4) インフォームドコンセントに係わる状況

抹消血採取に関しては、千葉工業大学のスタッフ(医師)が直接本研究の趣旨を説明し、抹消血提供の同意を得られた方のみ同意書に署名していただいた。この際、説明を行った医師名を明記し、同意書は千葉工業大学において厳重に保管している。

C. 研究結果

ピューロマイシン耐性遺伝子またはピューロマイシン耐性遺伝子及びHIV-1 gag遺伝子を導入した組み換えバキュロウイルス(AcCAGgag-puro及びAcCAG-PGKpuro)を作製した。このウイルスを293T細胞に感染させ、Gagタンパク質の発現をウエスタンブロットにて確認した。またウイルス粒子表面にHIV-1 gagタンパク質を提示した組み換えバキュロウイルス(Ac/gp64-gag)を作製し、ウエスタンブロットにてGagタンパク質の発現を確認しAcCAG-gag-puroまたはAcCAG-PGKpuroをヒト抹消単核球由来樹状細胞に感染させ、活性化マーカーであるMHCクラスI、II、CD80及びCD86の発現上昇がFACSにて確認された。また、ELISAにて培養上清中のサイトカインIL-15、IFN-α及びIFN-γの産生が認められた。さらに、抗ウイルス因子であるAPOBEC3F及び3Gの発現がRT-PCR及びウエスタンブロットにて発現上昇が認められた。ヒト樹状細胞にAcCAGgag-puroまたはAcCAG-PGKpuroを感染させのち、この細胞へHIV-1 JR-CSF株を感染させ、p24タンパク質を定量しAcCAGgag-puro感染樹状細胞において、非感染AcCAG-PGKpuro感染樹状細胞と比較して、優位にp24量の減少が認められた。

D. 考察

HIV-1 gag遺伝子及びピューロマイシン耐性遺伝子を導入した組み換えバキュロウイルス(AcCAGgag-puro、AcCAG-PGKpuro及びAc/gp64-gag)を作製し、ウエスタンブロットによりGagタンパク質の発現を確認した。作製した組み換えバキュロウイルスをヒト樹状細胞に感染させることにより、MHC分子及び共刺激分子の発現上昇、IL-15、IFN-α及びIFN-γの産生が認められた。さらに抗ウイルス因子であるAPOBEC3F及び3Gの発現上昇をウエスタンブロットにより確認した。上記の結果は組み換えバキュロウイルスの感染により樹状細胞から産生されたIFNによるものと推測される。さらにAcCAGgag-puro感染樹状細胞にHIV-1 JR-CSFを感染させるとp24量の減少が認められた事からAcCAGgag-puro感染樹状細胞から産生されたIFN及びAPOBEC3F、3Gの発現上昇により抗HIV-1効果が表れたものと考察される。

E. 結論

HIV-1 Gag発現バキュロウイルスはヒト樹状細胞を活性化させ、HIV-1の抑制効果が認められたことから、この組み換えバキュロウイルスが新規のHIV-1ワクチンとしての可能性が示唆された。

F. 健康危機情報

該当なし

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III. 研究成果の刊行に関する一覧表

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Immunogenicity of newly constructed attenuated vaccinia strain LC16m8Δ that expresses SIV Gag protein

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ABSTRACT

We developed the method to efficiently construct recombinant vaccinia viruses based on LC16m8Δ strain that can replicate in mammalian cells but is still safe in human. Immunization in a prime-boost strategy using DNA and LC16m8Δ expressing SIV Gag elicited 7–30-fold more IFN-γ-producing T cells in mice than that using DNA and non-replicating vaccinia DIs recombinant strain. As the previous study on the DNA-prime and recombinant DIs-boost anti-SIV vaccine showed protective efficacy in the macaque model [Someya K, Ami Y, Nakasone T, Izumi Y, Matsuo K, Horibata S, et al. Induction of positive cellular and humoral responses by a prime-boost vaccine encoded with simian immunodeficiency virus gag/pol. J Immunol 2006;176(3):1784–95], LC16m8Δ would have potential as a better recombinant viral vector for HIV vaccine.

1. Introduction

As vehicles for delivering antigens of HIV-1, replication-defective viral vectors have been extensively studied because of their safety. For example adenovirus and vaccinia virus-based vectors expressing Gag, Nef, and other components of HIV-1 have been evaluated in monkeys [1,3] and human trials [2,4,5]. They, however, generally have not induced sufficient level of immunity nor protected human from HIV-1 infection although they elicited considerable anti HIV/SIV immunities in animal models [6]. Moreover, controversial results have been reported on containment of challenged viruses depending on SIV or SHIV, a hybrid virus between HIV-1 and SIV, in monkey models [1,7]. Therefore more effective vehicles may be needed for HIV vaccine development.

Replication-competent vaccinia virus that has been proven to be safe in human vaccination against small pox could be a good candidate for a better vehicle. Vaccinia LC16m8 strain has been shot to 100,000 people without any serious adverse effects [8]. The LC16m8, however, has been found to be genetically unstable and to generate spontaneously more virulent revertants from stock of LC16m8 viruses. To improve LC16m8, we identified the B5R gene responsible for the reversion, and constructed genetically stable LC16m8Δ, which is essentially as same as LC16m8 in antigenicity and safety in mice, and approximately 1000-fold more immunogenic than non-replicating vaccinia, MVA strain. In particular, LC16m8Δ never elicited any symptoms in severe combined immunodeficiency disease mice even at 10^7 pfu dose [9]. Therefore LC16m8Δ could be a better vehicle for vaccines against HIV and other human diseases.

Gag proteins of HIV-1 and SIV are major antigens to elicit cytotoxic T lymphocyte (CTL) responses. Activity of anti Gag CTL in HIV-1-infected people inversely correlates with their viral loads [10]. In some monkey experiments of SIV infection, the strength of anti Gag CTL has been reported to correlate with the containment of SIV [11]. Therefore, we constructed LC16m8Δ that expresses the *gag* gene of SIVmac239 to compare its ability to elicit anti Gag immunity with replication-defective vaccinia virus DIs strain, which has been reported to be immunogenically similar to MVA [12], and to evaluate its potential as a recombinant vector for HIV vaccine development.

During the course of constructing LC16m8Δ-based recombinant viruses, we encountered a drawback, such as inefficient incorporation of the foreign gene by conventional method in which an *in vivo* recombination process is involved. Therefore, in this paper we firstly describe our new device including construction of a new strain m8Δvnc110, which prompts construction of m8Δ express-

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ing the *gag* gene of SIV by *in vitro* ligation of the vaccinia genome with foreign DNA.

2. Materials and methods

2.1. Cells and viruses

Rabbit RK13 cells were cultured in RPMI1640 supplemented with 10% FCS. Human HeLa, mouse L929, NIH3T3, hamster BHK, and primary chicken embryo fibroblast (CEF) cells were maintained in DMEM supplemented with 10% FCS. Canarypox virus (a kind gift of National Institute of Animal Health) [13], and LC16m8Δ [9] and DIs [14] strains of vaccinia virus were used. Viral titers were calculated on the basis of the number of plaques on CEF. The titer of LC16m8Δ was similar when titrated on RK13 and CEF monolayers.

2.2. Construction of pJWSIVgag

To construct the plasmid that expresses the SIV *gag* gene under the cytomegalovirus promoter, the *gag* coding region was amplified with a pair of primer SIVGAGF1 (GCCAAGCTTGCCACC-ATGGGCGTGAGAAACTCCGTCTTGTCAGG; the underlined sequence is HindIII site) and SIVGAGR1 (CGCGCCCGGGCTACTGGT CTCCTC-CAAAGAGAGAATTGAGGTGCAGC; the underlined sequence is XmaI site) using pSIVmac239 [15] as a template under the condition: 2 min at 94 °C, 20 cycles of 30 s at 94 °C, 60 s at 60 °C, 2 min at 72 °C, and a final extension for 5 min at 72 °C. The *gag* fragment generated was digested with HindIII and XmaI, and then ligated with the enzyme-digested pJW322, which harbors the cytomegalovirus promoter derived from pJW4303 [16] (a kind gift of Dr. Y. Takebe).

2.3. Construction of m8Δvnc110

To generate a transfer plasmid pVNC110, the vnc/KE sequence (5′-GGTACCCGCCCGGGCCGGACCGGCCGGCCGAATTC-3′) containing four restriction enzyme sites (SrfI, SfiI, RsrII, and FseI), which are not present in the vaccina virus genomes, was inserted between KpnI and EcoRI sites of pSFJ1-10, which harbors a strong composite promoter consisting of the cowpox A-type inclusion body (ATI) and multiple mutated-p7.5 promoters (PSFJ1-10 promoter), which are sandwiched with the segments of the vaccinia hemagglutinin (*HA*) gene [17,18]. pVNC110 resultant was verified by sequencing to harbor these sites downstream of PSFJ1-10 promoter, which is sandwiched by the segments of the vaccinia *HA* gene.

Next, we transfected 1.5 μg of pVNC110 to 1×10^5 BHK cells, which had been infected with LC16m8Δ at 0.05 moi, to construct m8Δvnc110. After the culture at 33 °C for 2 days, the progeny viruses were harvested and their plaques were formed on RK13 cell monolayer. The candidate viruses were selected on the basis of HA$^-$ phenotype [19]. To ascertain whether the virus contains the expected sequences of pVNC110 in the *HA* gene, the virus-enriched fraction was prepared by disruption of the infected cells by repeated freeze and thaw followed by clarification by low speed centrifugation and concentration by centrifugation at 15,000 rpm for 30 min in microcentrifuge at 4 °C. Then, it was used as a template for PCR with a pair of the primers vvHA867s (GGATC-TACACATTCACCAGA) and vvHA1009as (CTAGTGTATGTGACGGTGT), the sequences of which were present in the *HA* gene, under the condition: 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 60 s at 54 °C, 60 s at 72 °C, and a final extension for 5 min at 72 °C. Virus containing the sequence of VNC110 produced a 1 kb fragment of PCR product.

2.4. Construction of m8ΔSIVgag

Viral particles of m8ΔVNC110 were purified by method including banding in sucrose gradient centrifugation [20], and then viral DNA was isolated by phenol extraction method. The vaccinia DNA was digested with CpoI and FseI followed by purification with phenol extraction and ethanol precipitation. The *gag* region in SIVmac239 genome was amplified by PCR using pSIVmac239 [15] as a template with a pair of the primers CPO-SIV gag f2 (TTTCGGACCGCCACCATGGGCGTGAGAAACTCCGTCTTG; underlined sequence is CpoI site) and FSE-SIV gag r1 (TATGGCCGGCCTAC-TGGTCTCCTCCAAAGAGAGA; underlined sequence is FseI site) under the condition: 2 min at 94 °C, 20 cycles of 30 s at 94 °C, 60 s at 60 °C, 2 min at 72 °C, and a final extension for 10 min at 72 °C. The gag fragment was digested with CpoI and FseI followed by purification with PCR purification kit (Qiagen, Hilden, Germany). The digested vaccinia genome (5 μg) and gag fragment (0.3 μg) were ligated using a ligation kit (Takara, Otsu, Japan) according to the manual, purified by phenol extraction and concentrated by ethanol precipitation. The ligated DNA was transfected with lipofectamine LTX (Invitrogen, Carlsbad, USA) to 3.5×10^5 BHK cells that had been infected at 10 moi with canarypox virus. Usage of avipox viruses as a helper virus has been well established [21]. After 2 days culture at 33 °C, the progeny viruses were harvested by repeated freeze and thaw and titrated on the monolayer of RK13 cells. m8ΔSIVgag was cloned from single plaque and its homogeneity was evaluated by staining the plaques with sera of monkey infected with SIVmac239 and alkaline phosphatase-conjugated anti monkey IgG antibody followed by NBT/BCIP coloring reaction. All plaques were positively stained.

2.5. Construction of rDIs/PSFJ/SIVgag

To construct a complementary transfer vector for the deleted region of DIs, we used a pDIsgptmH5 plasmid (a kind gift of Dr. K. Ishii) that possesses both the modified H5 promoter and the *E. coli* guanine phosphoribosyltransferase (gpt) gene driven by a P7.5 promoter, which are sandwiched with the DIs fragments adjacent to the deleted region [22]. A vaccinia synthetic PSFJ1-10 promoter sequence [17] was amplified by PCR at 52 °C of the annealing temperature using a pair of the primers: PSFJ1-10s (ACATGCATGCATGAAGTTGAAGATGATG: underlined sequence is SphI site) and PSFJ1-10r (GATATCCTCGAGCA-GCACACCGTGCAATAAATT: underlined sequence is EcoRV and XhoI sites). To substitute the PSFJ1-10 promoter for the mH5 promoter, the PCR product was inserted into the SphI and EcoRV sites of pDIsgptmH5, generating pUC/DIs/PSFJ that could express the foreign antigen gene under the control of the PSFJ1-10 promoter. A DNA fragment encoding the full-length gag gene of SIVmac239 was amplified by PCR at 55 °C of the annealing temperature using a pair of the primers: gag-s (CCCCCCGGGATGGGCGTGAGAAACTCC: underlined sequence is SmaI site) and gag-r (CCGGAGCTCCTACTGGTCTCCTCCAAAGAG: underlined sequence is SacI site), and inserted into the SmaI and SacI sites of pUC/DIs/PSFJ to generate the transfer vector, named pUC/DIs/PSFJ/SIVgag. This plasmid (10 μg) was transfected by Gene-Pulser (Bio-Rad Laboratories, Inc. Hercules, USA) to CEF infected with DIs at 1.0 moi. Recombinant DIs clones expressing the SIV *gag* gene were selected in the presence of gpt [23].

2.6. Western blotting

m8ΔSIVgag and rDIs/PSFJ/SIVgag were infected to various cells at 3 or 5 moi and cultured for 24 h at 33 °C. Then the infected cells and culture medium were collected and their protein amounts were quantified by BCA assay. Appropriate amounts of the cell lysates and medium fraction indicated in the figure legends were subjected to 12% SDS-PAGE and immunologically detected using 500-fold-diluted sera from SIVmac239-infected monkey and alkaline phosphatase-conjugated anti monkey IgG antibody (Promega, Madison, USA) followed by NBT/BCIP coloring reaction.

2.7. Immunization

Seven-week-old female C57/BL6 mice were purchased from CLEA Co. Ltd. (Tokyo, Japan). Fifty microgram of pJWSIVgag was intramuscularly injected into the right and left quadriceps, and 1×10^6 pfu of vaccinia viruses were inoculated intrademally according to the schedule indicated in Figs. 3A and 4A. All mice were maintained according to the institutional animal care and the guidelines of Hokkaido University. The study was conducted in a biosafety level 2 facility under the approval of an institutional committee for biosafety and in accordance with the requirements of the World Health Organization.

2.8. Assay of cellular immune response by IFN-γ ELISPOT

SIV Gag specific IFN-γ producing cells were quantified 2 or 3 weeks after the final immunization using an ELISPOT kit for mouse IFN-γ (R&D Systems, Minneapolis, USA). The excised spleens were disrupted with a syringe plunger and passed through a cell strainer (Becton Dickinson, Franklin Lakes, USA). Isolated spleen cells were suspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Aliquots (100 μl) were plated into wells that were coated with anti-mouse IFN-γ antibody and stimulated with SIV Gag-specific 15 mer overlapping peptide pools (0.5 μg of peptides/10^5 cells) (A gift of AIDS Research and Reference Reagent program. Catalog #6204). Cells mock-stimulated with medium alone served as a negative control while cells treated with 50 ng/ml of phorbol 12-myristate (PMA) and 0.5 μg/ml of calcium ionomycin were used as a positive control to ascertain the number of viable T cells. After 24 h incubation, IFN-γ secreting cells were detected according to the manufacturer's instructions. Numbers of spot forming cells (SFC) were determined using the ImmunoScan Plate Reader with ImmunoSpot software (Cellular Technology Limited, Cleveland, USA).

2.9. Proliferation assays

Lymphocyte proliferation was measured by incorporation of BrdU into the stimulated-lymphocytes using cell proliferation ELISA BrdU kit (Roche Applied Science, Mannheim, Germany). Isolated spleen cells (1×10^5) were cultured in a 96-well assay plate (BD Falcon, Franklin Lakes, USA) in the presence or absence of recombinant SIV Gag protein (SIVmac251 p27; Advanced Biotechnologies, Inc., Columbia, USA) at 5.0 μg/ml for antigen-specific stimulation. The plates were incubated for 2 days at 37 °C, and then another 24 h in the presence of BrdU (100 μM). Uptake of BrdU was determined using luminometer (Wallac 1420; PerkinElmer, Branchburg, USA). The results were expressed as the stimulation index (SI), which was calculated as a ratio of relative light unit per second in the presence to that in the absence of the antigen.

2.10. Statistical analysis

Data were expressed as arithmetic mean ± standard error of means (mean ± S.E.M.). The data analysis was carried out by using Student's *t*-test (EXCEL version 11.5, Microsoft). A *P*-value of <0.05 was considered significant.

3. Results

3.1. Construction of m8ΔSIVgag

Fig. 1 illustrates the outline for construction of m8ΔSIVgag. Firstly we constructed m8ΔVNC110 strain by usual method in which pVNC110 was transfected to BHK cells that had been infected with LC16m8Δ. Resultant m8ΔVNC110 harbors PSFJ1-10 promoter followed by the multi-cloning sites containing the restriction enzyme sites which are not present in the vaccinia genome. To construct m8ΔSIVgag, the genomic DNA extracted from ΔVNC110 virions was digested with CpoI and FseI, which do not cut the other part of the vaccinia genome, and ligated with SIV *gag* fragment *in vitro*. Then the ligation mixture was transfected to BHK cells that had been infected with canarypox virus, which cannot replicate in mammalian cells. A clone, named m8ΔSIVgag3, that was isolated from one among six plaques formed by the progeny viruses produced SIV Gag protein judged by staining the plaques with sera derived from a SIV-infected monkey.

3.2. Comparison of Gag production by LC16m8Δ and DIs-based recombinants

Previously Honda's group constructed replication-deficient vaccinia DIs strain-based recombinant, which had immunogenicity similar to MVA-based recombinant [12]. We now compared by

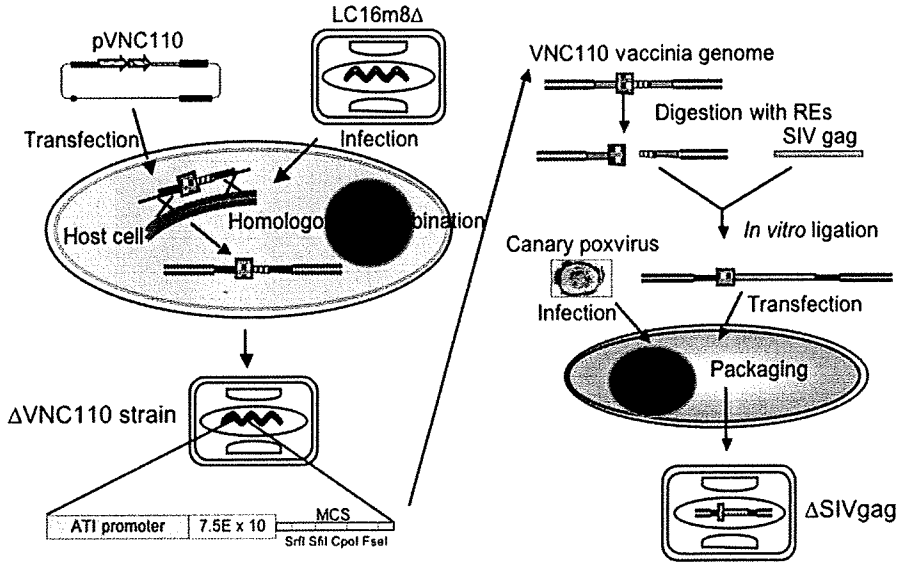

Fig. 1. Schematic presentation for construction of m8ΔSIVgag.

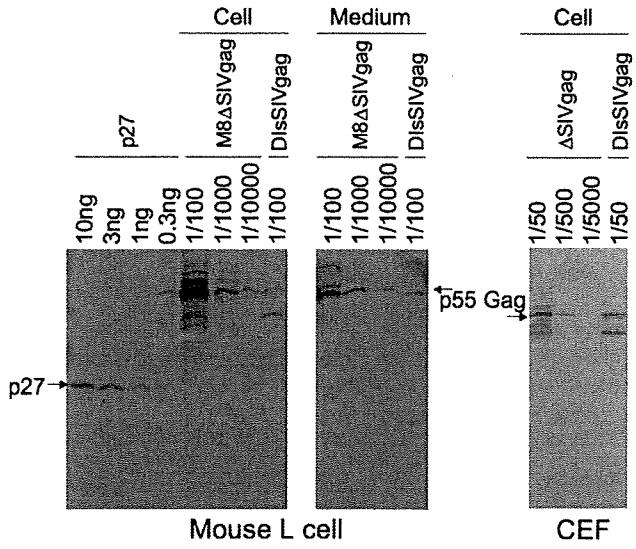

Fig. 2. Western blotting for p55 Gag produced by vaccinia recombinants. Appropriate fractions (1/50–1/10,000) of the cell lysates and medium prepared from m8ΔSIVgag- or rDIs/PSFJ/SIVgag-infected cells were subjected to Western blotting. One hundredth of the cell lysates contains approximately 1 μg of proteins.

Western blotting the amount of Gag protein produced by LC16m8Δ and DIs, both of which used the same promoter for expression of the foreign gene (Fig. 2). In mouse L cells, where DIs is not able to replicate, approximately 100-fold more amount of Gag protein was detected in both medium and cells infected at high multiplicity with m8ΔSIVgag than that in the cells infected with rDIs/PSFJ/SIVgag, whereas m8ΔSIVgag produced Gag protein just several fold more than rDIs/PSFJ/SIVgag in CEF in which both viruses replicate. In human HeLa, mouse NIH3T3, and rabbit RK13 cells m8ΔSIVgag again produced Gag protein 100-fold more than rDIs/PSFJ/SIVgag (data not shown). These results suggest that production of Gag is affected by not only the promoter just upstream of the foreign gene but also the replication capability of the vector virus.

3.3. Immunogenicity of LC16m8Δ and DIs-based recombinants

Next, we evaluated the immunogenicities of these recombinant viruses in mice by priming with plasmid pJWSIVgag expressing the *gag* gene followed by boosting with these recombinant viruses. Considering the preceding reports that viral vectors failed to elicit enough immunities in human although they were nicely antigenic in mice under optimal immunization schedule [4,24], we compared their immunogenicities under the suboptimal condition that includes two or three priming with pJWSIVgag followed by boosting once with 1×10^6 pfu of the recombinant viruses. We have assessed the number of IFN-γ producing cells by ELISPOT assay 2 weeks after a shot of the recombinant viruses, and found that sevenfold more cells were induced by prime-boost vaccination with pJWSIVgag and SIVm8Δgag than that of rDIs/PSFJ/SIVgag (Fig. 3). We also evaluated the induction of Gag specific IFN-γ producing cells by single immunization with pJWSIVgag, SIVm8Δgag or rDIs/PSFJ/SIVgag. In contrast to prime-boost regimen, significant positive spots were not detected by ELISPOT assay (data not shown). When assayed 3 weeks after final immunization with the viruses, differences were more prominent in that only one among four mice immunized with rDIs/PSFJ/SIVgag were ELISPOT positive com-

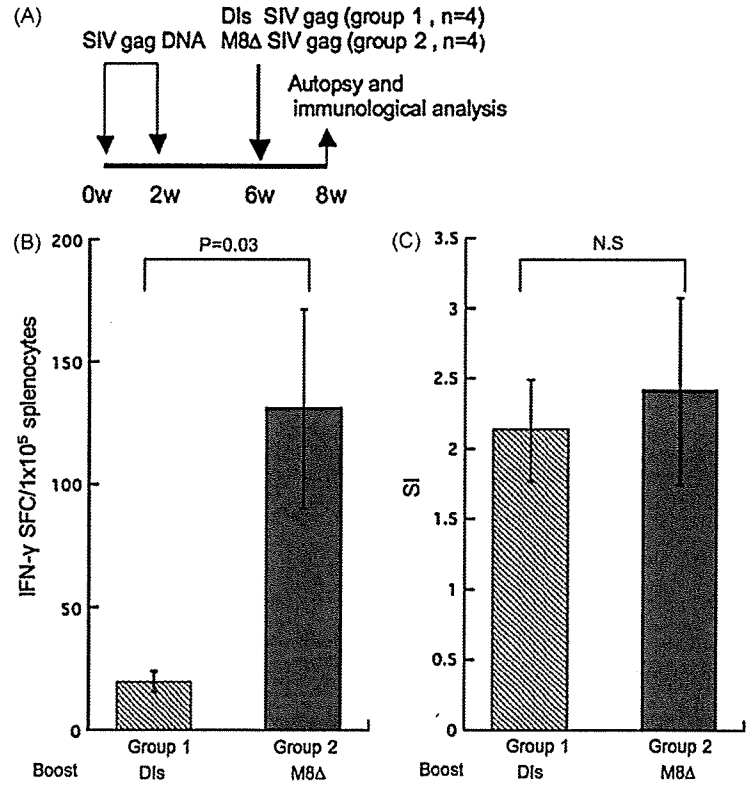

Fig. 3. Comparison of booster effect by m8ΔSIVgag and rDIs/PSFJ/SIVgag. (A) Schematic drawing of experimental design for immunization. Mice were immunized twice with SIVgag DNA followed by one boost with rDIs/PSFJ/SIVgag (group 1) or m8ΔSIVgag (group 2). (B) Frequency of SIV Gag-specific IFN-γ-producing cells in immunized mice. Spleen cells were stimulated with pooled SIV Gag peptides, and IFN-γ-producing cells were detected by IFN-γ-specific ELISPOT assays. Data are expressed as the mean number of SFC per 10^5 splenocytes ± S.E.M. (C) Induction of SIV Gag-specific lymphocyte proliferatives response. Spleen cells were cultured in the presence or absence of SIV p27 antigen, and incorporation of BrdU was measured as described in Section 2. Proliferative responses were presented as the value of the stimulation index (SI). Data are mean ± S.E.M. N.S. means not significant.

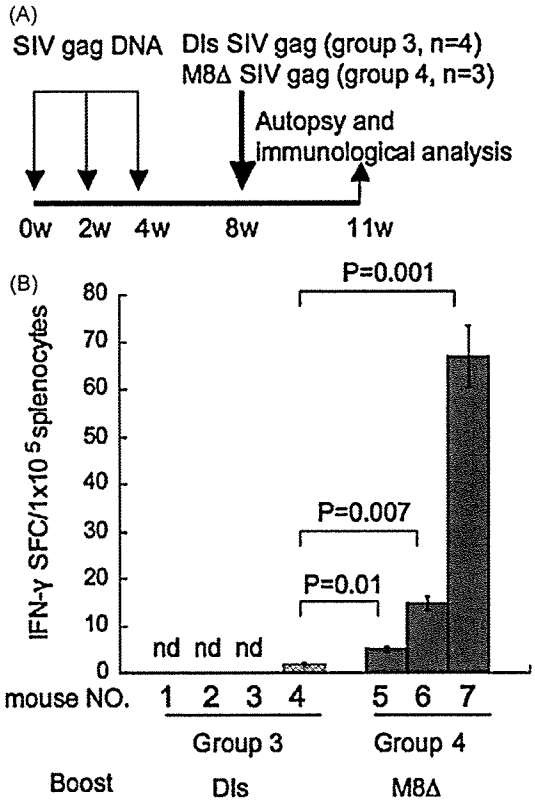

Fig. 4. Comparison of immunogenicities of m8ΔSIVgag and rDIs/PSFJ/SIVgag. (A) Schematic drawing of experimental design for immunization. Mice were immunized three times with SIVgag DNA followed by one boost with rDIs/PSFJ/SIVgag (group 3) or m8ΔSIVgag (group 4). (B) Frequencies of SIV Gag-specific IFN-γ-producing cells in individual immunized mice were presented as the number of SFC per 10^5 splenocytes. SFC of individual mouse was counted in triplicate and presented as the means ± S.E.M.

pared with all positive mice with m8Δgag and average of ELSPOT was approximately 30-fold more in mice immunized with m8Δgag than rDIs/PSFJ/SIVgag (Fig. 4). To monitor the sensitivity of ELISPOT assays, we always included positive controls that were splenocytes stimulated with PMA and ionomycin, and ascertained that they produced 300–500 spots/10^5 splenocytes in every experiment (data not shown).

Proliferation capacities of the lymphocytes derived from the immunized mice were also compared based on BrdU incorporation. Splenocytes from both immunized groups showed low levels of T-cell proliferation in response to stimulation with SIV Gag protein (Fig. 3). But we did not find significant difference between the mice immunized by either virus in contrast to the results of ELISPOT assay described above.

4. Discussion

In this study, we devised a new method involving *in vitro* ligation to efficiently construct recombinant vaccinia viruses expressing the foreign genes. We could construct SIV Gag expressing m8Δ only by this technique but not by the conventional method, which involves *in vivo* ligation. Moreover, we have successfully constructed two additional recombinant viruses expressing the chimeric genes, which contain rat MHC class I with an epitope sequence fused with β2 microglobulin. Approximately 60% of the progeny viruses expressed the transgenes even when no methods were used to enrich the recombinants (They will be published elsewhere.), suggesting that this new technique is generally applicable to construct m8Δ-based recombinant viruses.

Here, using the same promoter in both recombinant DIs and LC16m8Δ strains of vaccinia, we have demonstrated a much more efficient expression of SIV Gag transgene by the latter in several mammalian cells, which were infected at a high dose of inoculum. In contrast, less difference was observed in the level of Gag protein expression in CEF probably because both recombinant viruses propagated at comparable level in this cell type. These results suggest that vaccinia viruses, which propagate better, could provide more efficacious expression of immunogens of interest.

The propagation capability and related efficacy of Gag production by these recombinant vaccinia viruses may reflect their immunogenicity. IFN-γ producing T cells evaluated by ELISPOT were more efficiently elicited by m8ΔSIVGag and lasted longer than those by rDIs/PSFJ/SIVgag. Since several non-replicating vaccinia virus vectors including DIs, MVA, and NYVAC have been shown to be similarly immunogenic in mice [12,25], replication-competent vector such as LC16m8Δ may be more immunogenic than general non-replicating vectors. Since anti-SIV vaccination comprising the DNA-prime and recombinant DIs-boost has been reported to elicit protective immunity in the macaque model [26], it may be expected that m8ΔSIVGag would confer better protection against SIV challenge.

Our results are in contrast with reports by Hirsch et al., that showed similar level of immunogenicity between SIV Gag recombinant MVA and a replication-competent vaccinia vectors when a very high dose of vaccine was applied [27]. However, it should be important to evaluate the immunogenicities of vaccinia recombinants under the suboptimal immunization schedule, including a single boost with a low dose of vaccinia recombinants, which was adopted in this study, given that unsuccessful outcome of the human trials by the vaccines [2,4] that had been appreciated based on protective immune responses elicited by optimal immunization schedule in model animals [24,28].

Mucosal immunity has been suggested to be important for protection against HIV, because it sexually transmit in most cases. Since history of exposure of replicating virus in mucosal tissues has been reported to prime the mucosal immune system and lead to the induction of secretary IgA [29], it is expected that LC16m8Δ vaccination via the mucosal route may induce effective mucosal immunity. Moreover, replicating adenovirus vector has also been reported to be more effective than non-replicating one [30].

In contrast to the more efficient induction of IFN-γ producing T cells by m8ΔSIVGag, the Gag-specific lymphocyte proliferation responses were similarly elicited by both recombinants. Vaccinia viruses produce various kinds of immunomodulatory factors, which may tend to elicit uneven immunities [31]. Therefore, it is conceivable that quantitative and qualitative differences of the factors produced by LC16m8Δ and DIs strains may cause the diverged immune responses.

In summary, we devised an efficient method to construct a recombinant virus based on LC16m8Δ and evaluated it as a vaccine candidate. This replication-competent virus vector showed merits for further development in the viewpoint of its ability to elicit enhanced cell-mediated and hopefully humoral and mucosal immune responses.

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Postinfection passive transfer of KD-247 protects against simian/human immunodeficiency virus-induced CD4$^+$ T-cell loss in macaque lymphoid tissue

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Background: Preadministration of high-affinity humanized anti-HIV-1 mAb KD-247 by passive transfer provides sterile protection of monkeys from heterologous chimeric simian/human immunodeficiency virus infection.

Methods: Beginning 1 h, 1 day, or 1 week after simian/human immunodeficiency virus-C2/1 challenge (20 50% tissue culture infective dose), mature, male cynomolgus monkeys received multiple passive transfers of KD-247 (45 mg/kg) on a weekly basis for approximately 2 months. Concentrations and viral loads were measured in peripheral blood, and CD4$^+$ T-cell counts were examined in both peripheral blood and various lymphoid tissues.

Results: Pharmacokinetic examination revealed similar plasma maintenance levels ranging from 200 to 500 μg/ml of KD-247 in the three groups. One of the six monkeys given KD-247 could not maintain these concentrations, and elicitation of anti-KD-247 idiotype antibody was suggested. All monkeys given KD-247 exhibited striking post-infection protection against both CD4$^+$ T-cell loss in various lymphoid tissues and atrophic changes in organs compared with control group animals treated with normal human immunoglobulin G. The KD-247-treated groups were also partially protected against plasma viral load elevation in peripheral blood samples, although the complete protection previously reported with preadministration of this mAb was not achieved.

Conclusion: Postinfection passive transfer of humanized mAb KD-247 with strong neutralizing capacity against challenged virus simian/human immunodeficiency virus-C2/1 protected CD4$^+$ T cells in lymphoid organs.

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Keywords: anti-V3 mAb, CD4$^+$ T cells, HIV-1, KD-247, lymphoid tissue, passive immunization, simian/human immunodeficiency virus

Introduction

Elicitation of virus-specific humoral immune responses, with their strong CD4$^+$ and CD8$^+$ T-cell immune responses, are critical to good control of HIV-1 [1,2]. Although recent vaccine candidates based on active immunization are intended to stimulate CD4$^+$ and CD8$^+$ T-cell responses, induction of broadly neutralizing

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antibodies by active immunization has been limited to date [3,4]. In contrast, passive immunization with neutralizing antibody effectively induced sterilizing immunity by preventing the establishment of chronic infection. We and others have reported that chimpanzees can be protected against acute infection with the T-cell line-adapted strain HIV-1_{IIIB} by passive transfer of a mouse–human chimeric anti-HIV-1 V3 mAb [5]. Furthermore, we produced a high-affinity cross-neutralizing humanized mAb, KD-247, by sequential immunization with peptides derived from the V3 region of HIV-1 clade B primary isolates and found that KD-247 yields sterile protection of monkeys against the highly pathogenic simian/human immunodeficiency virus (SHIV) [6,7]. KD-247 is thus considered a promising new immunotherapeutic agent for HIV-1-infected patients [8].

It was demonstrated that intensive, short-term postinfection therapy with neutralizing immunoglobulin G (IgG) against simian immunodeficiency virus (SIV) can have long-term beneficial effects on disease in a pathogenic primate lentivirus model [9]. Passive transfer of neutralizing antibodies also conferred postinfection prophylaxis against pathogenic SHIVs in macaques [10,11]. Furthermore, passive immunization of pregnant or neonatal monkeys with combinations of mAbs has been reported to completely or partially neutralize SHIV in animal models of mother-to-child transmission of HIV [12,13]. However, whether neutralizing antibody plays a significant role in controlling established HIV infection is unclear. The current aim of antiretroviral therapy remains the maintenance of plasma HIV-1 RNA levels below the limit of detection [14]. In a clinical trial, three passively transferred mAbs, 2G12, 2F5, and 4E10, were shown to delay the rebound of HIV-1 after cessation of antiretroviral therapy; the delay was particularly pronounced in acutely infected individuals [15]. In this study, we evaluated the postinfection effect of KD-247 against $CD4^+$ T-cell loss and increased viral loads in the SHIV model.

Materials and methods

Preparation of KD-247

A high-affinity humanized mAb, KD-247 [Chemical Abstracts Service (CAS) Registry Number: 914257-21-9], was prepared as previously described [6]. Briefly, the mouse mAb C25 was elicited by immunization with six synthetic peptides derived from the V3 region of HIV-1 primary isolates. The complementary-determining regions and partial framework regions of C25 were transferred into the variable region of human IgG. Cells producing the humanized C25, KD-247, were expanded in large-scale culture, and the antibody was purified from the culture supernatants by ion exchange and affinity chromatography.

Pathogenic simian/human immunodeficiency virus challenge to monkeys and postinfection transfer of KD-247

All animals used in this study were mature, male cynomolgus monkeys (*Macaca fascicularis*) from the Tsukuba Primate Center, the National Institute of Infectious Diseases (NIID) (currently known as the Tsukuba Primate Research Center, National Institute of Biomedical Innovation), Japan. They were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, 1987, under the Japanese Law Concerning the Protection and Management of Animals, and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of NIID, Japan.

The pathogenic chimeric SHIV-C2/1 is an SHIV-89.6 variant isolated by in-vivo passage in cynomolgus monkeys [16]. Cynomolgus monkeys injected intravenously with SHIV-C2/1 exhibited high levels of viremia and marked $CD4^+$ T-cell depletion within 2 weeks after challenge [16,17]. Six naive monkeys were intravenously inoculated with 20 50% tissue culture infective dose ($TCID_{50}$) of SHIV-C2/1 and were then given 45 mg/kg weight of KD-247 at 1 h (Cy-1 and Cy-2), 1 day (Cy-3 and Cy-4), or 1 week (Cy-5 and Cy-6) after viral challenge; a single preinfection administration of the mAb at this dosage had exhibited sterile protection against SHIV-C2/1 infection [7]. Two control monkeys (Cy-7 and Cy-8) received 45 mg/kg of purified human normal immunoglobulin (control IgG; Nihon Pharmaceutical, Tokyo, Japan) instead of KD-247 at 1 day after viral challenge. Additional multiple (seven or eight) administrations of the same concentrations of KD-247 or control IgG were given weekly from day 7 for a period of 2.5–3 months. Blood samples were drawn to examine the plasma concentrations of KD-247, SHIV RNA copy numbers, and $CD4^+$ T-cell counts. At approximately 11–13 weeks after viral challenge, necropsies were performed and histological examination and flow cytometric analyses of lymphoid organs were conducted. The schedules of KD-247 administration, blood drawing, and necropsy are shown in Fig. 1(a).

Plasma concentration of KD-247

KD-247 concentrations in macaque plasma were measured by ELISA. Ninety-six-well ELISA plates (MaxiSorp, Nunc A/S, Roskilde, Denmark) were coated with a KD-247-specific antigen, SP13 peptide (GPGRAFGPGRAFGP GRAFC). After blocking and washing, monkey plasma at appropriate dilutions was added and the plates incubated. KD-247 was diluted to concentrations ranging from 2.5 to 40 ng/ml and used as a reference. The wells were washed and then incubated with a detection antibody solution consisting of peroxidase-conjugated antihuman IgG mAb (Kaketsuken, in-house preparation). After final washes, peroxidase substrate was added and the reaction was