculated infants and permitted revaccination by another smallpox vaccine strain without complications, demonstrating a safety advantage over the use of MVA as a recombinant vaccinia vector (Fujii and Minamitani, 1968).

In order to assure the safety of the T-J CAVRP candidate vaccines, the pilot products of these vaccines are evaluated in baby monkeys, as well as in adult monkeys. Studies showed that as much as 800 mg of the recombinant BCG-based HIV vaccines by oral administration, or repeated subcutaneous injections of 50 mg of the vaccine, did not cause any detectable adverse effects when compared directly with the standard BCG vaccine (Sukpanichnant *et al.*, Department of Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, December 2003, unpublished data). Similar safety experiments are underway to evaluate the recombinant non-replicative vaccinia DIs-based HIV vaccines.

FUTURE PLANS

The above-mentioned first generation candidate vaccine incorporates the *gag* genc only, and does not induce sterile immunity because the immunized monkeys do not suppress primary viremia completely after the SHIV challenge. The *gag* gene product cannot induce neutralizing antibodies that block virus entry. Therefore, introduction of the *env* gene to current constructs may be important to enable the vaccines to prevent both HIV-1 infection and disease progression. Furthermore, the construct of a single *gag* gene would have a restricted range of CTL responder population in which the HLA type is involved. To address these issues, future research may be conducted to improve the capacity and expression level of incorporated HIV-1 *env* genes in rBCG and rDIs.

To improve immunogenicity of the vaccine constructs, antigen expression in BCG or in DIs should be optimized. Regarding rBCG, we have already found that the codon-optimized *gag* p24 gene is expressed at a drastically higher level than the native *gag*; this strategy can be applied to other rBCG constructs to make them more immunogenic (Kanekiyo *et al.*, unpublished data). Such investigations are continuing now through collaboration between Japan and Thailand. These approaches are of particular importance to obtain an HIV vaccine that is highly effective.

ACKNOWLEDGEMENTS. We would like to thank Drs. Bonnie Mathieson, Jorge Flores and Rebecca Sheets, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA; Dr. Bernard Moss, Laboratory of Viral Diseases, NIAID, for his helpful discussions; and Drs. Hidemi Takahashi, and Yohko Nakagawa, Department of Microbiology and Immunology, Nippon Medical School, Tokyo, for their helpful discussions. Japan Science Technology Incorporation, the Organization of Pharmaceutical Safety and Research, the "Panel on AIDS" of the US-Japan Cooperative Medical Science Program, the Human Science Foundation of Japan, and the Japanese Ministry of Health, Labor and Welfare also supported this work. This study was also supported by the AIDS vaccine project in conjunction with the Japan Science and Technology Cooperation.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 20, Number 3, 2004, pp. 000–000
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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/μl (range: 8–1449/μl) and 747/μl (range: 98–3079μl) 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; SD, standard deviation.
[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.

ACKNOWLEDGMENTS

We gratefully acknowledge the dedicated field work of Ms. Saiyud Moolphate, Ms. Pornpimon Saksoong, and other members of the TB/HIV Research Project, the RIT-JATA, the Regional Medical Sciences Centre in Chiang Rai, and the Mae Chan Hospital. We also thank Dr. Kruavon Balachandra for her kind technical support and advice. This investigation was cosponsored by grants from the Sasakawa Memorial Health Foundation, the Japanese Foundation for AIDS Prevention, and the Japan Health Sciences Foundation.

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Prompt tumor formation and maintenance of constitutive NF-κB activity of multiple myeloma cells in NOD/SCID/γc^{null} mice

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(Received March 1, 2004/Revised April 28, 2004/Accepted May 10, 2004)

Clinically and biologically relevant animal models are indispensable to evaluate both the pathophysiology and strategies for diagnosis and treatment of multiple myeloma (MM). We examined the tumorigenicity of MM cell lines KMM-1 and U-266 in an *in vivo* cell proliferation model using NOD/SCID/γc^{null} (NOG) mice. Two cell lines were inoculated either subcutaneously (s.c.) in the post-auricular region or intravenously (i.v.) in the tail of NOG mice. The KMM-1 cell line produced a progressively growing large tumor with infiltration of the cells expressing human λ-chain in various organs of all NOG mice, while the U-266 cell line failed to do so. Tumor cells grown in NOG mice maintained the original histomorphology, as well as expression patterns of tumor markers human λ Ig light chain and VEGF. Tumor progression in mice also correlated with elevation of serum human soluble IL-6R and gp130. Tumor cells sustained a strong NF-κB activity *in vivo* and induced NF-κB components were indistinguishable from those in cells cultured *in vitro*. The rapid and efficient engraftment of the MM cell line in NOG mice suggests that this is a very useful animal model which could provide a novel system in which to clarify the mechanism of growth of cancer cells, as well as to develop new therapeutic regimens against MM. (Cancer Sci 2004; 95: 564–568)

Multiple myeloma (MM) is a B-cell neoplasm characterized by clonal expansion of plasma cells in the bone marrow, and is associated with neoangiogenesis and often with severe bone disease.[1–4] MM has a poor prognosis and a very short survival time, mainly because of poor efficacy of conventional and transplantation therapy. To develop novel therapies and to study the pathophysiology, a good animal model of this disease is needed. Such a model would require high engraftment efficiency and conservation of relevant tumor features.

Severe combined immunodeficiency (SCID) mice have been utilized in studies on the mechanism of MM and on therapeutic strategy.[5–10] However, this model has five major drawbacks, i.e., that tumor formation requires a long period of time, repeated transplantation, total body irradiation, and human fetal bone and anti-gp130 mAbs. Depending on the type of treatment, immunosuppressive conditioning releases a cascade of pro-inflammatory cytokines,[11] which strongly influence the activities of various cell types of tumor stroma, such as fibroblasts, myoepithelial cells, and macrophages, that are in close functional interaction with adjacent tumor cells.[12–14] Therefore, such treatments could lead to changes in relevant histomorphologic or functional features of tumors implanted into respective recipients. Furthermore, immunosuppressive pretreatment, such as irradiation, alters the expression patterns of adhesion molecules in peripheral tissues.[11] This may influence immune cell migration into such sites, causing difficulties in the interpretation of cellular transfer studies in such models.[11] In the present study, we have used a newly developed SCID mouse strain, the NOD/SCID/γc^{null} (NOG) mouse, in order to overcome such problems. This is a unique type of animal, lacking both T- and B-cells and having defects in NK activity, macrophage function, complement activity, and dendritic cell function.[15,16] MM cell lines were inoculated either subcutaneously (s.c.) in the post-auricular region or intravenously (i.v.) in the tail of NOG mice enabling both macroscopic and microscopic observation of the mechanism of tumorigenesis and malignant growth of MM.

We show here that a progressively growing large tumor was rapidly and reproducibly induced in all mice inoculated with cells of the MM cell line within only 2 to 3 weeks, and infiltration of the tumor cells was observed in various organs of NOG mice. Tumor cells sustained a strong NF-κB activity *in vivo*, which might play an important role in the cell proliferation of MM. These results suggest that the NOG mouse model of MM should be useful for investigating the *in vivo* molecular pathogenesis, infiltration into different organs, and therapeutic measures for MM patients.

Materials and Methods

Mice and cell lines. NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific-pathogen-free conditions in the Animal Center of National Institute of Infectious Diseases (Tokyo). The Ethical Review Committee of the Institute approved the experimental protocol.

KMM-1 and U-266 cell lines (both of which produce λ chain), established from MM patients, and a T-cell line, Jurkat, were cultured in RPMI-1640 medium (Nikken Bio-laboratory, Kyoto, Japan) with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 2 m*M* L-glutamine, 100 U of penicillin per milliliter, and 100 µg of streptomycin per milliliter at 37°C and 5% CO_2.

Inoculation of cell lines into NOG mice. Cells were washed twice with serum-free RPMI-1640 medium. These cells were re-suspended in serum-free RPMI-1640 medium. Mice were anesthetized with ether and cells were inoculated either i.v. in the tail or s.c. in the post-auricular region of NOG mice at a dose of 2×10^6 to 10×10^6 cells per mouse.

Growth measurement of s.c. tumor and histological and immunological examination. Mice were sacrificed 40 or 60 days after in-

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oculation with a cell line. We measured the length, width, and height of the tumor according to the Somers scale. Blood was collected from the heart of mice with heparinized syringes. Peripheral blood mononuclear cells (PBMNCs) were isolated from the blood by density gradient concentration with Ficoll-Hypaque. Cytospin specimens of PBMNCs were prepared and fixed in methyl alcohol for May-Grunwald and Giemsa staining. Tumor tissues and various organs were fixed with Streck Tissue Fixative (S.T.F) and processed to paraffin wax-embedded sections for staining with hematoxylin and eosin (H&E) and immunostaining. Paraffinized cryosections of tumors and various organs were deparaffinized and hydrated in xylenes or clearing agents and graded alcohol series, and then rinsed for 5 min in water. Deparaffinized samples were incubated with 0.025% trypsin/phosphate-buffered saline (PBS) for 30 min followed by washing, and then incubated with 0.3% methanol for 30 min at room temperature and washed twice with PBS. Immunostaining was done as described in the Vector M.O.M. immunodetection kit (Cat.# PK-2200, Vector Laboratories, Inc., Burlingame, CA) for MM cells with a 1:500 dilution of primary mouse monoclonal antibody specific for human λ Ig light chain (M614, Dako) and rabbit polyclonal antibody specific for VEGF (A-20, Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by washing in PBS and then incubation with a secondary antibody, M.O.M. biotinylated anti-mouse and anti-rabbit IgG. The sections were washed in PBS and further incubated with VECTASTAIN *Elite* ABC for 20 min at room temperature. Positive staining was visualized after incubation of these samples with a mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 50 m*M* Tris-HCl buffer and 0.01% hydrogen peroxide for 5 min. The samples were counterstained with hematoxylin for 2 min, hydrated completely, cleaned in xylene and then mounted.

Measurement of serum human soluble IL-6 (sIL-6), sIL-6R, and sgp130 levels. Blood collected from the heart of mice during sacrifice was used for the measurement of human sIL-6, sIL-6R, and sgp130. Concentrations of sIL-6, sIL-6R, and sgp130 were determined using ELISA procedures.[17] Microtiter 96-well plates were coated with 2.5 μg/ml anti-human IL-6 (Cat.# IM-R109, Diaclone Research), 2.5 μg/ml anti-human IL-6R (Cat.# MAB227, R & D Systems), and 2.5 μg/ml anti-human gp130 (Cat.# 42228, Genzyme-Techne) for 2 h at 37°C followed by washing 3 times with PBS containing 0.1% Tween 20. The plates were blocked with 3% BSA at 37°C for 2 h and washed 3 times with the same washing buffer. sIL-6, sIL-6R, and sgp130 standards and unknown samples were added and incubated at 37°C for 2 h, and then the plates were washed. To detect bound sIL-6, sIL-6R, and sgp130, biotinylated anti-human IL-6 (50 ng/ml, Cat.# 44206, Genzyme-Techne), IL-6R (50 ng/ml, Cat.# 44227, Genzyme-Techne), and gp130 (50 ng/ml, Cat.# 44228, Genzyme-Techne) was added and the plates were incubated for 2 h at 37°C, followed by washing. Horseradish peroxidase-conjugated streptavidin was added and incubation was continued at 37°C for 30 min, followed by washing 3 times with washing buffer. Peroxidase activity was determined using TMB as a substrate. The reaction was stopped with 1.8 *M* H_2SO_4, and the absorbance was measured at 450 nm.

Electrophoresis mobility shift assay (EMSA). Nuclear extracts were prepared as described previously.[15] Nuclear extracts (5 μg of protein) were incubated in 12 μl of binding buffer (10 mmol/liter HEPES, pH 7.8, 100 mmol/liter NaCl, 1 mmol/liter EDTA, 2.5% glycerol), 1 μg of poly [d(I-C)] and ^{32}P-labeled κB probe derived from the H-2K promoter for 30 min at room temperature. To identify the subunits constituting the NF-κB complexes, specific Abs against p50, c-Rel (Santa Cruz Biotechnology), p65 (#1226), RelB (#13482) (from Dr. Nancy Rice), and p52 (#1319) (from Upstate Biotechnology) were used. Antibody was added to the nuclear extract, and the mixture was allowed to stand for 30 min at room temperature before incubation with the radiolabeled probe. DNA-protein complexes were analyzed by electrophoresis in 5% polyacrylamide gel in 0.5× TBE (44.5 mmol/liter Tris, 44.5 mmol/liter boric acid, 1 mmol/liter EDTA). After electrophoresis, the gels were dried and subjected to autoradiography.

Results

Efficient engraftment and rapid tumor formation of MM cells in NOG mice without change of histomorphology or tumor marker expression. To investigate the *in vivo* growth, MM cell lines [KMM-1 and U-266] were inoculated either s.c. in the post-auricular region or i.v. in the tail of NOG mice (Table 1). Mice inoculated s.c. with cell line KMM-1 produced a visible tumor

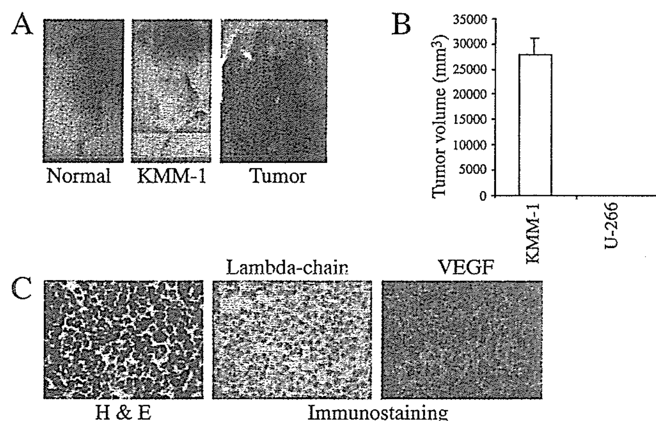

Fig. 1. Growth and tumor marker expression of multiple myeloma KMM-1 cells in NOG mice. A: Photograph of a normal mouse (left panel), a mouse inoculated with KMM-1 cells s.c. in the post-auricular region after 40 days (middle panel), and a s.c. KMM-1 tumor 40 days after inoculation of cells (right panel). B: s.c. tumor growth of mice inoculated with KMM-1 cells, shown as the mean±SEM from 19 mice. C: H&E and immunohistochemical staining of tumor tissue of KMM-1 cell-inoculated mice. The left panel represents H&E staining. Immunohistochemical staining was conducted using anti-human λ-chain (middle panel) and VEGF antibodies (right panel).

Table 1. ***In vivo*** **growth of multiple myeloma cell lines in NOG mice**

Cell lines	No. of cells inoculated per mouse[1]	Inoculation route[2]	Day of sacrifice after inoculation	No. of mice with tumor/ no. of mice inoculated[3]
KMM-1	10×10^6	s.c.	40	19/19
KMM-1	2×10^6	i.v.	40	3/3
U-266	10×10^6	s.c.	60	0/3
U-266	2×10^6	i.v.	60	0/3

1) Mice were inoculated with 2×10^6 to 10×10^6 cells per mouse.
2) s.c., subcutaneous; i.v., intravenous.
3) Number of animals in which tumor developed.

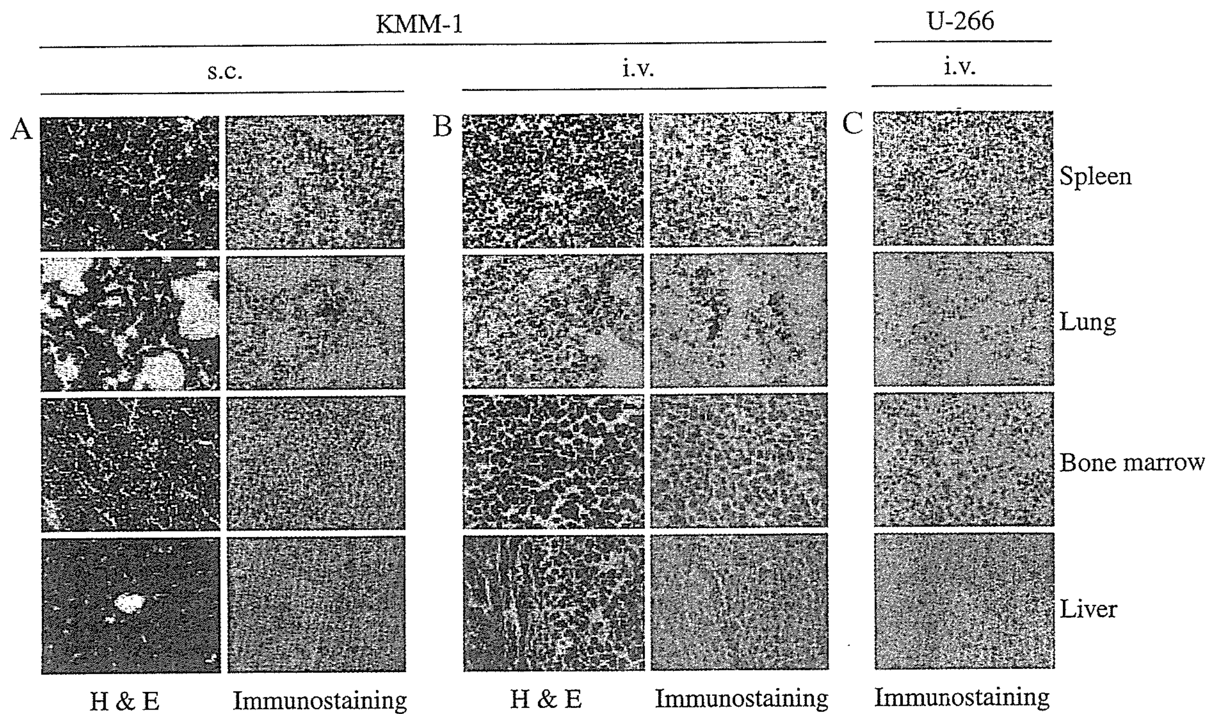

Fig. 2. Infiltration of MM cells in various organs of NOG mice. A and B: H&E and immunohistochemical staining of spleen, lung, bone marrow, and liver of mice injected with KMM-1 cells either s.c. or i.v. C: Immunohistochemical staining of spleen, lung, bone marrow, and liver of mice i.v.-injected with U-266 cells. Left and right panels of figure A and B represent H&E and immunostaining, respectively. Immunohistochemical staining was conducted using anti-human λ-chain antibody.

within 2 weeks in NOG mice while i.v.-inoculated mice were found to have a solid tumor in the peritoneal cavity after sacrifice. The KMM-1 cell line was most efficient in the formation of a large tumor (Fig. 1A), as well as development of severe anemia and clinical signs of near-death, such as piloerection, weight loss, and cachexia, in NOG mice at the time point of sacrifice, while U-266 was ineffective. The average tumor size (mean=27,893 mm^3) in NOG mice inoculated s.c. with KMM-1 was determined 40 days after inoculation (Fig. 1B). To test whether tumors maintain the original histomorphology and expression patterns of tumor markers in NOG, we performed H&E and immunostaining of tumor tissues obtained from mice inoculated with KMM-1 cells. Histological and immunological analysis revealed that *in vivo* tumor cells retained their morphology well, and expressed human λ Ig light chain and VEGF. These results showed that MM cell line-inoculated NOG mice generated large tumors very efficiently. This extremely rapid tumor formation in all mice without change of histomorphology or tumor marker expression is one of the hallmarks of our animal model.

Infiltration of MM cells into various organs of NOG mice. To assess the tissue distribution of MM cells, we carried out histological examinations of various organs of NOG mice after inoculation of the cells. Proliferation and infiltration of tumor cells were found not only in primary tumor tissues, but also in peripheral blood and to a lesser extent in spleen and lung of NOG mice inoculated s.c. with the KMM-1 cell line (Fig. 2A). We found that KMM-1 cells exhibited infiltration in spleen, lung, liver, and bone marrow of mice after i.v. inoculation (Fig. 2B), while U-266 cells did so to a lesser degree only in bone marrow (Fig. 2C). H&E and immunohistochemical staining showed a degree of infiltration of tumor cells at the site of inoculation and in various organs with KMM-1 and U-266 (Fig. 2, A, B, and C). These data suggest that MM cell line could invade different organs of NOG mice in a similar manner to myeloma cells in patients. Interestingly, MM cells appeared to

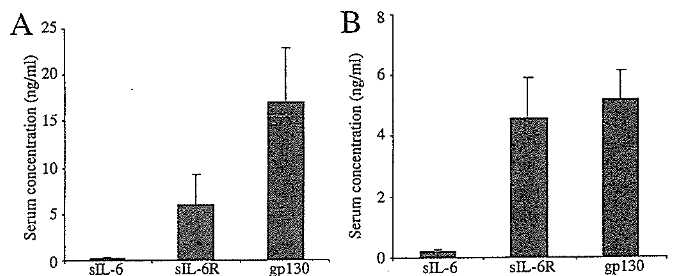

Fig. 3. Serum levels of sIL-6, sIL-6R, and sgp130 in NOG mice inoculated with KMM-1 cells, determined by ELISA. A and B: Data obtained from mice inoculated s.c. (12 mice) and i.v. (3 mice) with KMM-1 cells, respectively.

infiltrate various organs of mice more aggressively and massively after i.v. inoculation than after s.c. inoculation.

Serum soluble human IL-6R and gp130 levels as indicators of MM cell proliferation in NOG mice. Numerous studies have established that sIL-6R levels are significantly elevated in serum from MM patients.[18–21] MM cell uptake in SCID mice was associated with the presence of circulating sIL-6R and sgp130.[22] Serum human IL-6, IL-6R, and gp130 levels measured 40 days after inoculation of MM cells. sIL-6R [mean=6.42 ng/ml (s.c.) and 4.57 ng/ml (i.v.)] and sgp130 [mean=16.13 ng/ml (s.c.) and 5.17 ng/ml (i.v.)], but not IL-6, were markedly elevated in mice that were found to be successfully engrafted with KMM-1 cells (Fig. 3, A and B). Mice inoculated with U-266 cells showed no increase in serum sIL-6R or sgp130 (data not shown), even though histological analysis of bone marrow showed some infiltration of U-266 cells after i.v. inoculation (Fig. 2C).

NF-κB binding activity of KMM-1 cells inoculated in NOG mice. High NF-κB binding activity is thought to be crucial for maintaining the characteristics of MM cells in patients.[23,24] To determine whether or not NF-κB activity is changed *in vivo*, we

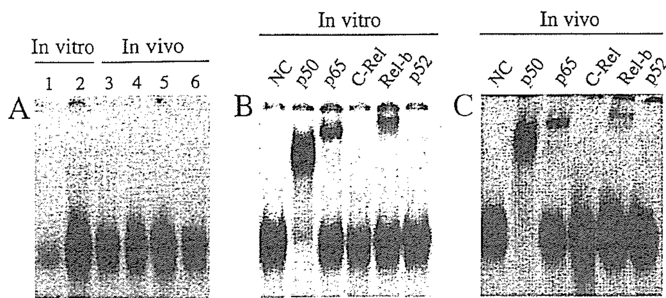

Fig. 4. Unaltered NF-κB binding activity of KMM-1 cells in NOG mice. A: Analysis by EMSA of NF-κB binding activity of the KMM-1 cells derived from cells cultured *in vitro* or tumor cells. Nuclear extracts (5 μg) were treated with ^{32}P-labeled wild-type NF-κB oligonucleotides. Lane 1 contains the negative control using Jurkat, and lane 2 contains *in vitro*-cultured KMM-1 cells. Lanes 3 to 6 show *in vivo* NF-κB DNA binding of KMM-1 cells from four different tumor-bearing mice. B and C: Analysis of NF-κB components of KMM-1 cells by super-shift assay. Super-shift using antibodies specific to p50, p65, c-Rel, RelB, and p52 subunits of NF-κB was conducted for both *in vitro* (B) and *in vivo* (C) samples. NC, negative control.

performed EMSA (Fig. 4A). Tumor cells from mice retained a strong NF-κB DNA binding activity, similar to that *in vitro*. We also investigated the DNA binding of NF-κB components in tumor cells in comparison with that of cells cultured *in vitro*. For this purpose, super-shift assays were performed with nuclear extracts of tumor tissues in the absence or presence of Abs that specifically recognize the following members of the NF-κB family: p50, p65, c-Rel, RelB, and p52. The results indicated that the induced NF-κB complexes contained p50, p65, and RelB both *in vitro* and *in vivo* in the case of KMM-1 (Fig. 4, B and C, respectively). These results also suggested that NF-κB activity is required for MM cell growth in NOG mice, and could be a therapeutic target for myeloma.

Discussion

Recent advances in the studies of MM have suggested new avenues for treatment and potential cure of this disease. To achieve these goals, it is essential to establish an effective animal model. Although several models, mainly using conventional SCID mice, are available, there are many problems. Representative data for the establishment of animal models for MM are as follows. a) By 3 months after inoculation of MM cell lines, a distinct solid tumor was formed at the inoculated site in one of five mice and re-transplantation was indispensable.[5] b) Mice were given whole-body irradiation followed by inoculation of MM cells.[6,7] c) When fetal bones were implanted in SCID mice to provide a human microenvironment, human MM cells grew in these bones.[8,9] d) Intraperitoneal tumors were observed in only 50% of animals when the mice received anti-gp130 mAbs.[10,22] The differences in behavior of MM cell lines in different types of SCID mice as regards the formation of tumors are dependent on the host immune system; natural killer cells might play an important role in the rejection of implanted tissues or cells in SCID mice.[25–28] In addition, recipient dendritic cells could have a role in transplant rejection.[29] In addition to the absence of T- and B-cells, NOG mice have no NK and there are functional defects in dendritic cells.[16] In the present study, MM cells inoculated s.c. in the post-auricular region over the skeleton of mice permitted us to quickly observe the tumor growth macroscopically and to measure the size of the tumor. By measuring the size of the tumor macroscopically, it was possible to easily compare the growth ability of inoculated tumor cells between the controls and mice treated with drugs or other agents. It is noteworthy that MM cells were found to infiltrate various organs of mice more aggressively and massively after i.v. inoculation than after s.c. administration. Indeed, circulating levels of sIL-6R may act as a useful prognostic marker, since high serum sIL-6R concentrations were associated with death of patients within 3 years of diagnosis.[19] Our results indicate that the presence of high levels of human sIL-6R and sgp130 in mouse serum is linked to development of tumor growth and clinical signs of imminent death. The implantation procedure is easily performed, the engraftment is highly efficient and expression levels of tumor markers, NF-κB activity and components are not altered. The present animal and inoculation system should be applicable to other malignant cells and cell lines of different origins that are unrelated to MM (unpublished results), and could be very useful in the study of tumorigenesis in general.

In summary, the reproducible growth behavior and preservation of characteristic features of MM cells suggest that our NOG mouse model can provide a unique opportunity to investigate the mechanisms of pathogenesis and malignant cell growth of myeloma, and to develop novel therapeutic regimens.

We thank S. Ichinose of Instrumental Analysis Research Center, Shu Endo of the Animal Research Center, Tokyo Medical and Dental University, and Peter J. Richard of Cardiff University, Wales, UK for their advice and assistance with the experiments. we also thank Y. Sato of the National Institute of Infectious Diseases for her skillful technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare of Japan.

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JOURNAL OF VIROLOGY, Sept. 2004, p. 9842–9853 Vol. 78, No. 18
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.18.9842–9853.2004
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A Consecutive Priming-Boosting Vaccination of Mice with Simian Immunodeficiency Virus (SIV) *gag/pol* DNA and Recombinant Vaccinia Virus Strain DIs Elicits Effective Anti-SIV Immunity

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Received 8 August 2003/Accepted 18 May 2004

To evaluate immunity induced by a novel DNA prime-boost regimen, we constructed a DNA plasmid encoding the *gag* and *pol* genes from simian immunodeficiency virus (SIV) (SIV*gag/pol* DNA), in addition to a replication-deficient vaccinia virus strain DIs recombinant expressing SIV *gag* and *pol* genes (rDIsSIV*gag/pol*). In mice, priming with SIV*gag/pol* DNA, followed by rDIsSIV*gag/pol* induced an SIV-specific lymphoproliferative response that was mediated by a CD4$^+$-T-lymphocyte subset. Immunization with either vaccine alone was insufficient to induce high levels of proliferation or Th1 responses in the animals. The prime-boost regimen also induced SIV Gag-specific cellular responses based on gamma interferon secretion, as well as cytotoxic-T-lymphocyte responses. Thus, the regimen of DNA priming and recombinant DIs boosting induced Th1-type cell-mediated immunity, which was associated with resistance to viral challenge with wild-type vaccinia virus expressing SIV*gag/pol*, suggesting that this new regimen may hold promise as a safe and effective vaccine against human immunodeficiency virus type 1.

As human immunodeficiency virus type 1 (HIV-1) continues to spread throughout the world (16, 27, 51, 42), the need for a safe and effective prophylactic vaccine is more urgent now than ever (16, 27, 43, 51). A realistic goal for such a vaccine is to limit HIV-1 infection by eliciting immune responses that reduce the viral load and prevent disease progression. With other viral diseases, T-helper-cell type 1 (Th1)-mediated immune responses and CD8$^+$ cytotoxic T lymphocytes (CTL) have been reported to provide protection and reduce disease progression (11, 17, 23, 34, 45, 55, 77). Moreover, Th1/CD8$^+$ T-cell responses have been shown to play an important role in controlling HIV-1 replication (9, 28, 41, 44, 50, 52, 56, 60, 61, 62). In previous studies, nonhuman primates and chimpanzees immunized with attenuated strains of simian immunodeficiency virus (SIV) or HIV-1 had strong antigen-specific immune responses and were protected from challenge with SIV, simian/human immunodeficiency virus (SHIV), or HIV-1 (1, 15, 66, 81). These studies demonstrate that an experimental immunogen is capable of mediating protection against intravenous and mucosal viral challenge in animal models of HIV and SIV, although attenuated HIV-1 vaccines are generally considered to be unsafe for use in humans (4, 21).

Recently, HIV-1 DNA-based vaccines have been shown to induce protective T-cell-mediated immune responses (12, 13, 33, 57, 75). To increase vaccine efficacy, DNA has also been modified by codon optimization, as well as by coinjection with cytokine-encoding plasmids, recombinant proteins, and other vaccine vectors (7, 10, 29, 32, 46). The immune response to DNA vaccines based on HIV-1 antigen genes was increased when innate and adaptive cytokine genes were combined (5, 14, 22). Furthermore, enhanced levels of protection were demonstrated with a combination regimen consisting of DNA priming (SIV *gag*, *pol*, *vif*, *vpx*, and *vpr*, and HIV-1 *env*, *tat*, and *rev*), followed by boosting with a recombinant modified vaccinia virus Ankara (MVA)-based vaccine (SIV*gag* and *pol* and HIV-1 *env*) (2).

Recombinant MVA has been used frequently as a booster vaccine in various combination regimens. In an effort to develop additional safe booster antigens, we generated a recombinant vaccine based on the vaccinia virus strain DIs, which has proven not to replicate in all mammalian cells tested (25). The virologic and immunologic properties of the DIs vector have been reported previously by our group (25, 26, 38, 70, 71). The vaccinia virus DIs vector expressing SIV Gag protein elicited immune responses able to suppress SHIV infection in macaques (26). In the present study, we have demonstrated enhanced Th1-type immune induction in mice primed with a plasmid DNA vaccine encoding SIV*gag/pol*, followed by boosting with a newly developed recombinant DIs strain that expresses SIV*gag/pol* (rDIsSIV*gag/pol*). Our result demonstrates that this new prime-boost regimen is both safe and effective at eliciting anti-immunodeficiency viral immunity, suggesting its promise as a potential vaccine against HIV-1.

MATERIALS AND METHODS

Animals. Female BALB/c mice (H-2^d) were obtained from Shizuoka Laboratory Center (Shizuoka, Japan) and were used at between 8 and 12 weeks of age.

Construction of SIV*gag/pol* encoding plasmid DNA. Plasmid DNA encoding SIV*gag/pol* was prepared by standard procedures as previously described (47, 48, 64). Briefly, SIV*gag/pol* DNA was derived from the eucaryotic expression vector pcDNA3.1(−) (Invitrogen, Carlsbad, Calif.). pcDNA3.1(−) was digested with XhoI and EcoRI and ligated to SIV*gag* and *pol* genes that were amplified from SHIV-C2/1 DNA (GenBank no. AF217181) with the primers 5′-AACTCGAG

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AAGATAGAGTGGGAGATGGG and AAGAATTCAGGCTATGCCACCT CTCTA-3'. *gag/pol* genes were derived from the molecular clone SIV_{mac239}. The correct insertion of SIV*gag/pol* DNA into the plasmid was confirmed by PCR.

Generation and propagation of rDIsSIV*gag/pol*. rDIsSIV*gag/pol* was constructed based on the previously described protocol (25, 26). The SIV*gag/pol* gene was first amplified from $SHIV_{NM-3rN}$ DNA (24) by using primers SGP-5 (5'-AATACCCGGGATGGGCGTGAGAAACTC) and SGP-3 (AATAGAGC TCCTATGCCACCTCTCTAG-3') and then subcloned into the pUCvvp7.5H vector (25, 26). A HindIII fragment encoding SIV*gag/pol* and the p7.5H promoter region were inserted into the HindIII site of a pUC/DIs transfer vector. rDIsSIV*gag/pol* and a control vector expressing the gene for LacZ (rDIsLacZ) were generated by homologous recombination and propagated in chicken embryo fibroblasts (CEF). Each virion preparation was purified by sucrose density ultracentrifugation and stored at −120°C. The expression of a 55-kDa protein corresponding to SIV Gag was confirmed by Western blotting with extracts from CEF infected with rDIsSIV*gag/pol* and anti-SIV Gag-specific monoclonal antibodies (IB6 or V10) (35).

SIV antigens. Overlapping 15-mer peptides spanning SIV Gag (with 11-amino-acid overlaps) were provided from the NIH AIDS Research and Reference Reagent Program (National Institutes of Health, Rockville, Md.). Peptides spanning Gag p27 and p15 regions were divided into eight pools and used as antigens. Purified native SIV p27 Gag protein (SIV Gag) was purchased from Advanced Biotechnologies, Inc. (Rockville, Mass.).

Lymphocyte proliferative assays. Lymphocyte proliferative assays were performed as previously described (19). One week after the final vaccination, the mice in each of five groups (see Fig. 2) were sacrificed. The spleens were removed, and the tissue was disrupted by compression through a cell strainer (Becton Dickinson, Franklin Lakes, N.J.). Isolated spleen cells were pooled and resuspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. The $CD4^+$-T-cell, $CD8^+$-T-cell, or $CD4^+/CD8^+$-T-cell fraction was then depleted by using magnetic cell sorting (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) (20, 63). Aliquots of cells (100 μl) were transferred to 96-well round-bottom plates in triplicate. Either 1 μg of native purified SIV p27 Gag protein or pooled peptides (1 μg/peptide/10^5 cells) per ml was added to each well. The cells were incubated at 37°C in 5% CO_2 for 2 days before the addition of 1 μCi of [^{3}H]thymidine. After an additional 24 h of incubation, the cells were harvested, and the uptake of ^{3}H was determined. The results are expressed as the stimulation index (SI), which was calculated as a ratio of the counts per minute in the presence or absence of antigen.

Analysis of antigen-specific cytokine production. To further characterize the type of immune response induced in the vaccinated mice, $CD4^+$ T cells were isolated from the total spleen cell population by using MACS as described above. The purity of the isolated $CD4^+$ T cells was determined by flow cytometric analysis to be >98% (20, 63). The purified $CD4^+$ T cells were cultured for 3 days at a density of 10^6 cells/ml in the presence of pooled peptides or SIV Gag protein at a concentration of 10 μg/ml, along with T-cell-depleted and irradiated feeder cells at a density of 10^6 cells/ml. Culture supernatants were collected, and the concentrations of gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, IL-5, IL-6, and IL-10 were measured by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Amersham, Arlington Heights, Ill.).

IFN-γ-specific ELISPOT assays. SIV-specific IFN-γ-producing cells were enumerated by using an enzyme-linked immunospot assay (ELISPOT) and a murine IFN-γ ELISPOT kit (Diaclone Research, Besacon, France). Aliquots (100 μl) of cell suspensions containing 10^5 spleen cells were transferred to 96-well plates that were coated with anti-mouse IFN-γ antibody. Then, 1 μg of either SIV Gag protein or pooled peptides (1 μg/peptide/10^5 cells) was added, and the plates were incubated for 24 h at 37°C in 5% CO_2. The plates were then washed three times and incubated with biotinylated anti-mouse IFN-γ antibody for 1 h at 37°C. IFN-γ-specific cells were detected by using streptavidin-alkaline phosphatase conjugate and BCIP (5-bromo-4-chloro-3-indolylphosphate) substrate (Roche, Mannheim, Germany). Wells were imaged, and spot-forming cells (SFC) were counted by using a KS ELISPOT compact system (Carl Zeiss, Oberkochen, Germany). An SFC was defined as a large black spot with a fuzzy border (40). To determine significance levels, a baseline for each peptide pool was established by using the average and standard deviation (SD) of the number of SFC for each peptide. A threshold significance value, which corresponded to this average plus two SDs, was then determined. A response was considered positive if the number of SFC exceeded the threshold significance level of the control wells with no added peptide.

SIV Gag-specific CTL assays. Lymphocytes from the vaccinated mice were evaluated for CTL activity by using ^{51}Cr release assays as previously described by Takahashi et al. (72). In brief, spleen cells removed from red blood cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, 2-mercaptoethanol, penicillin, and streptomycin. The cell suspensions containing 10^7 spleen cells were restimulated with 0.1 μg/peptide/10^7 cells of pooled peptides for 5 days. IL-2 containing rat T-cell-stimulated culture supernatant (T-STIM; Collaborative Res. Bedford, Mass.) was added 3 days after cell culture. Cells from the *H-2^d* haplotype line, M12.4.5, were used as targets. M12.4.5 cells were incubated with $Na_2^{51}CrO_4$ (3.7 MBq/10^7 cells) for 90 min at 37°C in 5% CO_2 before being pulsed with peptides (either 40 or 50 μg). After 1 h, the target cells were thoroughly washed with RPMI 1640 and then dispensed into 96-well V-bottom plates (10^4 cells/well). The in vitro-stimulated effector cells were added, and the plates were incubated for 4 h at 37°C. To determine the spontaneous or maximum release of ^{51}Cr, target cells were incubated with medium alone or treated with 2% Triton X-100, respectively. The percent specific lysis was calculated by using the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release).

SIV Gag-specific humoral responses. SIV Gag-specific antibody endpoint titer was measured by ELISA as previously described (67). ELISA plates were coated with 0.3 μg of SIV Gag protein (Advanced Biotechnologies) per well. Heat-inactivated pooled mice sera were serially diluted and then added to the ELISA plates. Gag-specific antibody bound to Gag protein was captured with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, Ala.).

Evaluation of vaccine-induced immunity by virus challenge. To address whether the immune responses induced by the vaccination were protective, mice were challenged with wild-type vaccinia virus recombinant expressing SIV*gag/pol* (vv9019; National Institute for Biological Standards and Control, Blanch Lane, Hertfordshire, United Kingdom). The viral challenge was performed by using the method set forth by Belyakov et al. (8) and Qiu et al. (54). At 6 days after final vaccination, four mice in each vaccinated group and four naive mice were intraperitoneally (i.p.) challenged with 10^7 PFU of vv9019. One week after the challenge with the recombinant vaccinia virus expressing SIV*gag/pol*, the mice were sacrificed, and their ovaries were removed. The ovaries were homogenized, sonicated, and assayed for the challenge virus titer by plating serial 10-fold dilutions on a plate of CEF. After 3 days of culture, the plates were stained with 0.2% of crystal violet, and plaque were counted at each dilution. The results were expressed as the fold of the reduction in vaccinia virus titer in vaccinated mice versus the titer in naive mice.

ELISPOT assay for gag, pol, or whole vaccinia virus antigen-specific immunity. The magnitude of T-cell responses against SIV Gag, Pol, and vaccinia virus antigens was also measured by ELISPOT assay based on the recombinant vaccinia virus stimulation method described previously (74, 80). Briefly, spleen cells isolated from normal mouse were infected with 10 PFU per cell of either SIV gag-expressing wild-type vaccinia virus (vvSIV gag; National Institute for Biological Standards and Control), SIV pol-expressing wild-type vaccinia virus (vvSIV pol; National Institute for Biological Standards and Control), or nonrecombinant wild-type vaccinia virus (Vaccinia WR; National Institutes of Health) for 16 h and then fixed with paraformaldehyde. The virus antigen-expressing cells were used as stimulator cells and cultured with spleen cells of vaccinated mice at a stimulator/responder ratio of 1:2, and each antigen-specific ELISPOT assay was performed by the method described above.

Statistical analysis. Data are expressed as the mean ± the SD, and data analysis was carried out by using the StatView program (SAS Institute, Cary, N.C.). A P value of <0.05 was considered significant. The comparative analysis of animal groups subjected to different vaccine regimens was performed by using the Kruskal-Wallis H-test, followed by the Student-Newman-Keul correction.

RESULTS

Construction and expression of SIV*gag/pol* DNA and rDIsSIV*gag/pol*. The *gag/pol* region of SIV_{mac239} was inserted into two selected vectors. The first, pcDNA3.1(−), a eukaryotic expression vector, was used as the backbone of the SIV*gag/pol* DNA vaccine (Fig. 1A), and the second, pUC/DIs, was used as a transfer vector to generate rDIsSIV*gag/pol* and the control vector, rDIsLacZ (Fig. 1B). PCR was used to confirm that the SIV*gag/pol* DNA had been correctly inserted into each vector and Gag-specific Western blots were used

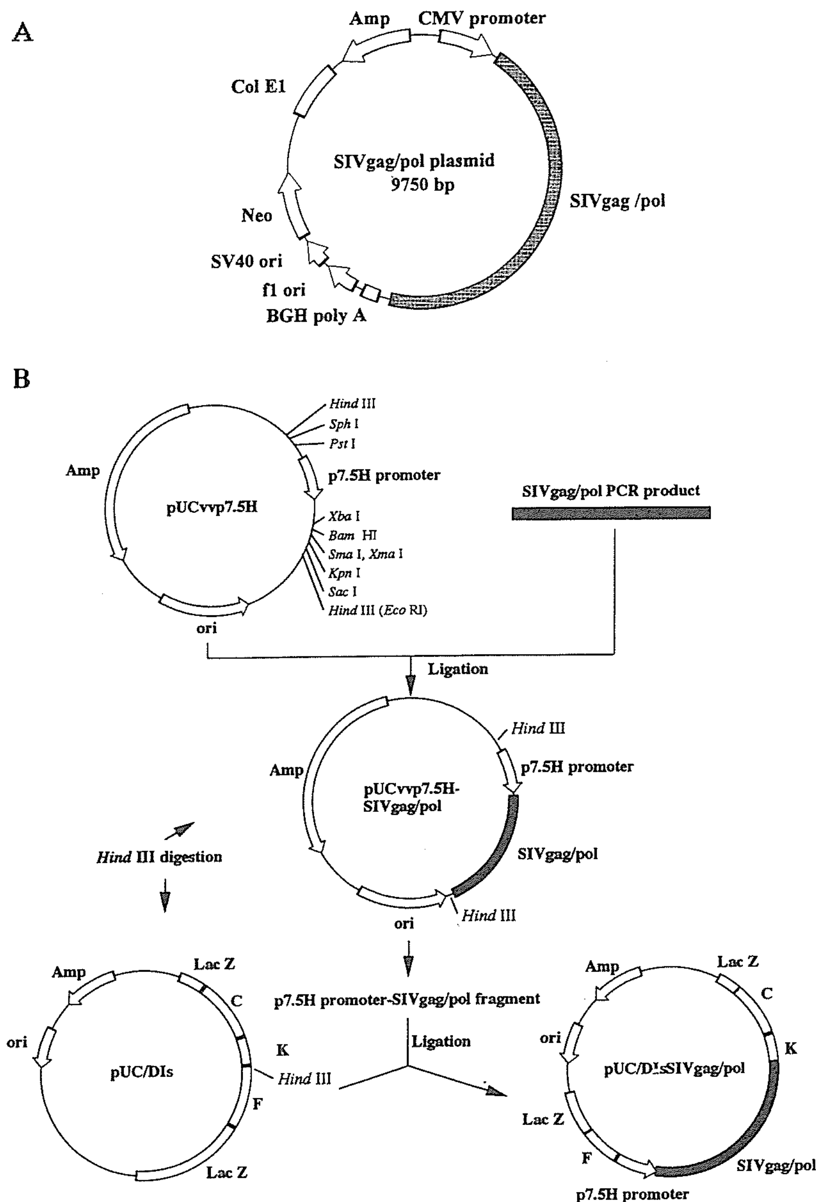

FIG. 1. Construction of SIV*gag/pol* expression vectors. (A) SIV*gag/pol* plasmid DNA; (B) construction and generation of recombinant DIsSIV*gag/pol*. The ampicillin resistance gene and vaccinia virus early/late promoter p7.5H are designated by Amp and p7.5H, respectively. Arrows indicate the direction of transcription. Hatched and white blocks represent the SIV*gag/pol* gene and HindIII fragments of vaccinia virus DNA, respectively.

to verify in vitro expression of the SIV Gag protein (data not shown).

Prime-boost regimen. A total of 50 BALB/c mice were divided into five groups of 10 mice each. Group 1 received three intramuscular injections (50 μg of each) of SIV*gag/pol* DNA at 2-week intervals, followed by two injections of rDIsLacZ (10^6 PFU each) at with 1-week intervals. Similarly, group 2 mice received three injections with 50 μg of control DNA pcDNA3.1 (−), followed by two injections of rDIsLacZ (10^6 PFU each) at 1-week intervals. Group 3 received three 50-μg intramuscular injections of SIV*gag/pol* DNA; 4 weeks later, the mice in group 3 were boosted with two intradermal injections of rDIsSIV*gag/pol* (10^6 PFU each) with a 1-week interval. The mice in group 4 received three injections of control DNA (50 μg of each), followed by two injections of rDIsSIV*gag/pol* (10^6 PFU each) (Fig. 2). In group 5, mice were immunized with SIV*gag/pol* DNA five times at the same intervals as described above. We confirmed the original data by the second run of the experiments with 50 additional animals and statistically summarized the challenge results (see Fig. 9).

The prime-boost vaccine regimen generates antigen-specific CD4⁺-T-lymphocyte proliferative responses. SIV Gag-specific T-lymphocyte proliferative responses were measured in splenocytes from immunized mice with either SIV Gag protein or peptides spanning the full-length Gag protein of SIV_{mac239}. Spleen cells from mice in groups 1, 3, 4, and 5 showed signif-

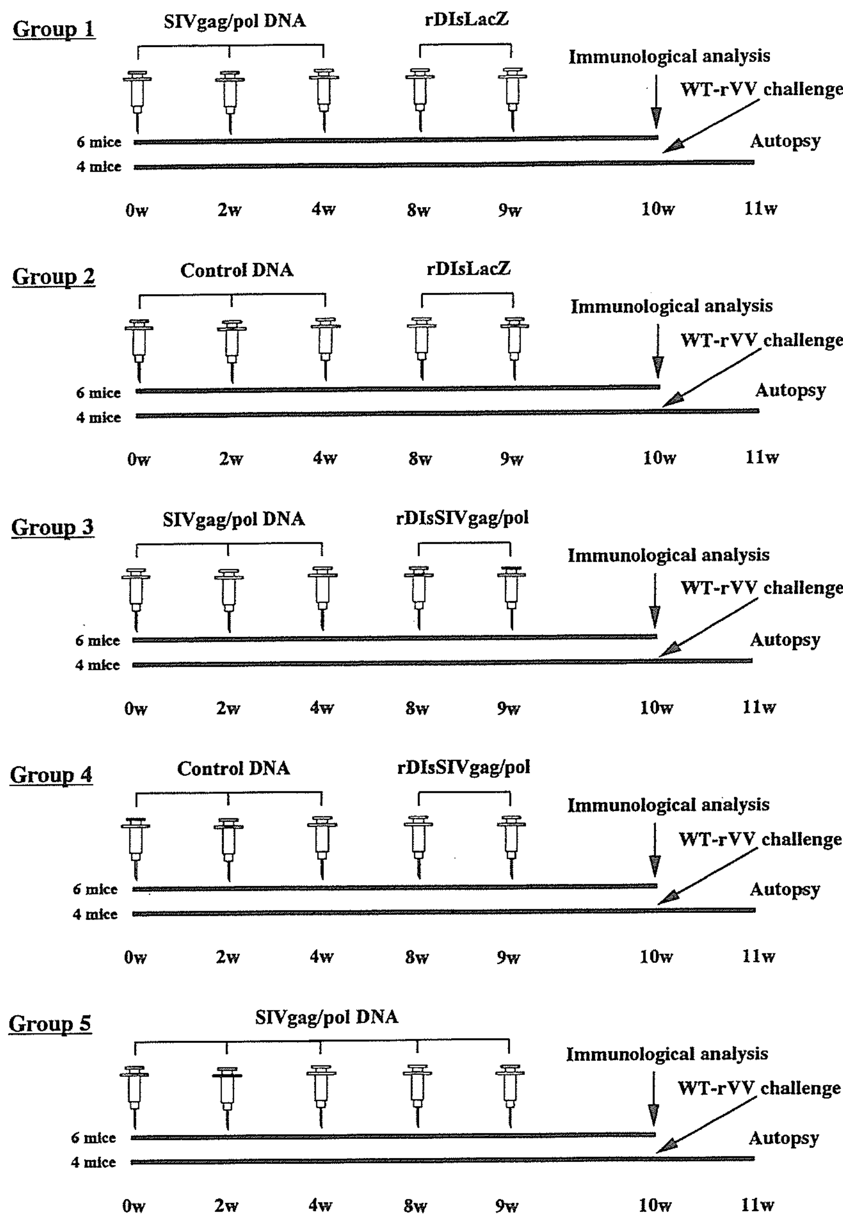

FIG. 2. Schematic of the experimental protocol for immunization and viral challenge. BALB/c mice were divided into five groups of 10 mice each (groups 1 to 5) and immunized three times consecutively with SIV*gag/pol* DNA and then twice with rDIsLacZ (group 1), three times with control DNA and then twice with rDIsLacZ (group 2), three times with SIV*gag/pol* DNA and then twice with rDIsSIV*gag/pol* (group 3), three times with control DNA and then twice with rDIsSIV*gag/pol* (group 4), or five times with SIV*gag/pol* DNA (group 5).

icant levels of proliferation in response to stimulation with SIV Gag protein and peptide pools (Fig. 3), whereas no proliferative activity (SI < 3) was seen with splenocytes from control group 2. Among the five animal groups, splenocytes from the mice in group 3 (immunized with a prime-boost regimen) showed the highest levels of T-cell proliferative responses against SIV Gag proteins ($P < 0.01$). The mean SIs for each of the five groups were 3.6 ± 1.2, 0.8 ± 0.4, 9.3 ± 2.3, 4.4 ± 1.5, and 3.4 ± 0.96, respectively (SIV Gag protein-stimulated group in Fig. 3A). Depletion of the $CD4^+$- or $CD4^+$ $CD8^+$-T-cell fraction from group 3's splenocytes dramatically reduced the proliferative responses to <10% (Fig. 3B). In contrast, the proliferative activity was not affected by the depletion of the $CD8^+$ fraction from the cell suspensions.

The splenocytes from the mice in group 3 also exhibited the highest proliferative responses against pooled peptides spanning the full-length SIV Gag protein (Fig. 3). Among the three positive peptide pools (4, 6, and 7), reactivity to pool 6 was the highest, with a mean SI of 5.4 ± 2.8 (Fig. 3A). Peptides in this pool correspond to the SIV p27 region and encode an SIV-specific $CD4^+$-T-cell epitope, which is recognized by the *H-2^d^* allele. Splenocytes from mice in control group 2 were not reactive with any of the SIV antigens (SI < 1.0).

Immunization with the prime-boost regimen induces Gag-specific Th1-type responses. To further characterize the type