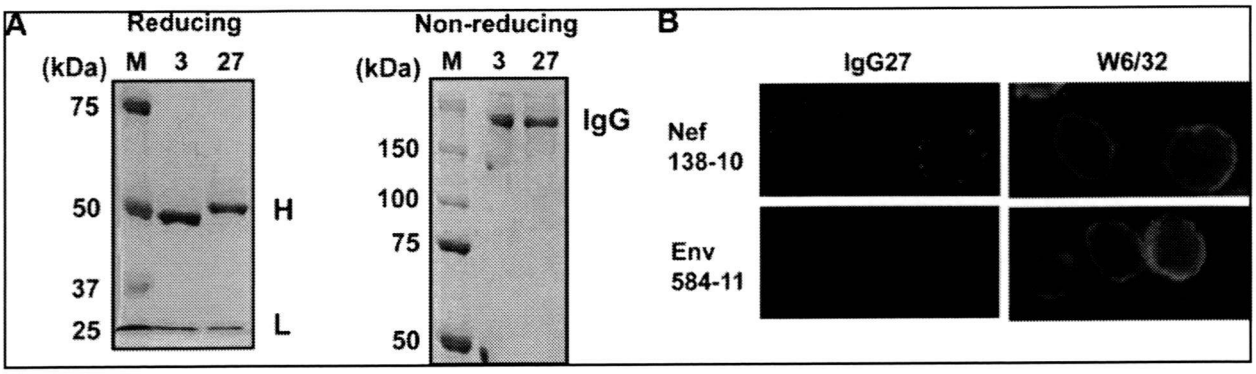

FIG. 3. Reconstitution of intact human IgG molecules by a baculovirus expression system. (**A**) CBB staining of expressed human IgG molecules under reducing and nonreducing conditions. Heavy and light chains are indicated by H and L, respectively. (**B**) A24-positive B-LCL cells were pulsed with 10 μM Nef138-10 or Env584-11 peptides, and stained with the reconstituted human IgG27. These cells were then stained with a biotinylated polyclonal rabbit anti-c-Myc-tag antibody (Santa Cruz Biotechnology Inc.), and finally with a PE-conjugated streptavidin. We also stained the cells with a PE-conjugated anti-HLA-ABC antibody (W6/32) to analyze the expression of HLA-ABC molecules on the cell surface. The stained cells were mounted and observed with an Eclipse E600 microscopy system. Two or three cells are shown in each image.

Nef138-10/A24 on the cell surface. The mean intensity of the fluorescent staining with scFv#27 was higher than the mean intensity seen with scFv#3 (Fig. 2, top panel).

Individual scFv clones were then converted to intact human IgG using the Bac-to-Bac Baculovirus expression system (Invitrogen Corporation, Carlsbad, CA). The human IgG1 heavy chain and Ig light chain expression cassettes from pAc-λ-CH3 and pAc-κ-CH3, respectively (PROGEN, Heidelberg, Germany), were inserted into pFastBac Dual under the polyhedrin promoter (light chain) and the p10 promoter (heavy chain). The VH and VL regions of each clone were inserted with the *ScaI*/*Hin*dIII site and *XhoI*/*Nhe*I site, respectively. Recombinant baculovirus was made according to the manufacturer's procedure. Titers were determined using the BacPAK Baculovirus Rapid Titer Kit (Clontech Laboratories, Inc., Mountain View, CA). Insect cells (Sf9 and High Five cells) were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 5 and incubated for 96–120 h. In preliminary experiments, we found out that expression was 2- to 3-fold higher in High Five cells than in Sf9 cells, and expression reached a plateau after 96 h when the insect cells were infected at an MOI of 5 (data not shown). The expression of human IgG molecules was confirmed by Western blotting with anti human IgG + IgM (H + L) antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Expressed proteins were purified by HiTrap Protein A columns (GE Healthcare UK Ltd.) and analyzed by SDS–PAGE under reducing and nonreducing conditions. The yield of human IgG expressed using this system was 1–5 mg/1-liter culture. SDS–PAGE analysis of the expressed human IgG showed two bands corresponding to H and L chains under reducing conditions and a single band corresponding to IgG under nonreducing conditions (Fig. 3A), suggesting that the molecules have a correct conformation. CBB staining also showed that we obtained pure human IgG after one-step purification using a protein A column.

To examine cell surface-specific reactivity, we incubated IgG27 with A24-positive B-LCL cells pulsed with Nef138-10 peptides and observed the stained cells under microscopy. The staining procedure was identical to the procedure used for flow cytometry, except that the antibody dilution buffer was FCS instead of NRS. Stained cells were mounted on Teflon Printed eight-well chamber glass slides (Erie Scientific Company, Portsmouth, NH) and observed with an Eclipse E600 microscopy system (Nikon Corporation, Tokyo, Japan). As shown in Fig. 3B (right panel), HLA-A, HLA-B, and HLA-C molecules were expressed with approximately equal frequency on the cells in the assay. Fluorescence signals appeared as dots on the cells pulsed with the Nef138-10 peptide (Fig. 3B). By contrast, cells pulsed with Env584-11 showed no fluorescence. These data suggest that the reconstituted human IgG was specific for Nef138-10/A24 on the cell surface.

We further characterized reconstituted human IgGs. ELISA analysis showed that clones IgG3 and IgG27 bound specifically to Nef138-10/A24 in a dose-dependent manner but not to Env584-11/A24 or BSA (Fig. 4). At a lower concentration clone IgG27 showed higher affinity than IgG3, consistent with staining results seen earlier with the corresponding scFvs.

FIG. 2. Flow cytometric analysis of scFvs for their binding specificity on cells pulsed with peptides. A24-positive B-LCL cells (HLA-A24, A26, B7, B52, CW7) were pulsed with 10 μM of A24-restricted epitope peptides; the Nef138-10 (RYPLTFGWCF), Env584-11 (RYLRDQQLLGI), Gag28-9 (KYKLKHIVW), and CMV pp65-328 (QYDPVAALF) were stained with each scFv clone. These cells were then stained with a biotinylated polyclonal rabbit anti-c-Myc-tag antibody (Santa Cruz Biotechnology Inc.), and finally with a PE-conjugated streptavidin. We also stained the cells with a PE-conjugated anti-HLA-ABC antibody (W6/32) to analyze the expression of HLA-ABC molecules on the cell surface. The shaded histograms show specific staining with each scFv clone whereas open histograms show background staining with a rabbit anti-c-Myc-tag antibody and a PE-conjugated streptavidin. The ordinate indicates the relative cell number and the horizontal axis indicates fluorescence intensity in a log scale.

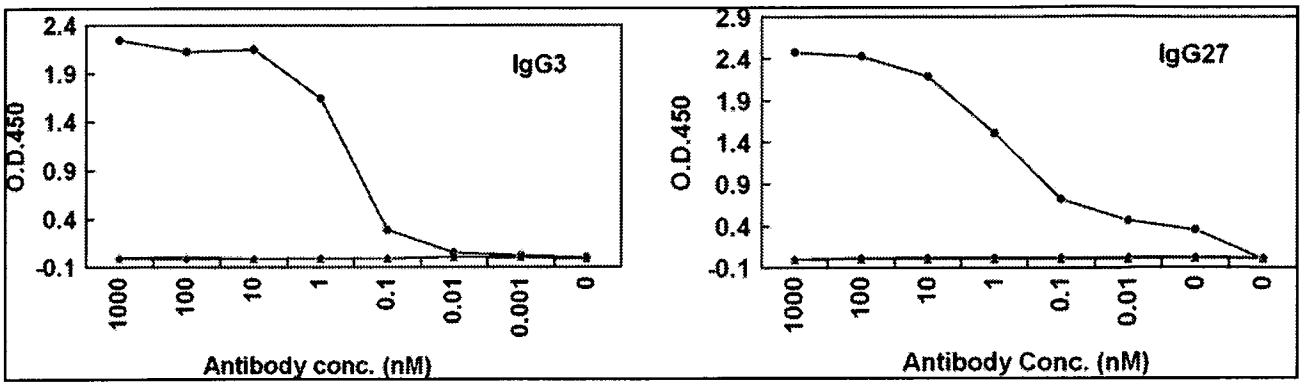

FIG. 4. Analysis of the molecular interaction of the reconstituted human IgG by ELISA. The binding specificity was analyzed by ELISA with antihuman IgG antibody (SIGMA). The bindings to Nef138-10/A24 (circle), Env584-11/A24 (triangle), and BSA(diamond) are shown. The ordinate indicates the optical density value at OD_{450}. The IgG concentration is shown below the horizontal line.

Therefore, we concluded that we had reconstituted intact human IgG molecules with the same high degree of specificity as the parental scFvs.

We also analyzed antigen–antibody interactions by surface plasmon resonance using BIAcore 1000 (GE Healthcare UK Ltd.). The analysis of the interaction between antigens and antibodies was done at 25°C under a flow rate of 20 μl/min. HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20; pH 7.4) buffer was used in all experiments. The antihuman IgG Fc region antibodies were immobilized about 3700 resonance units (RU) on a research grade CM5 sensor chip (GE Healthcare UK Ltd.) by standard amine coupling. The IgG molecules were flowed on the chip and captured about 1200 RU. Nef138-10/A24 were purified further on a Mono Q column for the analysis of antigen–antibody interaction by BIAcore. Nef138-10/A24s were diluted in HBS-EP buffer containing NSB Reducer (GE Healthcare UK Ltd.) and injected for 3 min. The chip was regenerated by injecting 10 mM glycine-HCl (pH 1.5). The human IgG captured cell was used as the reference cell. Data were collected at five different concentrations of Nef138-10/A24 and analyzed by BIAevaluation 3.0 software (GE Healthcare UK Ltd.). The binding and dissociation constants were determined using data from five different concentrations (Fig. 5A). The dissociation constants of IgG3 and IgG27 were approximately 23 μM and 20 μM, respectively (Fig. 5B). The dissociation constants of the IgG3 and IgG27 clones were in the expected range for TCR interactions, but were too low for antibody interactions.[26,27] IgG27 stained Nef138-10/A24 specifically on the surface of peptide-pulsed cells. We also tried to stain the cells with expression of whole Nef protein using Sendai virus vector but could not stain endogenously expressed HIV-1 Nef CTL epitope.

The low expression of the endogenously expressed CTL epitope and the low binding affinity of the antibodies might explain the inability to detect endogenously expressed pMHCs. We speculate that clones IgG3 and IgG27 could have originated from lower-affinity IgM clones in our pooled scFv library.

In this study, we successfully isolated scFvs directed against an HIV-1-specific pMHC, Nef138-10/A24, using a panning procedure with magnetic beads to select the specific antibodies from phage display libraries. Also, we successfully reconstituted the human IgGs directed against the HIV-1 Nef138-10 CTL epitope loaded on an HLA-A24 molecule using the baculovirus expression system. All the clones that reconstituted using this system kept the same specificity of parental scFvs and were easily obtained at a high amount and purity after one-step purification. These systems will provide us with a rapid generation of monoclonal antibodies that is difficult to generate using conventional hybridoma technology. Ours is the first report to describe the generation of

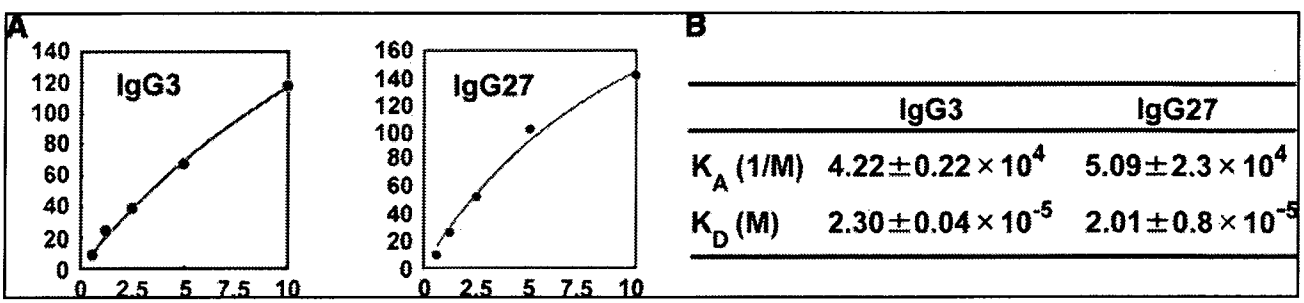

FIG. 5. Analysis of the antigen–antibody interaction using Biacore. The antibody–antigen interactions were analyzed by surface plasmon resonance. (**A**) The IgG molecules were captured on an antihuman IgG immobilized CM5 chip and Nef138-10/A24 was flowed on the chip at five different concentrations. (**B**) The data were collected and analyzed by BIAevaluation. The binding (K_a) and dissociation (K_d) constants of each IgG clone are shown in the panel.

monoclonal antibodies bound specifically to an immunodominant HIV-1 CTL epitope loaded on an HLA class I molecule. We were able to show that this particular CTL epitope had a B cell epitope. Efforts to isolate antibodies with higher affinities would be warranted. The escape phenotype associated with this particular CTL epitope may result either from structural differences of pMHCs, from aberrant processing of the mutant epitope, or from decreased numbers of pMHCs on the cell surface. To address these questions, further studies are underway using these monoclonal antibodies and some other techniques.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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Monitoring of HIV-1 envelope-mediated membrane fusion using modified split green fluorescent proteins

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ABSTRACT

A simple, cell-based, membrane fusion assay system that uses split green fluorescent proteins (spGFPs) as an indicator was developed. The attachment of the pleckstrin homology (PH) domain to the N-termini of each spGFP not only localized the reporter signal to the plasma membrane but also helped the stable expression of the smaller spGFP of seventeen amino acid residues. It was shown that this system allowed real-time monitoring of membrane fusion by HIV-1 envelope protein (Env) without the addition of external substrates. This method can be adapted to the analyses of other viral membrane fusion.

1. Introduction

Membrane fusion is the prerequisite event that allows enveloped viruses, some of which are linked to emerging infectious diseases such as avian influenza, severe acute respiratory syndrome, and acquired immunodeficiency syndrome (AIDS), to enter their host cells. Among these emerging diseases, AIDS has become a global threat to human health. The discovery of a membrane fusion inhibitor has made HIV-1 Env an important target for anti-HIV-1 chemotherapy (Chan et al., 1997; Eckert and Kim, 2001; Este and Telenti, 2007; Poveda et al., 2005; Weissenhorn et al., 1997). Recently, a new class of inhibitor that blocks the interaction between Env and its co-receptor, CCR5, has been developed (Santoro et al., 2004). A simple phenotyping method of Env-mediated membrane fusion will facilitate progress in the development of new inhibitors or in the evaluation of drug-resistant mutants (Olson and Maddon, 2003).

A phenotyping method of HIV-1 Env requires a system that generates a measurable signal upon membrane fusion either in a cell–cell or virus–cell system. The methods described employ materials, such as visible dyes, transcription factors, and self-complementing enzyme fragments, that produce a signal when they transfer from one compartment to another via membrane fusion (Barbeau et al., 1998; Blumenthal et al., 2002; Feng et al., 1996; Furuta et al., 2006; Holland et al., 2004; Huerta et al., 2002; Jun and Wickner, 2007; Lin et al., 2005, 2003; Monck and Fernandez, 1992; Sakamoto et al., 2003).

The development of a versatile, cell-based membrane fusion assay system is described in this report. The system employs a modified green fluorescent protein (GFP), split GFP (spGFP), which has been engineered to have the capacity for self-assembly (Cabantous et al., 2005). The pleckstrin homology (PH) domain (Lemmon, 2008) was fused at the N-termini of spGFPs so as to focus the signal in the membrane regions. These spGFPs become fluorescent only when they have reassociated with each other (Cabantous et al., 2005) during the membrane fusion. Unlike an enzyme-based assay, this spGFP system allows real-time monitoring of the membrane fusion process without any additional substrates.

2. Materials and methods

2.1. Construction of expression vectors

The PH domain based on human phospholipase Cδ was synthesized by assembling 10 oligonucleotides, each containing 79 nucleotides. The oligonucleotides were combined and assembled

Abbreviations: AIDS, acquired immunodeficiency syndrome; GFP, green fluorescent protein; spGFP, split green fluorescent protein; PH, pleckstrin homology; DMEM, Dulbecco's modified Eagle's medium; MSD, membrane-spanning domain.

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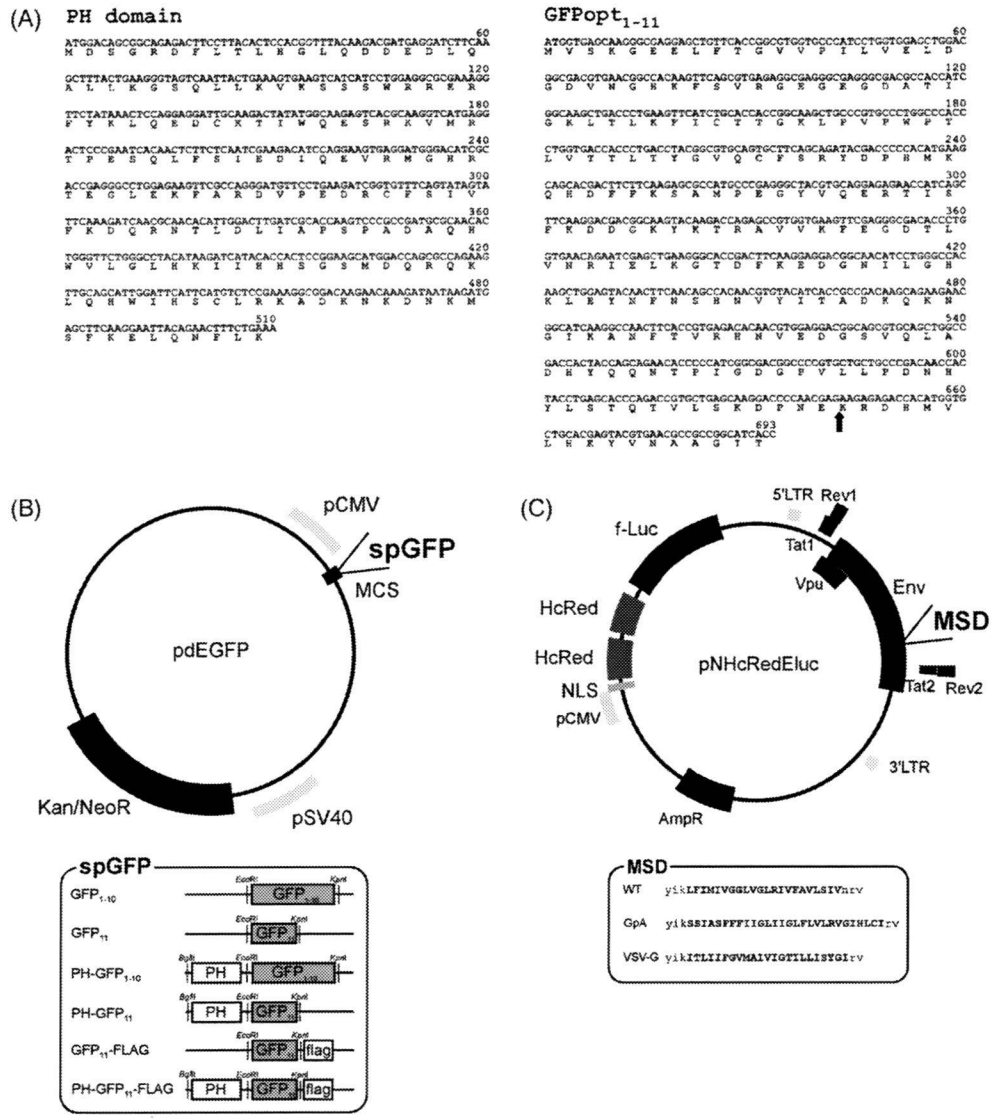

Fig. 1. Engineered proteins and expression vectors. (A) The amino acid and nucleotide sequences of PH domain and $GFPopt_{1-11}$ were shown. The amino acid residue was shown using the single-letter abbreviations. The split point between GFP_{1-10} and GFP_{11} is indicated by the arrow in $GFPopt_{1-11}$. (B) (upper panel) The expression vector for spGFPs. MCS: multiple cloning site; spGFP: the insertion point of spGFP; *Kan/NeoR*: kanamycin/neomycin resistance gene. (Lower panel) The different spGFPs. PH: pleckstrin homology domain. The restriction sites that were used are indicated. (C) (upper panel) The HIV-1 envelope expression vector, pNHcRedEluc. pCMV: human cytomegalovirus promoter, NLS: nuclear-localization signal, HcRed: a far-red fluorescent protein isolated from *Heteractis crispa*, f-Luc: firefly luciferase, MSD: membrane-spanning domain, *AmpR*: ampicillin resistance gene. (Lower panel) Primary structures of the MSDs used. WT: wild type, GpA: glycophorin A, VSV-G: vesicular stomatitis virus G protein. The predicted MSD regions are capitalized.

by PCR (94 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s for 30 cycles). Similarly, 30 oligonucleotides, which overlapped each other with 18 bases at the both ends, were used to assemble the optimized GFP gene, named $GFPopt_{1-11}$. The primary sequences of the PH domain and $GFPopt_{1-11}$ are shown in Fig. 1A. Both amplicons were cloned and sequenced in pCR4Blunt-TOPO (Invitrogen, Carlsbad, USA). $GFPopt_{1-11}$ was split into GFP_{1-10} (1–642 base pairs) and GFP_{11} (643–696 base pairs), at a point between the 10th and 11th β-sheets of the GFP. The subscripts 1–10 and 11 reflect this location. The PH-GFP_{1-10} and PH-GFP_{11} genes were generated by combining the PH domain gene with the spGFP genes. These genes were then cloned to pdEGFP, which was constructed by deleting the EGFP gene in pEGFP-N2 (BD Biosciences Clontech, Palo Alto, USA) (Fig. 1B). The expression vector for each protein was named by adding pd in front of the target protein, such as pdPH-GFP_{1-10}.

The FLAG tag sequence was added to the 3′-termini of the spGFP genes by using a 3′-primer that included the FLAG tag sequence during PCR. A new HIV-1 Env-expression vector called pNHcRedEluc, a derivative of pElucEnv (Miyauchi et al., 2005), was constructed by replacing the gene for EGFP with that of a tandem red fluorescent protein; HcRed (Evrogen, Moscow, Russia). This was preceded by a nuclear-localizing signal (Fig. 1C). Thus the nuclei of transfected cells became red. The transfection efficiency could then be measured by firefly luciferase activity. The pNHcRedEluc△NB vector in which most of the env gene had been deleted was prepared as a negative control.

2.2. Cell cultures and transfection

The 293FT (Invitrogen, Carlsbad, USA) and 293CD4 (Miyauchi et al., 2005) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, USA). The 293FT cells were cultured with 500 μg/ml of Geneticin (Gibco, Grand Island, USA),

as recommended by the manufacturer. Transient transfection was accomplished using Fugene HD (Roche, Indianapolis, USA). Stable cell lines expressing PH-GFP$_{1-10}$ were established after transfecting 293CD4 cells with pdPH-GFP$_{1-10}$ by electroporation (Biorad GenePulsar, Hercules, USA). Transfected cells were selected with 700 μg/ml of Geneticin in DMEM.

2.3. Fusion assay

The spGFP-mediated fusion assay was performed as follows. The expression vectors pNHcRedEluc and pdPH-GFP$_{11}$ were transfected into 293FT cells. The transfected 293FT cells were overlaid with 293CD4 cells which were transfected transiently or permanently with vector pdPH-GFP$_{1-10}$. In the case of transient transfection, the mixing of cells was started at 42 h after transfection. Fusion was monitored in real-time using an IN Cell Analyzer 1000 (GE Healthcare, Uppsala, Sweden) or was observed using a confocal microscope (Olympus FluoView FV1000, Tokyo, Japan) that examined fixed cells (4% paraformaldehyde) at designated time points.

The fusion assay using the mixing of the two different fluorescent proteins expressed in the Env(+)- and receptor(+) cells, respectively as an indicator was carried out as follows. The pElucEnv (Miyauchi et al., 2005) was transfected into 293FT cells to make Env(+) cells. Because pElucEnv expressed both the HIV-1 Env and GFP proteins, this generated "green" Env(+) cells. Meanwhile the expression vector for DsRed (Clontech/Takara, Otsu, Japan) was transfected into 293CD4 cells to generate "red" receptor(+) cells. These two types of cells were co-cultured and the extent of the fusion was monitored by the redistribution of the green and red signals by microscopy.

The inhibitor C34 was used to show the specificity of the new fusion assay. According to the previous study (Kliger et al., 2001), two different concentrations, 12 nM and 150 nM in a final concentration, of the peptide inhibitor, C34, was added at the beginning of the co-culture. The IC50 value of the C34 peptide was about 12 nM and more than 90% inhibition was observed with the concentration of 150 nM (Kliger et al., 2001). The cells were fixed and examined after 2.5 h of co-culture and analyzed as described above.

2.4. Protein analysis

Sample preparation and immunoblotting were done as described previously (Miyauchi et al., 2005). Anti-FLAG antibody (Sigma, Saint Louis, USA) and anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, USA) were used as primary antibodies for the analysis of GFP$_{11}$ and GFP$_{1-10}$, respectively. Chemiluminescence signals were detected using an LAS-3000Lite (Fujifilm, Tokyo, Japan).

2.5. Immunofluorescence assay

Transfected cells were fixed in an acetone:methanol solution (1:1) for 15 min at room temperature and stained with an anti-FLAG antibody (3 μg/ml) for 40 min at 30 °C. A secondary antibody, labeled with Alexa Fluor 555 (Invitrogen, Carlsbad, USA), was used. The fluorescent signal was observed using a confocal microscope (Olympus FluoView FV1000, Tokyo, Japan).

3. Results

3.1. Expression of modified spGFPs

3.1.1. Immunoblotting analysis

The expression vectors containing different spGFPs shown in Fig. 1B were transfected into the cells and the expressed proteins were then analyzed by immunoblotting. When probed with an anti-GFP antibody, GFP$_{1-10}$ and PH-GFP$_{1-10}$ were detected as approximately 25 kDa and 40 kDa bands, respectively (Fig. 2A). The observed molecular weights were consistent with those expected from the amino acid sequences. The cells transfected with pEGFP-N2 (BD Biosciences Clontech) were included as a positive control (Fig. 2A, EGFP lane). As for GFP$_{11}$, an anti-GFP antibody failed to detect any band (data not shown). A FLAG tag was added to GFP$_{11}$ with and without the PH domain because this failure could have been caused by the absence of anti-GFP's epitope in the seventeen-amino-acid-long GFP$_{11}$ portion. When probed with an anti-FLAG antibody, a band of 22 kDa was detected for PH-GFP$_{11}$-FLAG but not for GFP$_{11}$-FLAG (Fig. 2B). This result suggested that GFP$_{11}$, as only a seventeen-amino-acid-long peptide, was unstable without the PH domain.

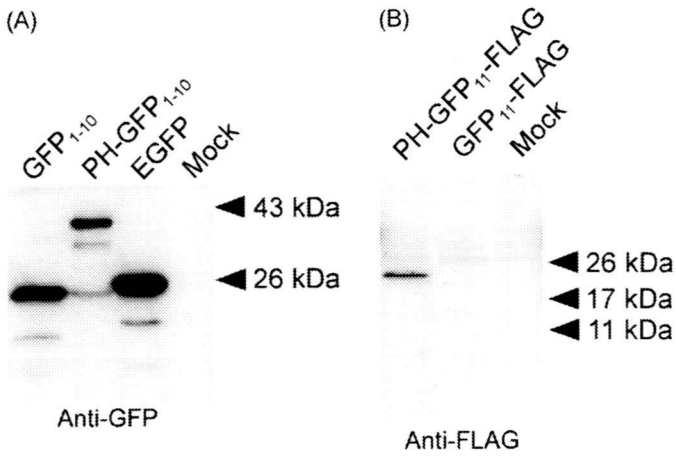

Fig. 2. The spGFPs expressed in the transfected cells. (A) Immunoblotting analysis of 293CD4 cells transfected with GFP$_{1-10}$ and PH-GFP$_{1-10}$ expression vectors and probed with an anti-GFP antibody. Cells transfected with pEGFP-N2 (BD Biosciences Clontech) were included as a positive control (EGFP lane). (B) The proteins expressed in 293FT cells transfected with the GFP$_{11}$-FLAG or the PH-GFP$_{11}$-FLAG expression vectors were probed with an anti-FLAG monoclonal antibody. Mock: the mock transfected cells.

3.1.2. Immunofluorescence analysis

Immunofluorescence analysis was used to examine the intracellular localization of the spGFPs. The GFP$_{1-10}$ distributed throughout the cell, but with the PH domain attached, the PH-GFP$_{1-10}$ localized to the periphery of the transfected cells (Fig. 3A). The expression of FLAG-tagged GFP$_{11}$ (Fig. 3B, top) was not detected. This finding is consistent with the results of immunoblotting (Fig. 2B). However, FLAG-tagged PH-GFP$_{11}$ was detectable at the cell periphery (Fig. 3B, middle). Without the FLAG tag, PH-GFP$_{11}$ showed no signal with anti-FLAG antibody (Fig. 3B, bottom).

3.2. Recovery of GFP function by reassociation of spGFPs

Next, pairs of the spGFPs were co-transfected into 293FT cells and their outcome was examined (Fig. 3C). Consistent with the data shown in Fig. 2, PH-GFP$_{11}$, but not free GFP$_{11}$, was able to generate a green signal (Fig. 3C). When PH-GFP$_{11}$ was co-transfected with GFP$_{1-10}$, a homogenous green signal was observed. This data suggests that the reassociation of two split GFPs could take place before they are localized to the plasma membrane. With the pair of PH-GFP$_{1-10}$ and PH-GFP$_{11}$, most of the green signal was detected in the rim of the co-transfected cells (Fig. 3C). As expected, neither the spGFPs nor the PH-spGFPs alone showed any fluorescence (Fig. 3D).

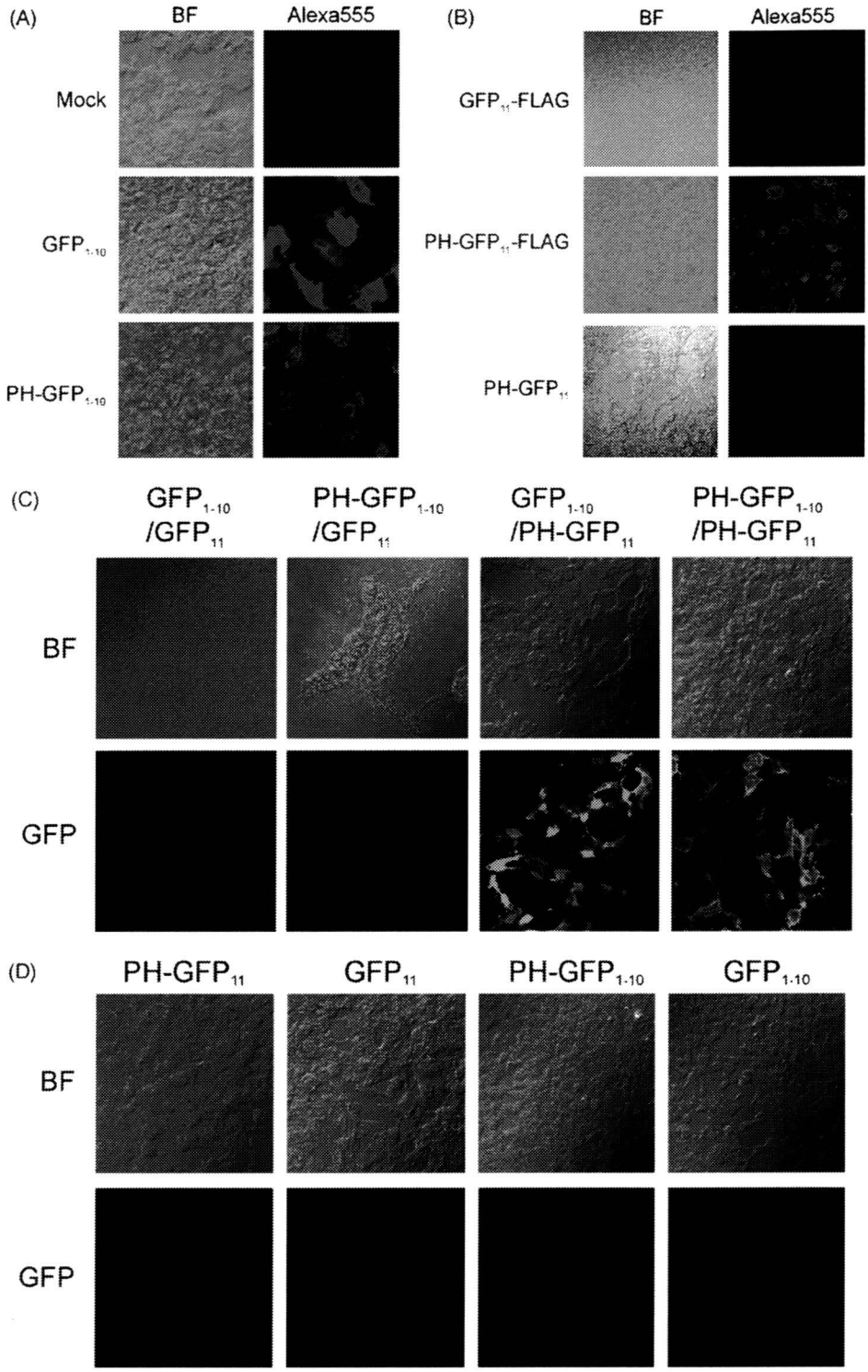

Fig. 3. Intracellular localization of spGFPs expressed with pdExpression vectors. (A) Immunofluorescence assay of GFP_{1-10} or PH-GFP_{1-10}-transfected cells using an anti-GFP antibody. Mock: mock transfected cells; BF: bright field view; Alexa555: the Alexa555-derived signal. (B) Immunofluorescence assay of GFP_{11}-FLAG, PH-GFP_{11}-FLAG, and PH-GFP_{11}-transfected cells using anti-FLAG antibody. The abbreviations used are the same as for (A). (C) Co-transfection of PH-GFP_{1-10} with PH-GFP_{11} and GFP_{11} and of GFP_{1-10} with PH-GFP_{11} and GFP_{11}. (D) Single transfection of different spGFPs. BF: bright field view; GFP: the GFP signal.

3.3. Membrane fusion assay using spGFPs

3.3.1. Analysis of the wild type HIV-1 Env-mediated fusion

PH-spGFPs was used for the analysis of membrane fusion induced by HIV-1 Env. For this, the 293FT cells were transfected with pNHcRedEluc and pdPH-GFP_{11} and then co-cultured with the 293CD4 cells that were stably expressing PH-GFP_{1-10}. The green signal was observed at the plasma membrane surrounding several red nuclei (Fig. 4A). The red nuclei were derived from the 293FT cells expressing Env as pNHcRedEluc expressed the nuclear-localizing HcRed proteins. The unique localization of green signal in the membrane region made it easy to differentiate the real signal from the non-specific autofluorescence background. Furthermore, the non-envelope-mediated spontaneous fusions, if they occurred, could be

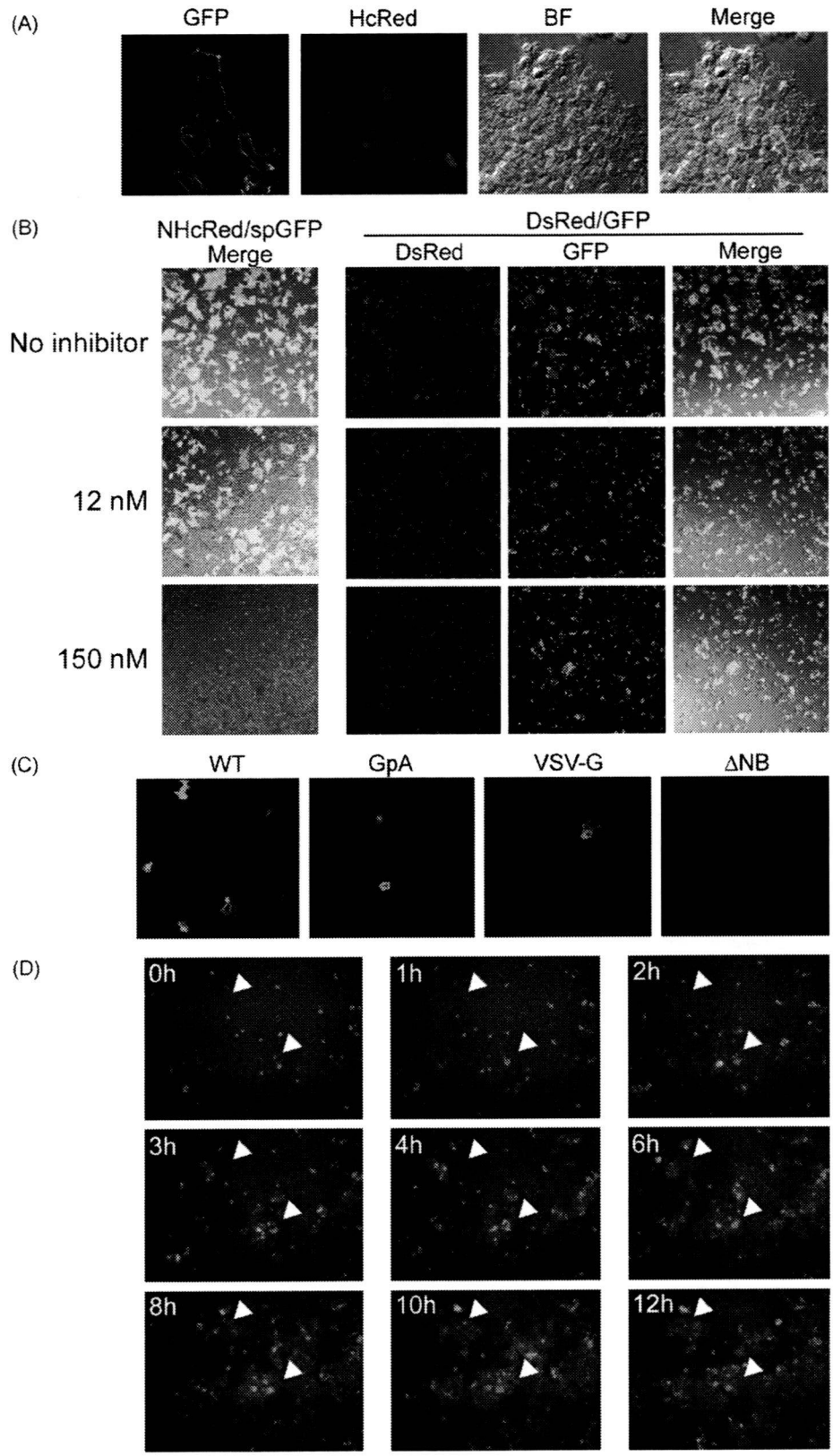

Fig. 4. Generation of the green fluorescent signal upon membrane fusion. Cell fusion between envelope- and receptor-expressing cells that harbor respective spGFP expression vectors were observed using a confocal microscope and IN Cell Analyzer. The HcRed signal was generated from the cells transfected with the expression vector for envelope and HcRed genes (Fig. 1C). (A) Detailed image of the localization of spGFPs and HcRed. BF indicates bright field; GFP: the green fluorescence signal; Merge: the merged images of GFP, HcRed, and BF. (B) The effect of the specific inhibitor and comparison with the fluorescent proteins-mixing assay. The specific inhibitor of the HIV-1 Env-mediated membrane fusion, C34, was used in the spGFP assay (left) and the conventional fluorescent protein-mixing assay (right). The final concentration of the inhibitor was indicated in nM. In the fluorescent protein-mixing assay (right), Env(+)-293FT cells expressing GFP and the receptor(+)-293CD4 cells expressing DsRed were co-cultured. Fused cells are seen as both GFP and DsRed signal-positive cells. (C) Comparison of the frequency of membrane fusion events between wild type (WT) and its MSD mutants (GpA and VSV-G) (details are in Fig. 1C). ΔNB indicates Env-deleted pNHcRedEluc. (D) The time-course analyses. Arrowheads indicate the regions of observed GFP signal. The time after co-culture is indicated in the upper left of each image.

ruled out by the absence of red nuclei in the syncytia. This result indicates that the simultaneous use of pNHcRedEluc and spGFPs allows us to monitor membrane fusion directly without the addition of dyes or substrates.

3.3.2. Analysis of HIV-1 Env-mediated fusion using an inhibitor and Env mutants

The specificity of the spGFP assay was examined by using the known inhibitor of the HIV-1 Env-mediated membrane fusion, C34 (Seo et al., 2005). The C34 peptide is known to inhibit the formation of 6-helix bundle. Two different concentrations, 12 nM (IC50) and 150 nM (IC90) (Kliger et al., 2001), were tested in spGFP fusion assays. For a comparison, in addition to the spGFP assay, the fusion assay using the Env(+)- or receptor(+)-cells expressing GFP and DsRed, respectively was used. In the spGFP assay, the number of the GFP signal-positive cells was decreased in a dose-dependent manner (Fig. 4B, left column). In a parallel assay, the number of the fused cells indicated by the presence of the both GFP and RFP signals in the fused cells was decreased similarly (Fig. 4B, right panel). The new spGFP assay was much easier to monitor, because the green signal was only observed when the actual fusion took place. In a conventional method relying on the mixture or redistribution of the two colors was more time consuming, because each cell has to be scored for the presence of either or both colors.

The previously described fusion-inefficient mutant Env that carries the mutation in the membrane-spanning domain (MSD) (Fig. 1C, lower panel) (Miyauchi et al., 2005) was also analyzed. Consistent with previous data (Miyauchi et al., 2005), the MSD mutants showed the fusion events but less frequently, as exemplified by the lower number of cells bearing the green signal (Fig. 4C).

3.3.3. Real-time membrane fusion assay

The membrane fusion in a real-time manner using spGFP system was monitored with an IN Cell Analyzer 1000. The green signal derived from the reassociated spGFPs gradually increased in the number and intensity over the observation period (Fig. 4D). When T7 RNA polymerase transfer assay was applied (Miyauchi et al., 2005), a corresponding increase in the reading of the reporter enzyme was observed (data not shown). Using the transient transfection system, we performed several tests to determine the timing needed to detect the green signal. Sometimes the signal was detected as early as 30 min after co-culturing. A more reliable result, however, was obtained after more than 1 h co-cultivation.

4. Discussion

A cell-based assay system of membrane fusion that uses spGFP has been developed. It was found that the PH domain not only localizes the signal resulting from the spGFPs reassociated to the membrane but also aids the stable expression of GFP_{11}.

The new spGFP-mediated system is cost- and labor-efficient. First, the same living co-cultured cells can be monitored for real-time monitoring over a prolonged period (Fig. 4D). Second, the reassociated spGFPs will produce a measurable signal without any additional reagents. The dye-mediated fusion assay requires preloading of dyes before the fusion reaction (Blumenthal et al., 2002). A quantitative fusion assay using enzymes, either as pre-expressed self-complementing enzyme fragments or as induced reporter enzymes, requires the addition of enzyme substrates to monitor the processes (Cavrois et al., 2002; Holland et al., 2004; Jun and Wickner, 2007). Furthermore, if a particular substrate is membrane impermeable, continuous monitoring of the same sample is impossible because the cells have to be lysed for the assay.

Simple assay systems described in this study are suitable for high-throughput analyses. The combination of dye-transfer and fluorescence-activated cell sorting can achieve this (Huerta et al., 2002; Lin et al., 2003). However, the system reported in this study is much simpler than these methods because it generates the detectable signal only when fusion actually occurs. In the dye-transfer assay or similar "color"-mixing assay shown in Fig. 4B, one has to discriminate the simple aggregation from real fusion because the dye signals are persistent throughout the assay.

As shown in the Fig. 4C, the lower incidence of membrane fusion induced by mutant Envs was detected as visible foci with the spGFP system. If one can clone the envelope genes from clinical samples into an appropriate expression vector, this system may be useful for detecting a minor population of Env that possesses the different co-receptor usage. Such a tropism assay can be easily adapted by using CCR5/CD4+ cells together with CXCR4/CD4+ cells. Similar identification of fusion foci can be achieved if GFP is used as a reporter gene in a transcriptional factor transfer assay, such as T7 RNA polymerase or Tat (Barbeau et al., 1998; Feng et al., 1996; Lin et al., 2005; Sakamoto et al., 2003), but the need for de novo transcription/translation steps may result in a longer lag time for signal generation. Of course, membrane fusion by viruses other than HIV-1 can be monitored easily.

This spGFP-based system does not require on-going transcription/translation steps during membrane fusion. Therefore, when the tag for a different intracellular compartments is applied, the system can be used to detect communication between two compartments in the cell, such as that which occurs in vesicular transport. The application of the spGFP in other biological systems has been described previously (Feinberg et al., 2008).

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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

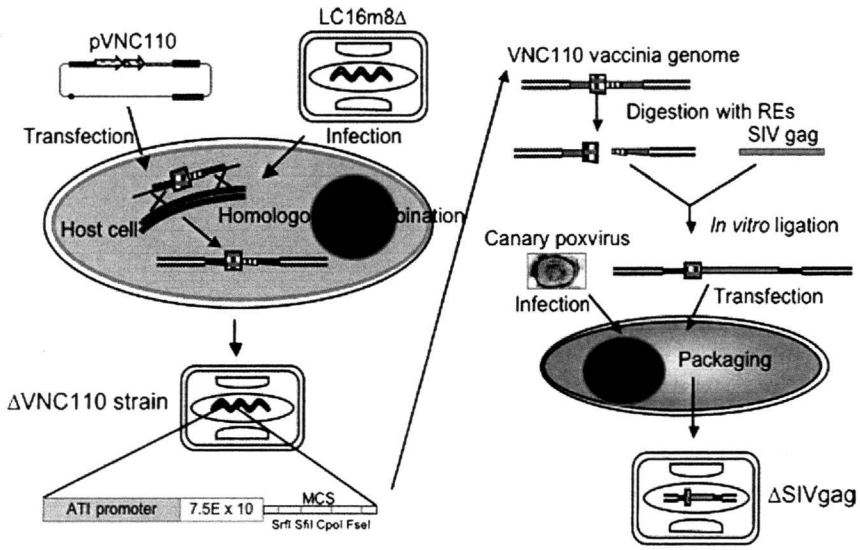

Fig. 1. Schematic presentation for construction of m8ΔSIVgag.

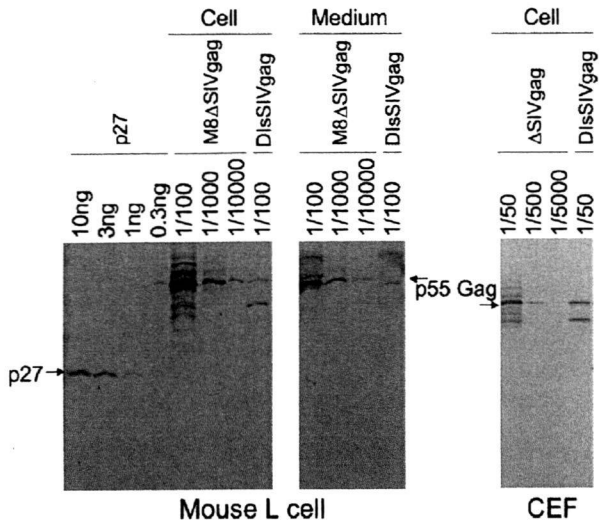

Fig. 2. Western blotting for p55 Gag produced by vaccinia recombinants. Appropriate fractions (1/50–1/10,000) of the cell lysates and medium prepared from m8ΔSIVgag- or rDIs/PSFJ/SIVgag-infected cells were subjected to Western blotting. One hundredth of the cell lysates contains approximately 1 μg of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

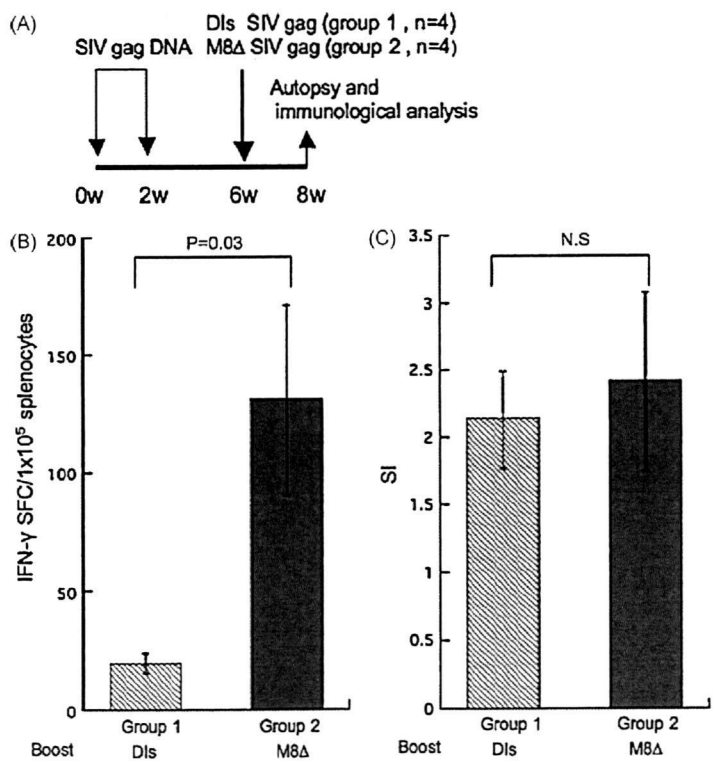

Fig. 3. Comparison of booster effect by m8ΔSIVgag and rDIs/PSFJ/SIVgag. (A) Schematic drawing of experimental design for immunization. Mice were immunized twice with SIVgag DNA followed by one boost with rDIs/PSFJ/SIVgag (group 1) or m8ΔSIVgag (group 2). (B) Frequency of SIV Gag-specific IFN-γ-producing 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.

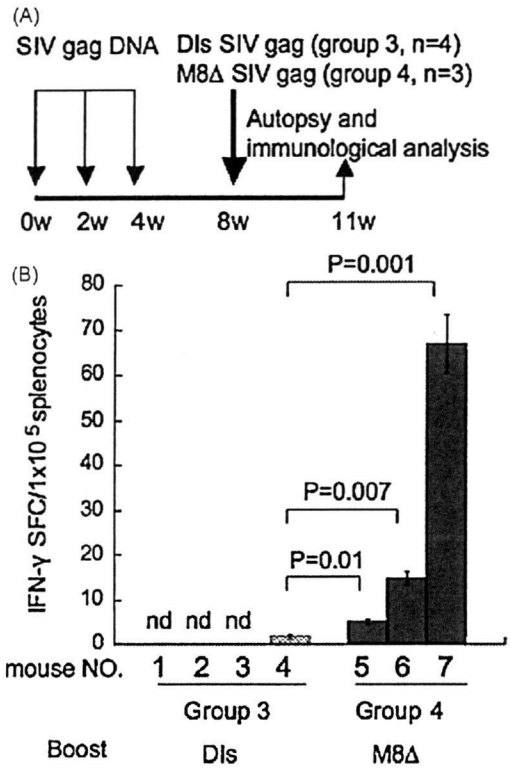

Fig. 4. Comparison of immunogenicities of m8ΔSIVgag and rDIs/PSFJ/SIVgag. (A) Schematic drawing of experimental design for immunization. Mice were immunized three times with SIVgag DNA followed by one boost with rDIs/PSFJ/SIVgag (group 3) or m8ΔSIVgag (group 4). (B) Frequencies of SIV Gag-specific IFN-γ-producing 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

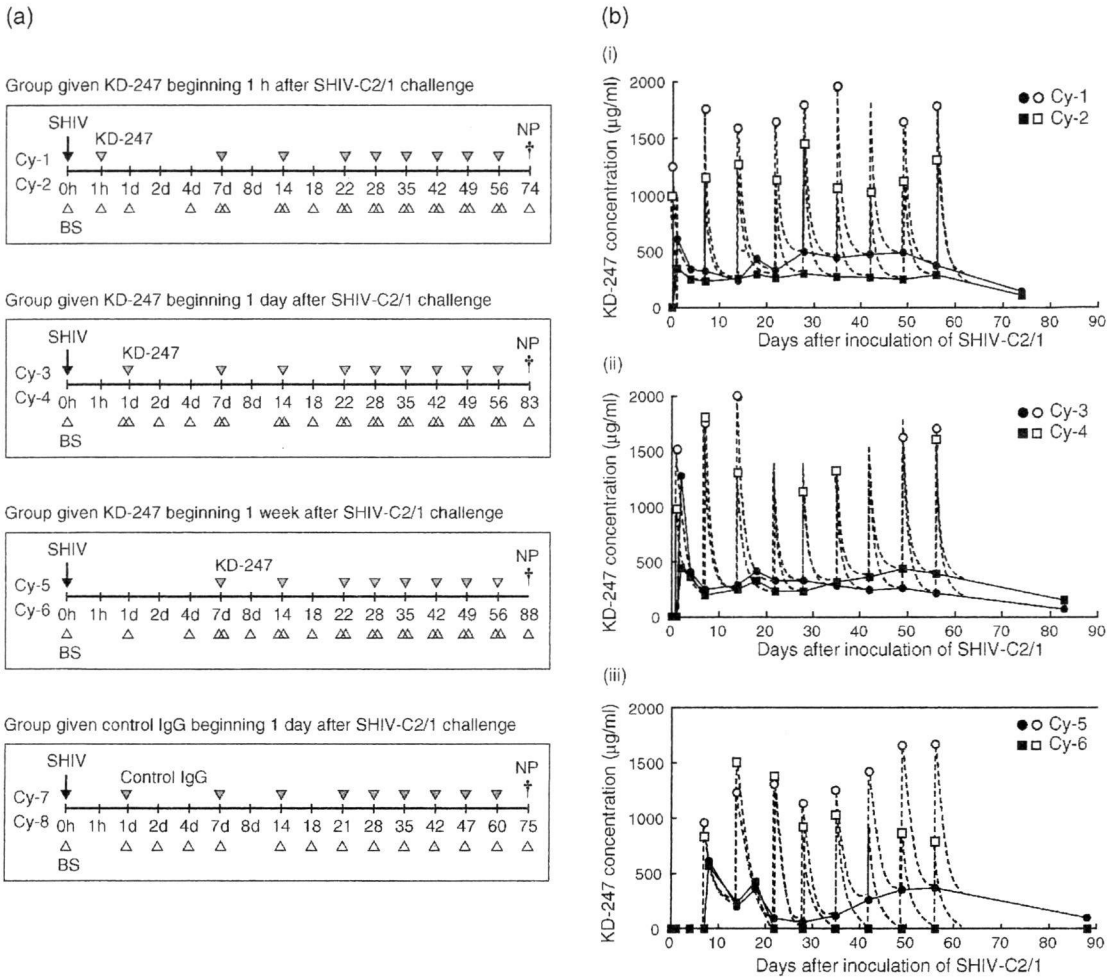

Fig. 1. KD-247 administration schedules and pharmacokinetic profiles. (a) Scheme of viral challenge and postinfection passive immunization with KD-247. A total of eight cynomolgus monkeys were used for viral infection studies with highly pathogenic SHIV-C2/1 (20 $TCID_{50}$). In the first group of two monkeys, 45 mg/kg of KD-247 was intravenously administered at 1 h after viral challenge and antibody administered once per week for 2 months. Monkeys in the second and third groups were injected with antibody at 1 day and 1 week after viral challenge, respectively, and similarly treated with the antibody. Monkeys in the fourth group were injected with 45 mg/kg of human normal immunoglobulin fraction at 1 day after viral challenge followed by passive transfer of this control IgG once a week for 2 months. Two other monkeys were used as positive controls for infection by SHIV-C2/1 with 20 $TCID_{50}$ without passive transfer of antibody, whereas 15 other monkeys were used as naive controls without viral infection and antibody transfer. (b) KD-247 concentrations in the plasma of monkeys treated 1 h (i), 1 day (ii), or 1 week (iii) after SHIV-C2/1 challenge. Closed and open symbols indicate KD-247 concentrations in plasma collected immediately before and 15 min after administration of KD-247, respectively. Broken lines show the estimated changes in KD-247 concentrations. BS, blood sampling; NP, necropsy; SHIV-C2/1, simian/human immunodeficiency virus C2/1; $TCID_{50}$, 50% tissue culture infective dose.

stopped. The plates were measured for optical density at 450 nm with a precision microplate reader (Emax; Molecular Devices, Menlo Park, California, USA). The concentrations of KD-247 antibody in the plasma were evaluated from a calibration curve drawn with software developed for the reader (SOFTmax; Molecular Devices).

Detection of anti-KD-247 antibodies

Anti-KD-247 antibodies in plasma were detected using 96-well ELISA plates (MaxiSorp) coated with KD-247. After washing and blocking, samples containing test monkey plasma at 1 : 400 dilution or a positive control were then added and incubated. A positive control was pooled with rabbit anti-KD-247 plasma at 1 : 4000 dilution. The wells were washed, incubated with biotinylated KD-247, and then washed again. Peroxidase-conjugated streptavidin (Sigma Chemical, St. Louis, Missouri, USA) was diluted and added to the wells for reaction.

Real-time reverse transcriptase-PCR quantitation of simian/human immunodeficiency virus RNA in plasma

Plasma viral loads were evaluated by real-time reverse transcriptase PCR (RT-PCR) as described previously