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Inhibition of HIV replication by a CD4-reactive Fab of an IgM clone isolated from a healthy HIV-seronegative individual

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HIV replication is restricted by some anti-CD4 mouse mAb *in vitro* and *in vivo*. However, a human monoclonal anti-CD4 Ab has not been isolated. We screened EBV-transformed peripheral B cells from 12 adult donors for CD4-reactive Ab production followed by functional reconstitution of Fab genes. Three independent IgM Fab clones reactive specifically to CD4 were isolated from a healthy HIV-seronegative adult (~0.0013% of the peripheral B cells). The germ line combinations for the V_H and V_L genes were V_H3-33/$_L$6, V_H3-33/$_L$12, and V_H4-4/$_L$12, respectively, accompanied by somatic hypermutations. Genetic analysis revealed a preference for V-gene usage to develop CD4-reactive Ab. Notably, one of the CD4-reactive clones, HO538-213, with an 1×10^{-8} M dissociation constant (Kd) to recombinant human CD4, limited the replication of R5-tropic and X4-tropic HIV-1 strains at 1–2.5 μg/mL in primary mononuclear cells. This is the first clonal genetic analysis of human monoclonal CD4-reactive Ab. A mAb against CD4 isolated from a healthy individual could be useful in the intervention of HIV/AIDS.

Key words: Autoimmunity · CD4-reactive Ab · IgM · Inhibition of HIV replication

Supporting Information available online

Introduction

CD4 is a T-cell marker that serves as a principal receptor for HIV. CD4-reactive Ab are detected in HIV-infected individuals (~13%) [1, 2] and HIV-exposed seronegative individuals (34%) [3]. In addition, some healthy individuals are positive for anti-CD4 Ab (~0.6%) [4]. Replication of multiple HIV clades is blocked by mouse mAb against CD4 *in vitro* and *in vivo* [5–12]. Thus, it is possible that anti-CD4 Ab play a role in protecting individuals from HIV infection and delaying AIDS disease progression. Similar arguments have been made for Ab against CCR5, a coreceptor for HIV [3, 10, 13]. Furthermore, some clinical studies suggest that CD4-reactive Ab, including a humanized mAb, has therapeutic potential against HIV infection and AIDS progression [5, 8, 10, 12]. However, the development and pathophysiological roles of self-recognizing Ab in healthy individuals are still largely unknown, and a human mAb against CD4 has not yet been isolated.

To gain insights into the genesis of auto-reactive Ab and to characterize the nature of CD4-reactive auto-Ab, we conducted experiments to isolate human monoclonal anti-CD4 Ab from PBMC of 12 HIV-seronegative adult donors. We succeeded in isolating

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three independent IgM clones recognizing CD4 from a healthy donor. Analysis of the V-region sequences of CD4-reactive Ab revealed a preference for V gene usage to give rise to CD4-reactive Ab. This is the first report describing CD4-reactive human mAb.

Results and discussion

Isolation of CD4-reactive IgM clones from a healthy individual

PBMC were collected from 12 HIV-seronegative adult volunteers, including two healthy and ten with autoimmune disorders, and B-lymphoblastoid cell lines (B-LCL) were established by infecting the cells with EBV (for experimental procedure, see Supporting Information Fig. 1). B-LCL were propagated in oligoclonal pools. In 790 cultures from one healthy donor, we identified two cultures positive for recombinant human CD4 (rhCD4) reactivity, HO538 and HO702, using ELISA (Fig. 1A). This donor may have a unique Ab repertoire, as auto-reactive B-LCL cultures were identified significantly more frequently in this donor than in the others (Fig. 1A). The rhCD4 reactivity was specific, as no binding was observed to 72 other viral, bacterial, and auto-Ag screened in parallel (Supporting Information Fig. 2). We amplified the Ig genes encoding the Fab regions by RT-PCR and cloned them into the bacterial expression vector pFabI-His2 that produces Fab fragments of an inserted set of V_H and V_L genes. We expected that some clones should reconstitute the CD4-reactive Fab present in the original B-LCL cultures. After screening by ELISA, one CD4-reactive Fab clone, HO538-213, was isolated from the HO538 culture, and two independent clones, HO702-001 and HO702-016, were isolated from the HO702 culture. These Fab clones originated from IgM, as determined by the sequence analysis. The estimated efficiency of peripheral B cells producing CD4-reactive Ab was ~0.0013% (three clones/2.4×10^5 estimated screened B cells × 100 (%), given that the B cells compose 10% of PBMC and that EBV immortalization is 30% efficient on average) [14]. According to the ELISA data, the Fab concentrations that yielded 50% maximal binding were ~8 μg/mL for HO538-213, and ~1 μg/mL for HO702-001 and HO702-016 (Fig. 1B). Consistent with these data, the BIACORE assay revealed that the dissociation constant (Kd) of HO538-213, HO702-001, and HO702-016 to rhCD4 was 6.5×10^{-8}, 7.7×10^{-9}, and 2.7×10^{-9} M, respectively (Fig. 1C), which is relatively weak compared with average Ab–Ag interactions (*e.g.* the Kd of mouse mAb Leu-3a to rhCD4 is 2.2×10^{-10} M).

Genetic analysis of CD4-reactive IgM clones

The Fab sequences were analyzed by the Kabat database (http://www.ncbi.nlm.nih.gov/igblast/) in GenBank, as previously described [15, 16]. The Ig gene family of each gene and the most homologous germline are indicated (Fig. 2A). All the three clones were of the IgM class and had a κ-chain for V_L. Comparison of the

A

Donor	Number of tested cultures	CD4	Nuclear antigen	Blood type i-glycolipid
Healthy 1	790	2 (0.3%)*	21 (2.6%)*	28 (3.5%)*
Healthy 2	160	0	0	0
Autoimmune (10 individuals)	435	0	3 (0.7%)	0

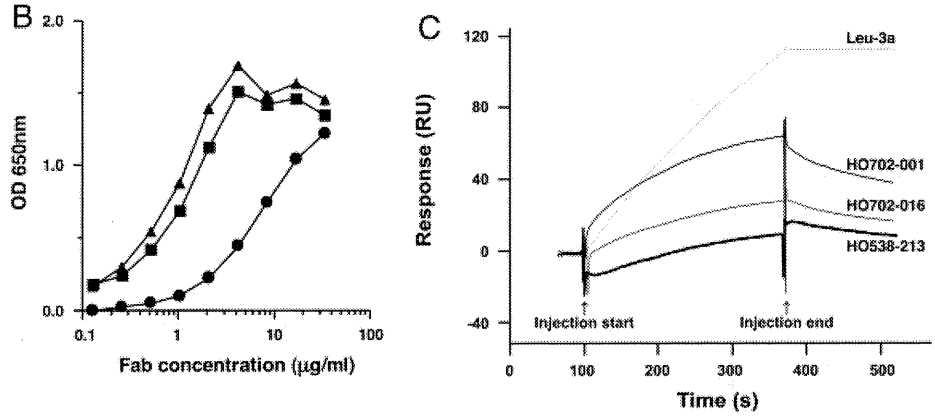

Figure 1. Isolation and characterization of healthy human-derived CD4-reactive Ab. (A) Summary of the frequency of B-LCL cultures that reacted with representative auto-Ag. The number of cultures positive for rhCD4 reactivity, HeLa cell nuclear staining, and blood type i-glycolipid are shown. *$p<0.05$, compared with other donor groups, Fisher's exact test. (B) CD4-binding kinetics of CD4-reactive IgM Fab. Serial dilutions of HO538-213 (circles), HO702-001 (triangles), and HO702-016 (squares) were incubated in microtiter plates pre-coated with rhCD4. (C) Surface plasmon resonance analysis of CD4-reactive IgM FAb HO702-001 (black), HO702-016 (dark gray), HO538-213 (bold), and mAb Leu-3a (gray) binding to immobilized rhCD4. The concentration of Ab was 0.3 μg/mL, flow rate 20 μL/min, and reaction time 270 s. RU, resonance units.

heavy chain with the germlines revealed that the μ-chains of HO538-213 and HO702-001 were 95 and 97% homologous to germ line V_H3-33, respectively, while HO702-016 was 96% homologous to germline V_H4-4 [17]. For the light chains, the κ-chain Vkappa3 of HO538-213 was 97% homologous to germline L6 [6, 18, 19], and κ-chain Vkappa1 of both HO702-001 and HO702-016 was 97% homologous to the germline L12 [6, 18, 19]. These data suggest that there is a preferential use of V_H and V_L genes to develop CD4-reactive Ab, considering the number of V_H and V_L genes present before the Ig gene rearrangement. According to the sequence analysis, the V_H amino acid sequences of HO538-213 and HO702-001 carried distinct mutations, although both were derived from the same germline V_H3-33. The mutations were more frequent in the CDR regions (Fig. 2B and C, Supporting Information Fig. 3), which is characteristic of somatic hypermutation (SHM) associated with affinity maturation. Unlike most SHM, however, mutations involving G/C were not dominant.

A

Clone	Ig class	VH family	Closest germline	% identity	VL family	Closest germline	% identity
HO538-213	IgM	VH 3	VH3-33	95%	Vk3	L6	97%
HO702-001	IgM	VH 3	VH3-33	97%	Vk-1	L12	97%
HO702-016	IgM	VH 4	VH4-4	98%	Vk-1	L12	97%

B

Clone			Number of mutations associated with: A	G	C	T	ins[1]	del[2]	GC involvement (%)[3]	Total	aa changes[4]	(%)
HO702-001	heavy chain	FR	3	2	1	0	0	0	66.6	6	2	33.3
		CDR	0	0	0	0	5	0	0.0	5	0	0.0
	light chain	FR	0	0	0	2	0	0	0.0	2	1	50.0
		CDR	1	0	0	0	6	0	0.0	7	0	0.0
		Total	4	2	1	2	11	0	33.3	20	3	15.0
HO702-016	heavy chain	FR	1	0	0	0	0	0	0.0	1	0	0.0
		CDR	0	2	1	0	11	0	100.0	5	0	0.0
	light chain	FR	0	0	0	2	0	0	0.0	2	1	50.0
		CDR	1	0	0	0	6	0	0.0	7	0	0.0
		Total	2	2	1	2	17	0	42.9	24	1	4.2
HO538-213	heavy chain	FR	2	1	1	4	0	0	25.0	8	3	37.5
		CDR	2	2	5	1	15	0	70.0	25	5	20.0
	light chain	FR	1	3	1	0	0	0	75.0	4	3	75.0
		CDR	3	1	0	0	2	0	28.6	6	3	50.0
		Total	8	6	7	6	17	0	58.6	43	13	30.2

C

Light chain

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                    FR1                  CDR1          FR2           CDR2
HO538-213   EIVMTQSPATLSLSPGERATLSC  RASQSVNNYLA  WYQQRPGQAPSLLIY  DASNRAT
HO702-001/016 D-QL----S----A-V-D-V-IT-  -------ISSN--  -----K---K-----  K---SLES

                    FR3                  CDR3          FR4
HO538-213   GIPARFSGSGSGTDFTLTISSLEPEDFAVYYC  QQ.RG.NWPHT  FGQGTKLEIKRT
HO702-001/016 -V-S----------E----------Q-D----Y----  --YNSYSRTY-  ----------
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Heavy chain

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                    FR1                  CDR1          FR2           CDR2
HO538-213   QVQLVQSGGGVVQPGRSLRLSCA.ASG.FTFS  SYGMH  WVRQAPGKGLEWVA  VVCYDGRTTYYADSVKG
HO702-001   -----QEW-----------------------  ------  --------------  -IN----SNK-------
HO702-016   -----QE---P-L-K-SQT-S-T-TVSG-SISSG  --YWS  -I---PA-------IG  .RI-YSGS-N-NP-L-S

                    FR3                  CDR3          FR4
HO538-213   RFTISRDISENTLYLQMNSLRAEDTAVYYCAK  DLNKGYCNNGGCYG.VDC  WGQGTLVTVSS
HO702-001   ---------N-K-----------------R  -...RD.-SG-S---SWF-P  ----------
HO702-016   -V--SV-T-K-QFS-KLS-VT-A-------R  T..PTL.GR-L.F...Y  ----------
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Figure 2. Genetic analysis of CD4-reactive Ab. (A) Summary of the Ig class, V-gene family, closest germ line, and percentage identity of the closest germ line of CD4-reactive Ab. (B) The mutation profiles of the CD4-reactive IgM Fab fragments. (C) The deduced protein sequences of the V_H and V_L genes of the CD4-reactive Fab fragments are aligned. FR, framework region. Dashes and dots indicate identical residues and deletions, respectively. See Supporting Information Fig. 2 for further detail.

Inhibition of HIV replication by a Fab fragment of a CD4-reactive IgM

We next examined the potential impact of these CD4-reactive Fab Ab on HIV replication. Viral replication was monitored in PBMC by measuring $p24^{CA}$ viral Ag levels in the culture supernatant. Among the three IgM Fab clones, HO538-213 suppressed R5-tropic virus HIV-1_{JR-FL} replication by 3.5±1.5-fold at 1–2.5 μg/mL (average±SD from four independent experiments, Fig. 3A). There was a modest but consistent suppression of X4-tropic virus HIV-1_{HXB2} replication (1.4±0.2-fold, average±SD from three independent experiments). BIACORE and ELISA revealed that HO538-213 did not compete with the anti-CD4 mAb Leu-3a [20, 21] for CD4 binding. Leu-3a restricts HIV-1 replication by physically blocking the *Env*-CD4 interaction (data not shown), suggesting that the epitope recognized by HO538-213 is distinct from the *Env*-interacting domain of CD4 [7, 22, 23]. The monoclonal anti-CD4 Ab OKT4a does not block the *Env*-CD4 interaction, but restricts HIV-1 infection, although decreasing CD4 lateral diffusion on the cell surface [24–26]. We hypothesized that HO538-213 may have a similar mechanism of action. CD4 localizes to lipid rafts, and CD4-crosslinking activates signal transduction involving tyrosine kinases [27–29]. Thus, we treated MOLT-4 cells with HO538-213, and the lipid raft fraction was isolated by a membrane floatation assay as verified by the raft markers glycosphingomyelin 1 and sphingomyelin (Fig. 3B, left panel). Tyrosine kinase activitiy was examined by immunoblotting the lipid raft fractions using a PY20 anti-phosphotyrosine mAb (Fig. 3B, right panel, arrowhead). We detected a significant amount of tyrosine phosphorylation in the lipid raft fraction after HO538-213 treatment, indicating that HO538-213 can assemble cell surface CD4. This is consistent with our hypothesis that HO538-213 inhibits HIV-1 infection by decreasing the lateral movement of cell surface CD4.

Does CD4-reactive IgM function as a natural HIV resistance factor?

We then further characterized the donor from which the CD4-reactive Ab was isolated. The donor serum did not show a strong reactivity to rhCD4 at 1:10 dilution, where the non-specific effect was no longer detected. We analyzed the HIV-inhibition titer of the donor plasma. In a TZM-bl cell assay, the plasma did not block HIV replication at 1:50 dilution (data not shown). These data suggest that the CD4-reactive IgM circulates at very low titers in the donor and may not be sufficient to block HIV infection *in vitro*. However, it is possible that the CD4-reactive IgM may be able to limit HIV-1 propagation under *in vivo* conditions.

We next investigated the immunological status of the donor. IgG and IgM levels were within the normal range, and the plasma was negative for rheumatoid factor, anti-DNA, and anti-ribonucleoprotein Ab. However, the donor serum reacted to nuclear Ag at a titer of 1:160 (1:40 or less is considered normal), and the staining patterns were nucleolar (1:160) and speckled (1:80). Consistent with these data, the frequency of auto-reactive

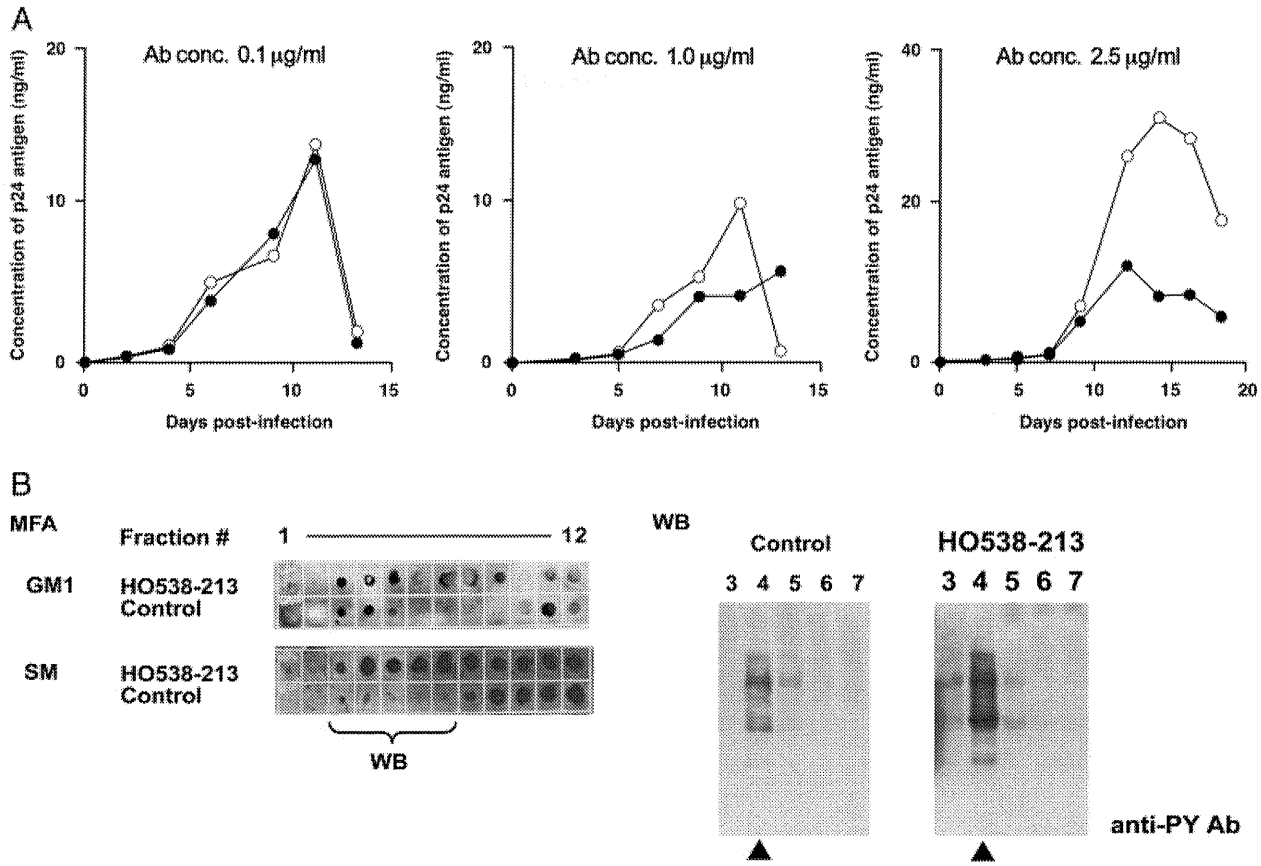

Figure 3. The effect of CD4-reactive Fab clone HO538-213 on HIV-1 replication. (A) The Fab clone HO538-213 (filled circles) was tested for its ability to inhibit HIV-1_{JR-FL} replication at a concentration of 0.1 (left), 1.0 (middle), and 2.5 (right) μg/mL. The CD4 non-reactive Fab clone 13-3 (open circles) was used as a negative control. Representative data from four independent experiments are shown. (B) Activation of the tyrosine kinase signaling cascade by HO538-213 in MOLT-4 cells. The detergent-resistant membrane fraction (arrowhead) was isolated by a membrane floatation assay (MFA) from MOLT-4 cells treated with HO538-213, and phosphotyrosine levels were examined by immunoblotting. GM1, glycosphingomyclin 1; SM, sphingomyelin.

Ab-producing cells from the same donor, namely against nuclear Ag and blood group i-glycolipid, was significantly higher than the other donors (Fig. 1A). In addition, we isolated anti-TNF-α IgG and IgM clones from this donor [16]. Although clinical manifestations of autoimmune disorders were lacking, it is likely that the donor may have an immunological background that generates auto-reactive Ab and tolerates them. Moreover, the donor has been healthy for 29 years, at the time the CD4-reactive Ab was first isolated, suggesting that such CD4-reactive Ab may not disturb host immunity.

Considering that the IgM-producing B cells we isolated went through positive/negative selection, their original target should not be CD4. It is thus likely that the IgM genes accumulated SHM that resulted in cross-reactivity to CD4 in the periphery after B-cell maturation. To better understand the unique immunological features of individuals with CD4-reactive Ab and their auto-reactive Ab repertoire, more human monoclonal self-reactive Ab are needed to analyze both their V-region sequences and cross-reactivities. Our experimental approach might be useful for addressing these issues. Unfortunately, however, we were unable to characterize the CD4-reactive Ab-producing cells, as the oligoclonal cultures of B-LCL were terminated after RNA extraction for our Ig gene cloning strategy. We speculate that B-1 cells could be the source of the CD4-reactive Ab, because B-1 cells produce IgM that often cross-reacts with auto-Ag.

Our genetic data indicated that only a fraction of the CD4-reactive Ab could have some HIV-inhibitory function. It is an open question whether such CD4-reactive HIV-inhibitory Ab may be present in the other healthy individuals, as well as in HIV-seropositive long-term non-progressors.

HIV-inhibitory CD4-reactive Ab are effective against multiple HIV clades, as CD4 is the major HIV receptor for all the viral clades [11]. A clinical trial is being conducted to examine the therapeutic efficacy of a humanized CD4-reactive mAb in patients with HIV infection [8, 12]. Although CD4-reactive Ab can be detected in healthy individuals, safety is always a concern when using self-recognizing Ab as therapeutic drugs. Given that HO538-213 was isolated from a healthy individual and that it recognized a different epitope than Leu-3a, HO538-213 might effectively inhibit HIV without disturbing $CD4^+$ T-cell functions. As noted above, the donor from which the three CD4-reactive IgM Fab were isolated has been healthy for more than 29 years since PBMC collection, suggesting that these Ab may not seriously inhibit $CD4^+$ T-cell functions *in vivo* and thus may be useful in treating HIV infection and other disorders [4].

Concluding remarks

This report provides the first clonal genetic analyses of human monoclonal anti-CD4 Ab. IgM is considered to function in "natural humoral immunity", as it has a relatively low affinity for pathogens and confers natural resistance to infectious agents. However, the pathogen-specific immunity function of IgM has not been demonstrated at a clonal level. Our data suggest that CD4-reactive IgM is present in healthy individuals and can contribute to natural resistance to HIV infection and AIDS progression. This is the first clear demonstration of a natural humoral immunity function of IgM against HIV.

Materials and methods

Functional cloning of heavy and light chain Ab genes

The establishment of Ab-producing cells, cloning of Ig genes encoding V regions, ELISA, and the purification of Fab fragments from *Escherichia coli* have been described previously [16]. The experimental procedure is schematically shown in the Supporting Information Fig. 1. In brief, PBMC from 12 donors, including two healthy individuals and ten individuals with autoimmune disorders, were infected with the B95-8 strain of EBV, and 1×10^4 cells were propagated in 96-well plates. The supernatant was analyzed by ELISA using rhCD4 derived from a baculovirus system (50 ng/well; INTRACELL) as an Ag. Other Ag tested, including viral, bacterial, and auto-Ag, are listed in the Supporting Information Fig. 2. Total cellular RNA was isolated from oligoclonal cell populations positive for anti-CD4 Ab production (RNeasy mini kit, Qiagen). cDNAs were synthesized and amplified by PCR with specific primers for human Ig μ-, γ-, λ-, and κ-chains. Only the μ- and κ-chains were amplified from HO538 and HO702 cultures and cloned into the pFab1-His2 vector, generating bacterial Fab-expression libraries [30]. The pFab libraries were screened for the production of CD4-reactive Fab by ELISA. The Fab fragments were purified using an anti-Fab Ab affinity column. The eluted Fab was dialyzed against PBS and concentrated by centrifugation (VIVASPIN concentrator, Vivascience AG). The purity of the Fab Ab was greater than 95% as determined by SDS-PAGE analysis (data not shown).

Surface plasmon resonance biosensor analysis

Surface plasmon resonance analyses were performed using BIACORE 3000 (GE Healthcare). The hrCD4 was immobilized onto CM5 sensor chips using standard amine-coupling chemistry. The purified Fab was diluted in a running buffer (10 mM HEPES, 0.15 M NaCL, 3 mM EDTA, surfactant P 20, pH 7.4) to 0.3–20 μg/mL and injected at a rate of 20–30 μL/min. The Fab was allowed to associate and dissociate for 120–270 s.

Cells

B-LCL and 293 T cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) supplemented with 10% fetal bovine serum (Japan Bioserum), penicillin, and streptomycin (Invitrogen). The primary mononuclear cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, 5 μg/mL plasmocin (InvivoGen), 10 mM HEPES, 5 μg/mL anti-CD3 mAb (OKT3, Janssen Pharmaceutical), 70 U/mL recombinant human IL-2 (Shionogi Pharmaceutical), GlutaMax-I (Invitrogen), insulin–transferrin–selenium-A (Invitrogen), and 10 mM HEPES (Invitrogen). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Other experimental procedures

Procedures for monitoring HIV-1 replication [31] and membrane floatation assays [32] were described previously. Standard auto-Ab was tested by the clinical laboratory testing service SRL (Tokyo, Japan).

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Abbreviations: **B-LCL:** B-lymphoblastoid cell lines · **rhCD4:** recombinant human CD4 · **SHM:** somatic hypermutation

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T cell-based functional cDNA library screening identified SEC14-like 1a carboxy-terminal domain as a negative regulator of human immunodeficiency virus replication

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ABSTRACT

Genome-wide screening of host factors that regulate HIV-1 replication has been attempted using numerous experimental approaches. However, there has been limited success using T cell-based cDNA library screening to identify genes that regulate HIV-1 replication. We have established a genetic screening strategy using the human T cell line MT-4 and a replication-competent HIV-1. With this system, we identified the C-terminal domain (CTD) of SEC14-like 1a (SEC14L1a) as a novel inhibitor of HIV-1 replication. Our T cell-based cDNA screening system provides an alternative tool for identifying novel regulators of HIV-1 replication.

1. Introduction

The molecular interaction between HIV-1 and the host is not fully understood. A systematic genome-wide approach provides the critical information for the completion of the HIV-1-host interactome. Many experimental genome-wide screening systems have been established to identify the cellular genes required for HIV-1 replication (Table 1, [1–8]). More than a hundred genes have been identified as being cellular factors that regulate HIV-1 replication. However, different screening systems do not identify the same set of genes, and many systems yielded non-overlapping candidates. These discrepancies are assumed to be due to differences in the experimental approaches, such as the virus, the cell line, or the genetic materials used.

For viruses, the wild-type HIV-1 [1,3–6] or a replication-incompetent HIV-1 pseudotyped with vesicular stomatitis virus (VSV)-G is used [2,7,8]. The VSV-G-pseudotyped "HIV-1-based vector" has been used to identify factors associated with the viral entry processes. However, in reality, it covers the events from post-membrane fusion to translation. One of the potential caveats in the use of the VSV-G-pseudotyped vector is that it enters cells via the VSV-G-restricted route, which is fundamentally different from the HIV-1 *Env*-mediated entry pathway [9–12]. The replication-competent HIV-1 should be ideal to cover the entire viral replication cycle; however, this may raise biosafety concerns.

For cells, non-T cells, such as a genetically engineered HeLa cells that ectopically express luciferase or beta-galactosidase (TZM-bl cells), are often used, since they are efficiently transduced with genetic materials [2,5–8]. Not many studies employ a T cell-based system, partly because genetic materials are not efficiently transduced into T cells [1,3,4]. To identify HIV-1 replication regulatory factors, it is preferable to perform the functional analysis in the natural targets of HIV-1 including T cells. The gene expression profile of non-T cells is apparently different from that of T cells as exemplified by the absence of T cell specific markers on non-T cells such as CD4. It is possible that a candidate gene isolated in the non-T cell-based system might not be expressed in T cells. It is impossible to identify T cell-specific factors in the non-T cell-based screening using the siRNA library or in the screening using cDNA libraries derived from non-T cells. Also, the effect or functions of some genes may not be identical in distinct cell types. The potential risk of a non-T cell-based assay is that we may falsely score a gene as a regulator of HIV-1 replication, although many genes have been discovered using non-T cell-based screening systems including the viral receptors. Ideally, the primary CD4-positive T cells, dendritic cells, macrophages, or NK/T cells should be used.

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Table 1
Summary of genome-wide screening strategies to identify regulatory factors of HIV-1 replication.

Genetic material	Transduction approach	Cell line	Replication competency of HIV-1	Reference
cDNA library	Retroviral, stable	TE671	Incompetent	[2,8]
siRNA library	Transfection, transient	HeLa or 293T	Competent or incompetent	[5,6,7]
cDNA library	Lenti- or retroviral, stable	MT-4	Competent	[1,3,4]

Given technical limitations, this is currently unrealistic for genetic screening experiments.

As for the genetic material, cDNA libraries are often used [1–4,8]. Recent studies utilized siRNA libraries [5–7]. The cDNA approach is advantageous for providing genetic diversity. Expression of the full-length open reading frame of a gene can upregulate the function of the gene, whereas cDNA fragments can function in a diverse fashion. The gene silencing approach downregulates gene expression; however, the silencing efficiency of a gene varies in different cell types and at different time points in the assay (reviewed in [13]). As mentioned above, the gene silencing approach is unable to score the contribution of genes that are not expressed in the cells used in the assay.

The screening can be performed in cells that are either transiently [5–7] or stably [1–4,8] transduced with genetic materials. In the transient transfection assays, it is possible that the dysregulation of a gene function can damage the physiology of the cells. In such a case, the inhibition of HIV-1 replication can be observed, but may not be a direct inhibitory effect of the gene of interest. Such a risk can be minimized by using cells stably transduced with the genetic materials.

We conducted a phenotype cDNA screen using a T cell line-based assay to identify cellular genes that render cells resistant to HIV-1 replication [3]. The advantage of our functional screening system is that cDNA libraries are stably transduced into cells, and that a replication-competent HIV-1 and a human T cell line MT-4 are used. With this system, we have successfully identified the SEC14-like 1a (SEC14L1a) C-terminal domain (CTD) as an inhibitor of HIV-1 replication that targets the late phase of the viral life cycle.

2. Materials and methods

2.1. Cells, transfection, cDNA selection

Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Tokyo, Japan). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The method of selecting human cDNAs that confer resistance to HIV-1 has been described previously in detail [1,3].

2.2. Plasmids

The SEC14L1a CTD1 was amplified from MT-4 polyA RNA by reverse transcriptase PCR (RT-PCR) using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACCCTGCGTGCGCCGCCAGCAGC-3′ and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-3'. The SEC14L1a CTD2 was amplified by PCR from human lymph node cDNA library (Takara, Otsu, Japan) using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACTGCGAAGTGCCAGAGGGTGGAC-3' and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-3'. Full length (FL) SEC14L1a was amplified by PCR from a plasmid containing the SEC14L1a open reading frame (ORF, CS0DL004YN18, Invitrogen), using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACGTGCAGAAATACCAGTCCCCAG-3' and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-3'. The AgeI-MfeI fragments of the PCR products were cloned into the XmaI-MfeI sites of the pEGFP-C3 plasmid (Clontech, Palo Alto, CA), generating pEGFP-SEC14L1a-CTD1, -CTD2, and -FL. The XhoI-MfeI fragments from the resulting plasmids were cloned into the corresponding restriction sites of the pCMMP KRAB vector, creating pCMMP GFP-SEC14L1a-CTD1, -CTD2, and -FL. The HIV-1 *tat* was amplified by PCR using the primers 5′-AACCGGTCTCGAGCCACCATGGAGCCAGTAGATCCTAGAC-3′ and 5′-GGATCCTCAGTCGTCATCGTCTTTGTAGTCTTCCTTCGGGCCTGTCGGGTC-3′. A Tat expression vector pCMMP Tat was constructed by cloning the AgeI-BamHI fragment of the PCR product into the corresponding restriction sites of the pCMMP KRAB vector. The HIV-1 *Env* and GFP expression vectors (pIIIex and pCMMP GFP, respectively) are described previously [3,12,14]. To construct the pCMMP GFP-FLAG (GFPf), pCMMP CXCR4 d-10 [15] was digested with AgeI and XhoI to remove CXCR4 d-10 ORF and self-ligated after blunting with T4 DNA polymerase. The HIV-1 *gag-pol*, *tat*, and *rev* expressing plasmid pCMVR8.91 was a generous gift from Dr. Trono's group [16].

2.3. Western blotting

Western blotting was performed according to techniques described previously [17]. The following reagents were used: anti-FLAG (rabbit polyconal, 600-401-383, Rockland, Gilbertsville, PA), anti-p24 (183-H12-5C, NIH AIDS Research and Reference Reagent Program), anti-gp120 (vA-20 and vT-21 antibodies, Santa Cruz Biotech, Santa Cruz, CA), biotinylated anti-goat antibody (GE Healthcare Bio-Sciences, Piscataway, NJ), horseradish peroxidase-conjugated streptoavidin (GE Healthcare Bio-Sciences), and EnVision+ system (Dako, Glostrup, Denmark). Signals were visualized with an LAS3000 imager (Fujifilm, Tokyo, Japan) and quantified by Multi Gauge ver 3.0 software (Fujifilm).

2.4. Confocal microscopy

293T cells transiently transfected with expression vectors for SEC14L1a derivatives were grown on glass plates, fixed in 4% formaldehyde in phosphate buffer saline (PBS) for 5 min at 24 h post-transfection, stained with Hoechst 33258 (Sigma), mounted (Vectorshield, Vector Laboratories, Burlingame, CA), and imaged using a confocal microscope META 510 (Carl Zeiss, Tokyo, Japan). For MT-4 cells, live cells were incubated with Hoechst 33258 and imaged unfixed. Image brightness and contrast were processed by META510 software (Carl Zeiss).

2.5. Immunoprecipitation

Cells expressing FLAG-tagged proteins were harvested and washed twice with PBS and then lysed in the lysis buffer (50 mM Tris–HCl, pH 8.0, 0.5% IGEPAL CA630, protease inhibitor cocktail from Sigma) on ice for 30 min. The soluble fraction was obtained by centrifugation at 15,000 rpm for 30 min at 4 °C, and was incubated with 20 μl of Red-Anti-FLAG M2 Affinity Gel (Sigma) with gentle mixing overnight at 4 °C. After washing the agarose beads for five times with the lysis buffer, the bound complexes were eluted with the FLAG peptide, and analyzed by Western blotting.

2.6. Flow cytometry

Cells were labeled with PE-Cy5-conjugated anti-CD4 antibody or PE-conjugated anti-CXCR4 antibody (Beckton Dickinson, San Jose, Calif.) for 30 min at 4 °C. Cells were washed once with PBS supplemented with 1% FBS and analyzed by FACS Aria (Beckton Dickinson). The GFP-positive cells were sorted using FACS Aria.

2.7. Monitoring HIV-1 replication

For HIV-1 infection, 1×10^5 cells were incubated at the room temperature with the HIV-1_{HXB2}-containing culture supernatant, which had approximately 1.0 ng of $p24^{CA}$, for approximately 30 min. The culture supernatants were collected at 4 d post-infection and subjected to ELISA to measure the $p24^{CA}$ antigen, using a Retro TEK p24 Antigen ELISA Kit according to the manufacturer's protocol (Zepto Metrix, Buffalo, NY). The signals were measured with an ELx808 microplate photometer (BIO-TEK®, Winooski, VT).

2.8. PCR analysis

The cellular DNA and RNA were extracted from cells infected with VSV-G-pseudotyped HIV-1 vector produced by using pNL-Luc plasmid, as described previously [17]. The Alu-LTR PCR and RT-PCR were performed as described previously [3,17] using the following primers: for the first Alu-LTR PCR reaction, 5'-AACTAGGGAACCCACTGCTTAAG-3' and 5'-TGCTGGGATTACAGGC-GTGAG-3'; and for the second Alu-LTR PCR reaction, 5'-AACT-AGGGAACCCACTGCTTAAG-3' and 5'-CTGCTAGAGATTTTCCACA-CTGAC-3'. For amplification of HIV-1 mRNA, 5′-ATGGAGCCAGTAG-ATCCTAGAC-3′ and 5′-CTATTCCTTCGGGCCTGTCGGG-3′ primers were used. For the control, we amplified beta-globin and cyclophilin A using the following primers: beta-globin, 5'-TATTGGTCT-CCTTAAACCTGTCTTG-3' and 5'-CTGACACAACTGTGTTCACTAGC-3'; and cyclophilin A, 5′- CACCGCCACCATGGTCAACCCCACCGTGTTCT-TCGAC-3′ and 5′- CCCGGGCCTCGAGCTTTCGAGTTGTCCACAGTCA-GCAATGG-3′. The amplicons were separated in a 2% agarose gel, stained with ethidium bromide, and imaged with a Typhoon scanner (GE Healthcare Bio-Sciences).

2.9. Collection of virus-like particle

Tissue culture supernatants containing virus-like particles (VLP) were passed through nitrocellulose filters (0.45 μm, Millipore, Tokyo, Japan) and the virions were collected by centrifugation (Optima™ TL, TLA 100.3 rotor, 541 k × *g* for 1 h; Beckman Coulter, Miami, FL).

3. Results

3.1. Identification of SEC14L1a as a potential regulator of HIV-1 replication

We prepared MT-4 cells that constitutively express cDNA transduced by a lentiviral vector or an MLV-based retroviral vector (Fig. 1A). The cDNAs were derived from human peripheral blood mononuclear cells (PBL) and *Oryctolagus cuniculus* (European rabbit) kidney-derived cell line RK13 cells. MT-4 cells transduced with cDNA were collected by FACS sorter using the green fluorescence as a marker since viral vectors encoded the GFP expression cassette. Then, cells were infected with HIV-1. Surviving cells were propagated and the genomic DNA was extracted to recover the transduced cDNA by PCR as previously described [3]. We isolated two clones encoding the carboxy terminal domain (CTD) of SEC14L1a (Gene ID 6397, Fig. 1B and C); one from the PBL cDNA library (1/65 independent clones, 1.5%), and one from the RK13 cDNA library (1/42 independent clones, 2.4%). The fact that the SEC14L1a CTD was successfully identified from two independent cDNA libraries strongly suggests that it is a negative regulator of HIV-1 replication. It is important to note that previous genome-wide screenings for HIV-1 regulators have not identified SEC14L1a CTD. This clearly suggests that our T cell-based cDNA screening system is unique, and should be able to complement the other genome-wide screening systems.

SEC14L1a belongs to the widely-expressed SEC14-superfamily that is involved in membrane trafficking and phospholipid metabolism [18–21]. The function of SEC14L1a is not well understood. The C-terminus of SEC14L1a encodes a Golgi dynamics (GOLD) domain (amino acids (aa) 523–674; Fig. 1C) that mediates the protein-protein interaction possibly involved in the maintenance of Golgi apparatus function and vesicular trafficking [22]. The only reported biological activity of SEC14L1a is to interact with cholinergic receptors AchT and CHT1 [23]. The GOLD domain is responsible for the physical interaction between SEC14L1a and cholinergic receptors. However, the functional significance of these interactions remains to be clarified. The conserved SEC14 domain directly interacts with lipid molecules [17–21]. However, the lipid ligand of SEC14L1a (aa 319–490, Fig. 1C) has yet to be identified.

3.2. Construction of expression vectors for SEC14L1a derivatives

The longest SEC14L1a cDNA recovered from the PBL cDNA library spanned nucleotides (nt) 2045–2492 of SEC14L1a mRNA (NM_003003.3), covering the CTD of the SEC14L1a open reading frame (ORF; Fig. 1B). We detected a potential translational start codon at nt 2188-2190 within the GOLD domain (asterisk, Fig. 1B). We speculated that the isolated cDNA might have expressed the carboxy half of the GOLD domain (aa 641–715) in MT-4 cells, leading to the inhibition of HIV-1 replication.

To test this, we constructed an expression plasmid for FLAG-tagged CTD (aa 642-715) fused to the carboxy terminus of GFP (CTD1; Fig. 1C). We also constructed GFP fusion proteins spanning the GOLD domain (CTD2, aa 493–715) or the full-length SEC14L1a (FL; Fig. 1C). Expression of these proteins was verified by Western blotting of transient transfected 293T cells (Fig. 1D). The confocal microscopy analysis indicated that the FL localized mainly in the cytoplasm, with some accumulation in the perinuclear regions (Fig. 1E), consistent with a previous report [23]. CTD1 was distributed in the cytoplasm and the nucleus, with a slight preference for the cytoplasm. CTD2 was evenly distributed to the nucleus and cytoplasm. When MT-4 cells constitutively expressing FL, CTD1, and CTD2 were analyzed, the subcellular distribution was less clear, due to the small cytoplasm (Fig. 1F). However, FL was distributed evenly to the nucleus and cytoplasm in MT-4 cells. In contrast, CTD1 was excluded from the nucleus in MT-4 cells (Fig. 1F). The distribution of CTD2 in MT-4 cells was similar to that in 293T cells (Fig. 1F). The differences of protein distribution in two cell types may be caused by the cell type-dependent regulation of protein trafficking and/or the effect of protein expression levels.

3.3. Verification of anti-HIV-1 activity associated with SEC14L1a CTD1

We introduced FL, CTD1, or CTD2 into MT-4 cells using the MLV vector, and isolated cells constitutively expressing FL, CTD1, or CTD2. Expression of SEC14L1a derivatives in MT-4 cells was verified by Western blotting (Fig. 2A). FL expression was verified by immuno-precipitation assay (Fig. 2A). The detection of FL by Western blotting was inefficient considering the fact that all the SEC14L1a derivatives are GFP-tagged, and the GFP intensity of FL-expressing MT-4 cells was not lower than that of CTD1-expressing

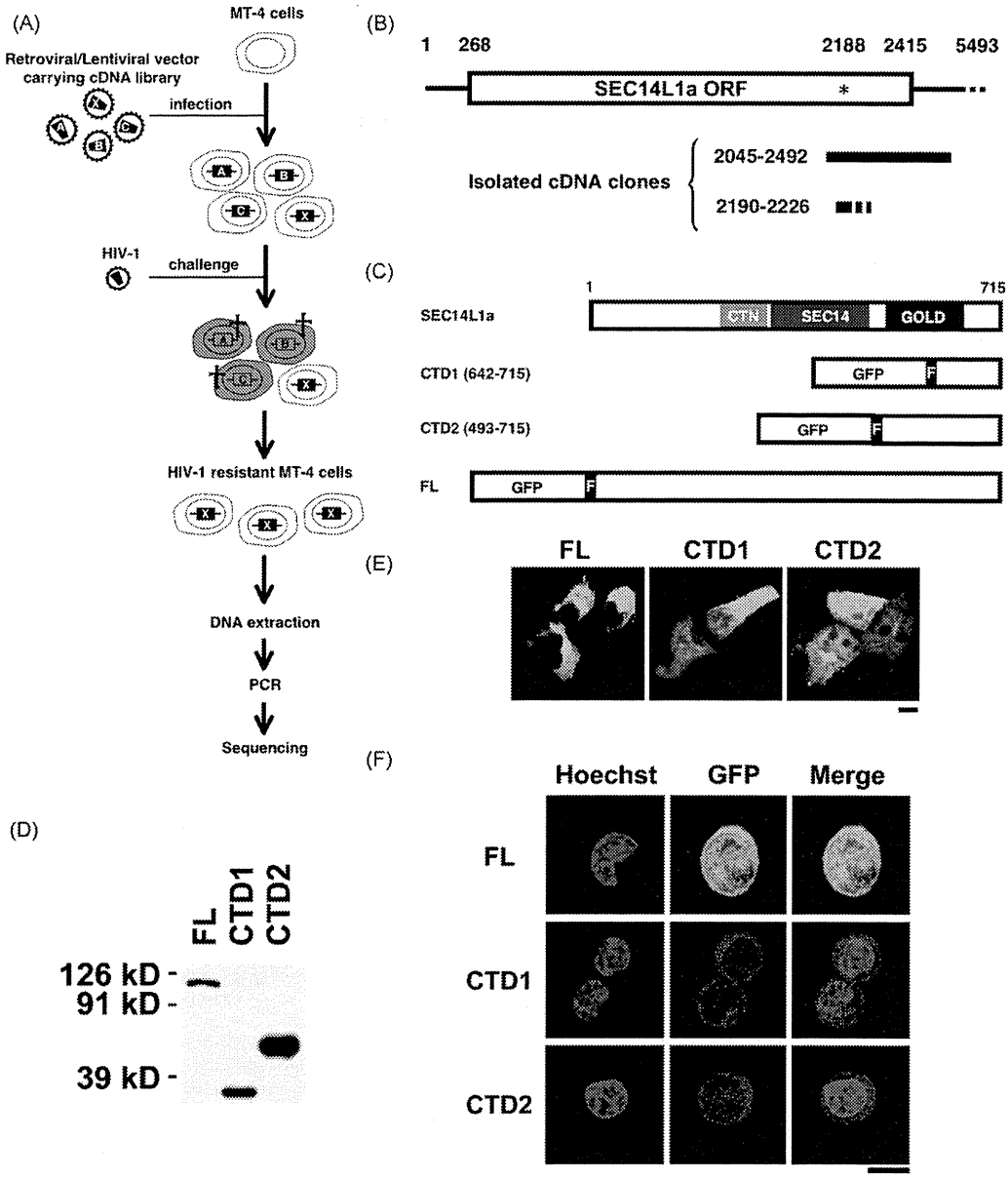

Fig. 1. Identification of SEC14L1a CTD as a potential regulator of HIV-1 replication. (A) The experimental strategy used to screen a cDNA library for genes rendering cells resistant to HIV-1. MT-4 cells were infected with a retroviral or lentiviral vector carrying cDNA libraries and were challenged with wild-type HIV-1_{HXB2}. The HIV-1-infected cells (gray with cross) quickly undergo cell death. The surviving cells were propagated, collected, and the transduced cDNA labeled X was determined. (B) Schematic representation of SEC14L1a mRNA (NM_00303.3) and the isolated gene fragments. The open reading frame (ORF) is assigned from nucleotides (nt) 268 to 2415. The potential internal translational initiation codon is marked with an asterisk. (C) Schematic representation of the SEC14L1a protein (NP_002994). SEC14L1a has a CRAL_TRIO_N domain (CTN, amino acids 241–313), a SEC14p-like lipid-binding domain (SEC14, amino acids 319–490), and a Golgi dynamics domain (GOLD, amino acids 523–674). The cloned fragments (CTD1 and CTD2) and full-length (FL) gene were tagged with a FLAG epitope (indicated with an "F") on their N-termini, and fused to the C-terminus of GFP. (D) Verification of FL, CTD1, and CTD2 expression in 293T cells by Western blotting using anti-FLAG antibody. (E) Confocal microscopy images of 293T cells expressing FL, CTD1, or CTD2. The green signal represents GFP fluorescence. Magnification, 630×; scale bar, 10 μm. (F) Confocal microscopy images of MT-4 cells constitutively expressing FL, CTD1, or CTD2. The blue signal represents the Hoechst-stained nucleus, and green represents GFP fluorescence. Magnification, 630×; scale bar, 5 μm.

cells (Fig. 1F). The MLV vector expressing GFP alone was used as a control. The cell proliferation, morphology, and cell surface levels of HIV-1 receptors were unaltered by any of the SEC14L1a derivatives (Fig. 1F, 2B, and data not shown). HIV-1 replication was tested in these cells. The level of HIV-1 replication was significantly inhibited in CTD1- and CTD2-expressing cells (69.1% and 69.8% on the average from seven independent experiments, respectively, $P < 0.05$, two-tailed Student's *t*-test), but was hardly inhibited in FL-expressing cells (86.4%, not statistically significant; Fig. 2C). This observation was reproducible in independently established MT-4 cells and SupT1 cells (data not shown). These data verified the original screening results, and suggest that the C-terminal half of GOLD domain of SEC14L1a serves as an inhibitor of HIV-1 replication. In contrast, it is suggested that FL is not a potent negative regulator of HIV-1 replication.

3.4. *SEC14L1a CTD1 and CTD2 target the late phase of the HIV-1 life cycle*

We analyzed the viral entry and production phases to determine which step of the HIV-1 life cycle CTD1 and CTD2 target.

The Alu-LTR PCR assay was performed to examine the effect of SEC14L1a derivatives on the viral entry phase. The MT-4 cells stably expressing GFP, FL, CTD1, or CTD2 were infected with VSV-

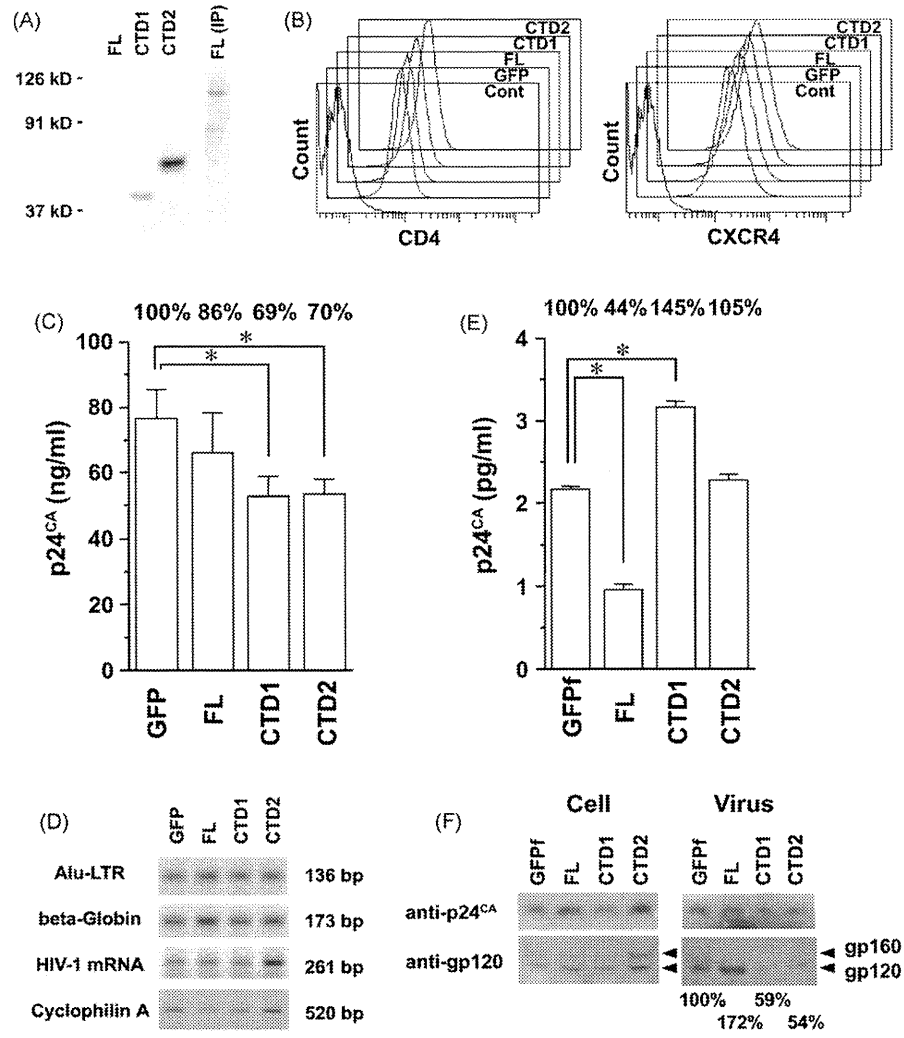

Fig. 2. Functional characterization of the SEC14L1a derivatives. (A) Detection of stable expression of FL, CTD1, and CTD2 in MT-4 cells by Western blotting using anti-FLAG antibody. FL was detected by the immunoprecipitation (IP) assay using agarose beads conjugated with anti-FLAG antibody. The flow cytometric analysis of the cell surface expression of HIV-1 receptors CD4 and CXCR4 in MT-4 cells stably expressing GFP, FL, CTD1, and CTD2. (C) Constitutive expression of CTD1 and CTD2 limited the replication of HIV-1 in MT-4 cells. The concentration of viral $p24^{CA}$ antigen in the culture supernatant was measured at 4 d post-infection. The results represent the average of seven independent experiments ± the standard error of the mean. The reduction of viral $p24^{CA}$ concentration relative to GFP was shown on the top. Asterisks indicate the statistical significance compared to GFP ($P < 0.05$ by two-tailed Student's *t*-test). (D) The PCR-based assay to examine the effect of SEC14L1a derivatives on the early phase of viral life cycle (top two panels) and the transcription from LTR promoter (bottom two panels). The HIV-1 entry efficiency was examined by Alu-LTR PCR. Beta globin was used as an internal control. The HIV-1 transcription efficiency was examined by RT-PCR targeting spliced viral mRNA. Cyclophilin A was used as a control. The expected length of each PCR amplicon was indicated. (E) The effect of SEC14L1a derivatives on the HIV-1 production. The 293T cells grown in a well of a 6-well plate were transfected with 200 ng of HIV-1 proviral DNA and 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The culture supernatant was recovered at 2 d post-transfection and the $p24^{CA}$ concentration was measured. The representative data from five independent experiments was shown. The results indicate the average ± the standard deviation. The relative $p24^{CA}$ concentration compared to GFPf was shown on the top. Asterisks indicate the statistical significance compared to GFPf ($P < 0.001$ by two-tailed Student's *t*-test). The *Env* incorporation onto the virus-like particles (VLP) produced by 293T cells expressing SEC14L1a derivatives. The 293T cells grown in a well of a 6-well plate were transfected with 1 μg of *gag-pol* (pCMVR8.91) and *Env* (pIIIex) expression vectors along with 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The cell lysates (Cell) and VLP fractions (Virus) were subjected to Western blot analysis detecting gp120 and $p24^{CA}$ harvested at 2 d post-transfection. The *Env* incorporation levels normalized to $p24^{CA}$ relative to GFPf were shown at the bottom.

G-pseudotyped HIV-1 vector, and the cellular genomic DNA was recovered at 4 d post-infection. The amount of Alu-LTR PCR products from FL-, CTD1-, or CTD2-expressing MT-4 cells was almost equal to that from GFP-expressing cells, suggesting that the early phase of the viral life cycle is not inhibited by any of the SEC14L1a derivatives (Fig. 2D). To examine the viral production phase, we examined the LTR-driven viral gene transcription by RT-PCR. Cellular RNA was extracted from the same MT-4 cells infected with VSV-G-pseudotyped HIV-1 vector, and RT-PCR was conducted to amplify LTR promoter-driven spliced HIV-1 mRNA. The amount of viral RNA expressed in FL-, CTD1-, or CTD2-expressing cells was not lower than that in GFP-expressing cells when the levels of the internal control was taken into account (Fig. 2D). Given that the similar number of viral genome was integrated as indicated by the Alu-LTR PCR, these data suggest that viral transcription is not inhibited by any of the SEC14L1a derivatives, and that the action point of CTD1 and CTD2 should be at post-transcriptional levels of the viral production phase.

Next, the FL, CTD1, or CTD2 expression vector was co-transfected with HIV-1 proviral DNA into 293T cells, and viral production was quantified by $p24^{CA}$ ELISA. The FLAG-tagged GFP (GFPf) was used as a control hereafter. We found that the FL expression significantly reduced the production of HIV-1 (44.2%, $P < 0.001$, two-tailed Student's *t*-test) compared to the GFPf control (Fig. 2E). In contrast, the CTD1 enhanced the production of HIV-1 (145.9%, $P < 0.001$, two-tailed Student's *t*-test; Fig. 2E). However, CTD2 did not measurably affect the HIV-1 production (105.1%, not statistically significant; Fig. 2E). As the ELISA assay examines the effect

of CTDs on *Gag* functions, we next tested the functional interaction between CTDs and *Env*. The *Env* incorporation onto the virion was examined by tripartite-transfection of expression vectors for *Env*, *gag-pol*, and SEC14L1a derivatives into 293T cells, and the VLP was collected by centrifugation. The immunoblotting against gp120 was performed on the cell lysate and the VLP fraction. The cellular *Env* and *Gag* expressions were not detectably affected by any of the SEC14L1a derivatives (Fig. 2F, left panel). The *Env* incorporation onto the VLP was slightly enhanced by FL (157%; Fig. 2F, right panel). In contrast, the VLP produced from CTD1- or CTD2-expressing cells incorporated substantially fewer *Env* than those from GFPf-expressing cells (59% or 54%, respectively; Fig. 2F, right panel). These data were reproducible in independently performed experiments. The densitometric analysis of Western blot image showed that the average ± the standard error of the mean of Env incorporation onto the virion was 129.7 ± 39.9%, 54.8 ± 24.7%, and 25.5 ± 10.3% for FL, CTD1, and CTD2 compared to GFPf, respectively (3–4 independent experiments). The *Env*-mediated cell-to-cell fusion assay indicated that SEC14L1a derivatives did not limit the cell surface targeting and function of *Env* (data not shown). In addition, the *Gag* processing in virion was unaffected by any of the SEC14L1a derivatives (data not shown). Collectively, these data suggest that the HIV-1 replication is inhibited by CTD1 and CTD2 due to the inefficient *Env* incorporation onto the virion. To test this possibility, we infected fresh MT-4 cells with the equal amount of HIV-1 propagated in CTD1- or CTD2-expressing MT-4 cells (1–2 ng $p24^{CA}$), and the viral replication was monitored at 3–4 days post-infection by measuring the $p24^{CA}$ concentration. The infectivity of HIV-1 propagated in CTD1- or CTD2-expressing cells was attenuated to 83.1 ± 17.9% or 82.4 ± 5.5% relative to the virus recovered from GFP-expressing cells, respectively (the average ± the standard error of the mean of 3 independent experiments). Altogether, these data suggest that the inhibition of HIV-1 replication by CTD1 and CTD2 is attributed to the attenuation of viral infectivity by lowering the *Env* incorporation onto the virion.

4. Discussion

In the present study, we provide the first evidence that the C-terminal fragment of SEC14L1a functions as an inhibitor of HIV-1 replication. The advantage of this system is that, since MT-4 cells are stably transduced with a cDNA library, the anti-HIV-1 function of a candidate gene is not due to a perturbed cell physiology. This system has been successful in identifying CD14, CD63, and Brd4-CTD as regulators of HIV-1 replication [1,3,4], and more candidates are being analyzed. Among the candidates, SEC14L1a CTD appeared to be one of the relatively modest inhibitors of HIV-1 replication. However, of note, the SEC14L1a derivatives have not been identified in other genetic screening systems. These facts point that our T cell-based system is sensitive in detecting the modest anti-HIV-1 activity of a gene, and is a unique tool in the pursuit of HIV-1 regulatory factors to complete the HIV-1-host interactome.

SEC14L1a may affect the Golgi-mediated vesicular trafficking since SEC14L1a lowers the cell surface levels of cholinergic transporters [23]. However, we do not have any data to suggest that SEC14L1a and its derivatives affect the cell surface targeting of membrane proteins including CD4, CXCR4 and *Env*. These data suggest that SEC14L1a's effect on cholinergic receptor expression is specific, and that the CTD's ability to inhibit HIV-1 replication is independent from SEC14L1a's regulatory functions on vesicular trafficking. The action point of CTD1 and CTD2 was shown to be the late phase of the viral life cycle. Given that CTD1 and CTD2 did not inhibit the biogenesis and the cell surface targeting of *Gag* and *Env*, the major mechanism of CTD1 and CTD2 to inhibit HIV-1 replication was to reduce the infectivity of HIV-1 by limiting the *Env* incorporation onto the virion. Consistent with this idea, the viral infectivity of virions produced in CTDs-expressing cells was attenuated. Then, how do CTDs block the *Env* incorporation onto the virion? We detected a weak interaction between *Gag* and CTD1 or CTD2 by immuno-coprecipitation analysis. Thus, we speculate that the interaction between *Env* and *Gag* at the plasma membrane is interfered by *Gag*-CTDs interaction, resulting in the reduction of *Env* incorporation onto the virion.

The CTD1 was an inhibitor of HIV-1 replication. While the CTD1 negatively affected the *Env* incorporation onto the virion, it positively affected the HIV-1 production. These observations may be seemingly controversial. However, the SEC14L1a derivatives' effect on HIV-1 replication is a summation of their effects of on each step of the viral life cycle. Therefore, it is conceivable that CTD1 can serve as a negative regulator of HIV-1 replication as well as a positive and negative factor on distinct steps of the viral life cycle. These seemingly controversial findings may be in part due to the cells in which the biological functions of SEC14L1a derivatives were examined. The effect of SEC14L1a derivatives on HIV-1 replication was investigated in MT-4 cells, whereas those on the HIV-1 production and *Env* incorporation onto the virion were examined in 293T cells. Although the basic biological features are largely shared among different cell types, it is possible that the SEC14L1a derivatives may function slightly differently in MT-4 cells from 293T cells given that the intracellular distribution of SEC14L1a derivatives in MT-4 cells was not identical to that in 293T cells (Fig. 1E and 1F).

Elucidating the molecular mechanism underlying CTDs' activity not only provides a hint to understand how the HIV-1 virion actively uptakes *Env* through the *Gag-Env* interaction, but also leads to the development of a novel anti-retroviral drug that lowers the infectivity of the virus by preventing *Env* incorporation onto the virion. This is the strength of our T cell-based assay since CTDs inhibit HIV-1 replication specifically. In the previous study, we proposed that a small portion of Brd4 may serve as a therapeutic molecular target for HIV-1 infection, since the constitutive expression of Brd4-CTD limited HIV-1 replication specifically [3], akin to the SEC14L1a CTDs. However, it remains to be examined whether the SEC14L1a and Brd4 derivatives inhibit HIV-1 replication in primary HIV-1 target cells.

The genome-wide screening has potential caveats, including a cDNA bias and a cell line bias. A cDNA library is not a perfect representation of mRNA expressed in the cells from which the library is constructed. For example, the longer the mRNA, the less efficiently the full-length cDNA is synthesized. In fact, we isolated Brd4-CTD from the PBL cDNA library as a potent inhibitor of HIV-1 replication [3]. However, although Brd4 (approximately 5000 nt mRNA in length) is expressed in MT-4 cells, we were unable to recover Brd4-CTD from the MT-4 cDNA library [3]. This clearly demonstrates the cDNA bias in the genetic screening. A cDNA library derived from non-T cells does not contain genes specifically expressed in T cells. Thus, we have to explore many more cDNA libraries to completely cover the genetic diversity of human cells. The cDNA libraries isolated from long-term non-progressors of HIV-1-seropositive individuals or from elite controllers might be of particular interest, considering that a dominant innate HIV-1 resistance gene, such as CCR5 delta 32, may partly account for the slow progression of AIDS. Similarly, use of a particular cell line and/or virus strain may bias the results. MT-4 cells are positive for HTLV-1, and are able to support robust HIV-1 replication. MT-4 cells do not express CCR5, and are unable to support R5-tropic HIV-1 strains. What if other T cell lines and R5-tropic viral strains are used? What if we assay the same cDNA library in TZM-bl cells? We plan to address these issues in the future studies.

In conclusion, genome-wide genetic screening is a powerful tool for identifying the regulatory factors of HIV-1 replication and innate HIV-1 resistance factors that limit HIV-1 infection and AIDS progression. The HIV-1-host interactome should also reveal poten-

tial therapeutic molecular targets that may be used to develop novel anti-AIDS drugs to tackle the emerging drug resistant viruses. However, the fact that different experimental systems often yield non-overlapping candidates suggests that we have to explore more experimental systems to fully understand the HIV-1-host interactome. Our T cell-based system provides an alternative tool for identifying novel HIV-1 regulatory factors, and should help us understand the HIV-1-host interaction in more detail.

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Conflict of interest statement

The authors state that they have no conflict of interest.

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Short communication

Purification and concentration of non-infectious West Nile virus-like particles and infectious virions using a pseudo-affinity Cellufine Sulfate column

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A B S T R A C T

Affinity column chromatography is a promising method for the purification of flavivirus particles that can supplement or potentially replace diafiltration and sucrose density centrifugation. In this study, the purification of West Nile Virus (WNV) antigens via Cellufine Sulfate column chromatography was examined. Virus-like particles (VLPs) produced by the expression of the prM and E genes were separated from most of the contaminant proteins with 0.2–0.4 M NaCl, but still retained their spherical forms and immunogenicity in mice. The column, with a 1 mL bed-volume, concentrated WN-VLPs a minimum of 15 fold from culture supernatants. A heparin analogue, suramin, competitively eluted WN-VLPs, but sulphated polysaccharides, such as heparin, heparin sulfate and dextran sulfate, did not. Furthermore, 2.4×10^9 plaque forming units of WNV and 196 μg of the viral antigens were recovered from 60 mL of infected culture medium at high yields (93% and 96%, respectively). These results indicate that, in addition to conventional methods, Cellufine Sulfate column chromatography is an effective preparation technique for WNV particulate antigens that does not impair the antigen virological characteristics.

West Nile virus (WNV) is a member of the family *Flaviviridae* and genus *Flavivirus*, which includes some viruses important to public health: Japanese encephalitis virus (JEV), tick-borne encephalitis virus, dengue virus and yellow fever virus. WNV often causes febrile illness with a risk of severe meningoencephalitis in humans and has emerged as several outbreaks worldwide (Dauphin and Zientara, 2007). There is no antiviral drug or prophylactic vaccine effective against WNV for human use.

WNV virions are spherical, enveloped particles about 50 nm in diameter and composed of the capsid, envelope (E), membrane (M) proteins and the RNA genome. The expression of the E and precursor of M (prM) proteins produces small (20–30 nm), noninfectious virus-like particles (VLPs) or recombinant subviral particles (Hanna et al., 2005; Takahashi et al., 2009). The particle morphology of flaviviruses is thought to be important for effective antibody induction and protection against viral challenge (Heinz et al., 1995). Many WNV vaccine candidates have been developed in particulate forms: inactivated whole virions (Lim et al., 2008), propagation-defective viruses (Widman et al., 2008) and chimeric flaviviruses replaced with the WNV prM-E gene region (Monath et al., 2006; Pletnev et al., 2006). In addition, WN-VLP antigens have been developed as a novel subunit vaccine candidate (Takahashi et al., 2009; Ohtaki et al., 2010).

Particle-based flavivirus vaccines are manufactured commonly by purification techniques such as diafiltration and sucrose density gradient centrifugation (Ehrlich et al., 2003; Monath et al., 2006; Toriniwa and Komiya, 2008). However, these purification methods depend on physical properties like molecular size, density and buoyancy and are occasionally unreliable. It has been proposed that unidentified host proteins in the mouse brain-derived JEV vaccine that could not be removed by conventional methods caused acute disseminated encephalomyelitis in some recipients (Ohtaki et al., 1995). Therefore, additional purification steps are needed to improve the safety of vaccines. Column chromatography that depends on biochemical characteristics would be advantageous for purifying vaccine antigens.

In this study, a pseudo-affinity Cellufine Sulfate column (Chisso, Tokyo, Japan) was tested for purification of WNV particle antigens. The Cellufine Sulfate column and its related products have been shown to be effective for the purification of several DNA and RNA

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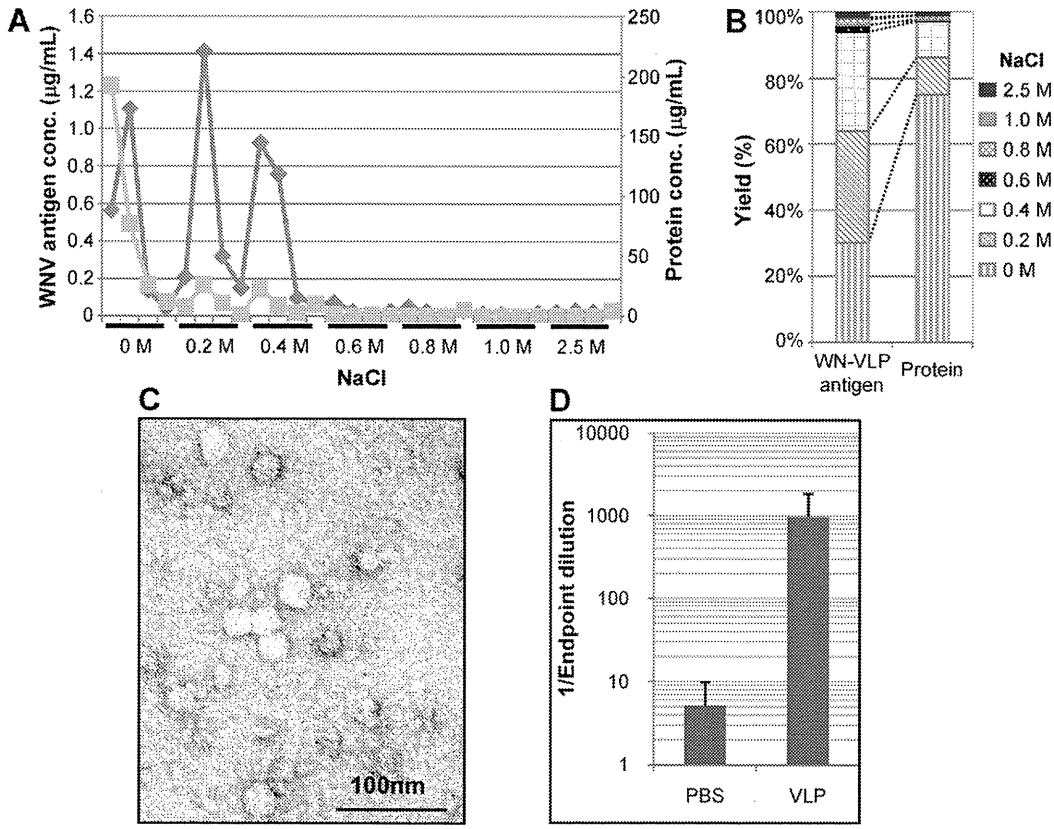

Fig. 1. Recovery of WN-VLPs by Cellufine Sulfate column chromatography. WN-VLPs released from #22.6 cells expressing constitutively the WNV prM-E gene were absorbed on a Cellufine Sulfate mini-column. The WNV antigens were eluted with the increasing NaCl concentration in 50 mM carbonate buffer (pH 9.2) and analysed by WNV-specific ELISA. The protein contents were determined by a Lowry protein assay. The peak fractions in 0.2 and 0.4 M NaCl were pooled, exchanged for PBS, concentrated and examined by electron microscopy and mouse immunization. (A) The elution patterns of WN-VLPs and proteins are represented by diamonds and squares, respectively. (B) Recoveries of WN-VLPs and proteins in (A) are summarized in build-up graphs where the total amounts of the eluted WNV antigens and proteins are defined as 100%. (C) An electron micrograph of the eluted WN-VLP antigens. (D) C3H/He mice were immunized twice with the eluted WN-VLP antigens in PBS. Sera were collected 7days after the second immunization. Titres of anti-WNV antibodies in mice were measured by IgG ELISA.

viruses, such as herpes simplex virus and measles virus (O'Neil and Balkovic, 1993), influenza virus (Opitz et al., 2009) and the human immunodeficiency virus (Bartz et al., 1994). Cellufine Sulfate is produced by the chemical modification of cellulose beads with a low concentration of sulfate ester and mimics sulphated polysaccharides, which are antiviral agents and are thought to interrupt viral entry into host cells by binding to the viral envelope proteins (Baba et al., 1988; Witvrouw and De Clercq, 1997). Indeed, heparin, heparan sulfate and dextran sulfate are reported to inhibit flavivirus infectivity proportional to their viral E protein-binding activity (Chen et al., 1997; Lee et al., 2006; Su et al., 2001). Thus, a sulphated cellulose column should be useful for the preparation of WNV antigen particles.

In an initial experiment, an attempt was made to determine whether WN-VLPs remain bound to Cellufine Sulfate in a carbonate buffer system, which is used for preparing formalin-inactivated JEV (Sugawara et al., 2002). WNV antigens were prepared from a CHO cell clone, #22.6, which expresses the WNV prM-E gene constitutively (Ohtaki et al., 2010). The #22.6 culture supernatant was concentrated by ultrafiltration, exchanged for 50 mM Tris–HCl (pH 7.5)-150 mM NaCl and applied to a Cellufine Sulfate mini-column (1 mL bed-volume). The bound WNV antigens were eluted in increasing concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.5 M) of NaCl in 50 mM carbonate buffer (pH 9.2). Fig. 1A shows the elution patterns of the WNV antigens and proteins measured in each fraction (0.75 mL) by an antigen-capture enzyme-linked immunosorbent assay (ELISA), as described previously (Takahashi et al., 2009), and by a Lowry assay with a bovine serum albumin standard, respectively. The recoveries were calculated and summarized in Fig. 1B. Almost a third (~30%) of the WNV antigens was lost in a wash step with NaCl-free (0 M NaCl) carbonate buffer, though almost 75% of the total proteins were eluted from the column in the wash fractions. However, with 0.2–0.4 M NaCl, mostly viral antigens were eluted, and more than 60% of the antigens were recovered in these fractions with only small amounts of contaminant proteins. These results suggest that if an adequate buffer is selected, Cellufine Sulfate is suitable for binding WN-VLPs and separating them from the contaminant proteins with a moderate NaCl concentration.

The WNV antigens recovered from the Cellufine Sulfate column were transferred from a carbonate buffer to phosphate-buffered saline (PBS) with a PD-10 column (GE Healthcare UK Ltd, Buckinghamshire, England), concentrated by ultrafiltration and observed by electron microscopy. As shown in Fig. 1C, the eluted antigens appeared to be spherical particles 20–30 nm in diameter, which corresponds to the intact forms of WN-VLPs. To examine the immunogenicity of the VLP antigens, groups of four C3H/He mice were immunized twice with the WN-VLP preparation in PBS, and titres of anti-WNV immunoglobulin G (IgG) were measured by antibody ELISA, as described previously (Takahashi et al., 2009). Fig. 1D shows that the WN-VLP antigens induced anti-WNV IgG in mice, indicating that the VLP antigens retained their immunogenicity after preparation by affinity chromatography. From these results, Cellufine Sulfate was expected to be a useful tool for purifying WN-VLPs, and additional experiments were performed to establish the optimal pH and buffer conditions for use of the affinity column.

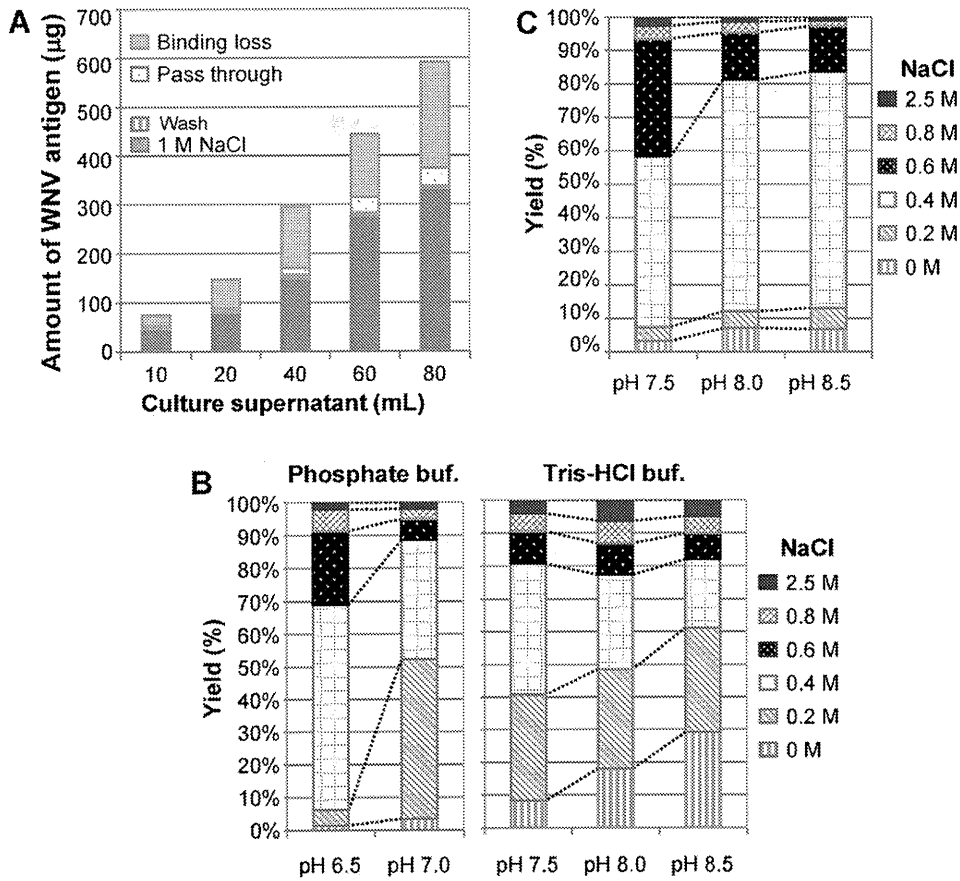

Fig. 2. The optimum conditions for preparing the WN-VLP antigens. Culture supernatants were collected from (A and B) serum-free medium of #22.6S cells or (C) FBS (10%)-containing medium of the parental #22.6 cells and applied to a mini-column. The amounts of WN-VLPs were determined by ELISA in the initial culture fluids and the effluents. (A) The mini-column was injected with increasing volume of the serum-free medium and washed with 50 mM Tris–HCl (pH 7.5). The bound antigens were eluted with 1 M NaCl in Tris–HCl. The "binding loss" represents the amount of WN-VLPs calculated by the total input minus the recovery in each fraction. (B) The effects of pH and NaCl concentration on the elution efficiency. A fixed volume (20 mL) of the serum-free culture medium of #22.6S cells was loaded to the column and eluted with various combinations of pH and NaCl concentration indicated. (C) Recovery of WN-VLPs from the FBS-containing culture medium of #22.6 cells was analysed at pH 7.5–8.5 as in (B).

The usefulness of the column for concentrating WN-VLPs was examined by direct injection of increasing volumes (10–80 mL) of the culture supernatant containing the VLP antigens (5–10 μg/mL), which was prepared from suspension cultures of the #22.6S cell clone adapted in serum-free SFM-II medium (Invitrogen, Grand Island, NY, USA) (Ohtaki et al., 2010). The binding capacity of the column (3 mg per 1 mL bed-volume) corresponds to the amount of WN-VLPs in 300–600 mL of the culture supernatant. The bound VLPs were washed with 4.5 mL of 50 mM Tris–HCl (pH 7.5) and eluted with 3 mL of buffer containing 1 M NaCl. The amounts of eluted WNV antigens increased proportionally with the increasing volumes of the culture medium. About 330 μg of the antigen was recovered from 80 mL of the culture supernatant and concentrated about 15 fold, from 7.4 to 110 μg/mL (Fig. 2A). These results indicate that Cellufine Sulfate can be effective for concentrating WN-VLPs from culture supernatants.

The influence of the elution buffer pH on WN-VLP recovery was examined by altering the ionic strength of the buffer to diminish the interactions between the VLP antigens and Cellufine Sulfate. Equal volumes (20 mL) of the #22.6S serum-free culture supernatant were applied to the mini-columns and eluted with 50 mM phosphate buffer (pH 6.5 or 7.0) or 50 mM Tris–HCl buffer (pH 7.5, 8.0 or 8.5) containing 0–2.5 M NaCl. Considerable amounts of WN-VLPs were eluted using phosphate buffer and (pH 7.0) 0.2 M NaCl. In addition, the highest amounts of WN-VLPs were recovered in phosphate buffer at pH 7.0 containing 0.4 M NaCl. However, without NaCl, increasing the pH from 7.5 to 8.5 in Tris–HCl buffer eluted increasing amounts of the VLP antigens in wash fractions (Fig. 2B). Alternative pH and NaCl concentrations were required to elute WN-VLPs that derived from parental #22.6 cell cultures in serum-containing medium (Fig. 2C). WN-VLP recovery was inefficient in Tris–HCl (pH 7.5–8.5) with 0.2 M NaCl. A higher NaCl concentration of 0.6 M was needed to achieve an 80–90% recovery of WN-VLPs at pH 7.5–8.5 (Fig. 2C). Taken together, phosphate buffer (pH 7.0) with 0.4 M NaCl seems to be adequate for recovery, and higher pH facilitates dissociation of WN-VLPs from Cellufine Sulfate. However, the producer cells and/or culture medium of the VLP antigens may still modulate the binding affinity.

The binding characteristics between WN-VLPs and Cellufine Sulfate were examined by competitive elution with sulphated polysaccharides (heparin, heparan sulfate and dextran sulfate) and a heparin analogue (suramin) (Sigma–Aldrich, St. Louis, MO, USA). Suramin is a symmetric molecule with the framework composed of 4 benzene and 2 naphthalene rings instead of polysaccharides (Fig. 3A). WN-VLPs bound to the column were eluted with increasing concentrations of the competitors from 1 to 100 μg/mL in PBS. All the competitors examined have been reported to inhibit flavivirus infection in the range of 1–100 μg/mL (Chen et al., 1997; Lee et al., 2006; Su et al., 2001). However, none of the sulphated glycans desorbed WN-VLPs by more than 20%. In contrast, suramin effectively eluted WN-VLPs, and more than 80% of the antigens were recovered with 100 μg/mL of this sulphated compound (Fig. 3B). These results suggest that the interaction of WN-VLPs with Cellufine Sulfate differs

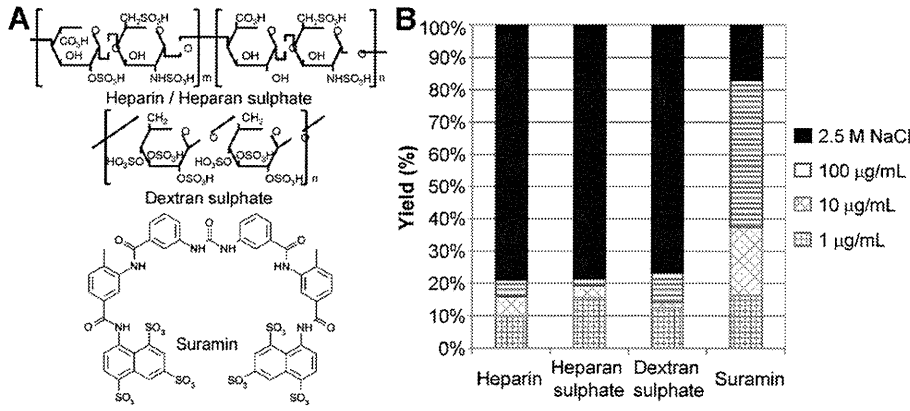

Fig. 3. Competitive elution of WN-VLPs from Cellufine Sulfate. (A) The chemical structures of heparin/heparin sulfate, dextran sulfate and suramin. (B) WN-VLPs from #22.6S cell cultures were adsorbed, washed with PBS and eluted with increasing concentrations of the sulphated competitors. All the remaining VLP antigens were desorbed with 2.5 M NaCl from a Cellufine Sulfate column. The results are expressed as % of the total amount of the bound WN-VLP antigens.

from that of the sulphated polysaccharides but mimics that of suramin.

Finally, the recovery of infectious WNV particles was examined. WNV was propagated on Vero E6 cell monolayers in serum-free VP-SFM (Invitrogen, Grand Island, NY, USA) medium. Viral infectivity and WNV antigen amounts in Tris–HCl (pH 7.5)–1 M NaCl effluents were measured by a plaque assay and ELISA, respectively. As depicted in Fig. 4A, the recoveries of both infectious virus and the viral antigens increased with the increasing volume of the infected culture fluids. About 2.4×10^9 plaque forming units (pfu) of WNV, corresponding to about 200 μg of the antigen, was obtained from 60 mL of the culture supernatant that contained initially 2.6×10^9 pfu of infectious WNV and 204 μg of the viral antigen. Thus, the yield of infectious virus particles was 70%, 63% and 93% for 15, 30 and 60 mL of the infected culture supernatant, respectively. The effects of pH and NaCl concentration on the yield of infectious WNV were analysed as described for non-infectious WN-VLPs in Fig. 2B. Only 20–30% of infectivity, corresponding to 10% of the viral antigens, was obtained with 50 mM Tris–HCl (pH 7.5)–0.2 M NaCl. At pH 9.0 in 50 mM carbonate buffer, however, 90% of the input infectivity and viral antigens was recovered in 0.75 mL of 0.2 M NaCl fraction (Fig. 4B). From these results, Cellufine Sulfate appears to be useful for concentrating and purifying infectious WNV virions from cultures without causing viral inactivation.

Initial viral materials from cell cultures usually contain virions or VLPs at a low concentration with large amounts of contaminant impurities. The first step for purifying the viral antigens is to concentrate them by reducing sample volume. Polyethylene glycol precipitation also collects host cell-derived contaminants. Diafiltration is easy to implement, but pores of the filters are sometimes clogged with aggregates from cell cultures. Sucrose density centrifugation, the most convenient method for virus preparation, is inadequate for purifying WN-VLPs because a sucrose concentration of over 30% disrupts WN-VLP particle structures and impairs their immunogenicity, as described previously (Ohtaki et al., 2010). For processing large volumes of culture fluids, continuous-flow ultracentrifugation has been used, but requires expensive centrifuge equipment and a large investment of time. In addition, ultracentrifugation can potentially generate considerable safety hazards when preparing infectious particles. Immunoaffinity chromatography using immobilized antibodies has the potential to be the most efficient method, but acidic elution buffers, often at pH 2.6, would likely impair infectivity of WNV and immunogenicity of WN-VLPs because flaviviruses are known to be highly sensitive to acidic pH (Schalich et al., 1996). Cellufine Sulfate chromatography can overcome these problems.

This study has demonstrated that Cellufine Sulfate can bind, concentrate and separate WNV particulate antigens from contaminant proteins under moderate salt and pH conditions. As 30% of WNV antigens were lost during wash steps with carbonate buffer (pH 9.2), used for preparing formalin-inactivated JEV (Sugawara et al., 2002), such a buffer seems to be unsuitable for efficient recovery of WN-VLPs. However, the recovered antigens retained their intact spherical forms and immunogenicity after the buffer change to PBS (Fig. 1). In addition, phosphate buffer (pH 7.0), a buffer advantageous for immunization, appears to be optimal for eluting

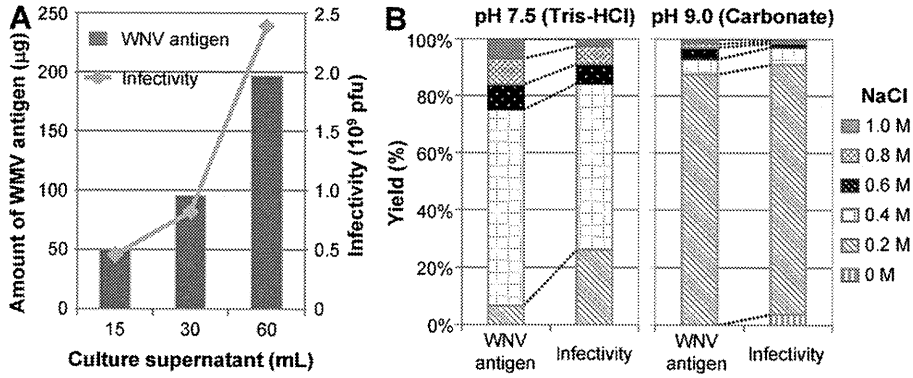

Fig. 4. The concentration and purification of infectious WNV virions. WNV was propagated on Vero cell monolayers in serum-free medium, bound to a Cellufine Sulfate column and eluted with 0–1 M NaCl. Viral infectivity and amounts of the WNV antigens were measured by a plaque assay and ELISA, respectively. (A) The viral infectivity and amount of WNV antigen in the 1 M NaCl effluents are presented by diamonds and bars, respectively. (B) The build-up graphs summarize the effects of pH and NaCl concentration on the yields of virus infectivity and WNV antigens.

WN-VLPs (Fig. 2). Thus, Cellufine Sulfate column chromatography is applicable to the production of non-infectious WN-VLPs and formalin-inactivated whole virions for particle-based WNV vaccines. Notably, infectious WNV was also recovered without loss of viral infectivity (Fig. 4), suggesting that a Cellufine Sulfate column could aid in the effective preparation of live vaccines, such as the promising chimeric virus and single-cycle virus vaccine candidates.

Sulfate ester residues on Cellufine Sulfate beads contribute to ionic interaction with the positively charged E protein on the surface of WNV antigen particles. A highly sulphated compound, suramin, competitively eluted WN-VLPs from the sulphated matrix, but other highly sulphated glycans, such as heparin, heparan sulfate and dextran sulfate, did not (Fig. 3). Although the reasons for the inefficient elution by the sulphated polysaccharides are unknown, it is possible that the difference in the backbone structures, a symmetric framework with 4 benzene/2 naphthalene rings or disaccharide polymer chains, might affect the desorption of WN-VLPs from negatively charged Cellufine Sulfate.

The ionic interaction between the WNV antigens and Cellufine Sulfate seems to depend on pH rather than NaCl concentration. WN-VLPs were eluted during wash steps at an increasing pH from 7.5 to 8.5 without NaCl and were more effectively eluted at a basic pH with 0.2–0.4 M NaCl (Fig. 2). Higher NaCl concentration, however, affected the yields slightly. Conversely, the yield at pH 6.5 was dramatically low compared to the yield at pH 7.0, suggesting that acidic pH conditions strengthen the binding affinity of the WNV antigens. The conformation of the E protein arranged on the surface of WNV particles is likely changed at a pH below 6.5, a phenomenon observed in other flavivirus E proteins (Schalich et al., 1996).

In conclusion, Cellufine Sulfate column chromatography is suitable for the effective purification and concentration of VLPs and live vaccine antigens of WNV. The technique preserves the virological properties of the antigens, the intact particulate forms, immunogenicity and infectivity. Cellufine sulfate chromatography can be easily used in addition to other conventional virus purification methods. Furthermore, this study will provide helpful information on how to prepare optimum conditions for the production of high-yield VLPs and the infective virions of non-WNV flaviviruses.

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Induction of Cross-Protective Immunity Against Influenza A Virus H5N1 by an Intranasal Vaccine With Extracts of Mushroom Mycelia

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The identification of a safe and effective adjuvant that is able to enhance mucosal immune responses is necessary for the development of an efficient inactivated intranasal influenza vaccine. The present study demonstrated the effectiveness of extracts of mycelia derived from edible mushrooms as adjuvants for intranasal influenza vaccine. The adjuvant effect of extracts of mycelia was examined by intranasal co-administration of the extracts and inactivated A/PR8 (H1N1) influenza virus hemagglutinin (HA) vaccine in BALB/c mice. The inactivated vaccine in combination with mycelial extracts induced a high anti-A/PR8 HA-specific IgA and IgG response in nasal washings and serum, respectively. Virus-specific cytotoxic T-lymphocyte responses were also induced by administration of the vaccine with extract of mycelia, resulting in protection against lethal lung infection with influenza virus A/PR8. In addition, intranasal administration of NIBRG14 vaccine derived from the influenza A/Vietnam/1194/2004 (H5N1) virus strain administered in conjunction with mycelial extracts from *Phellinus linteus* conferred cross-protection against heterologous influenza A/Indonesia/6/2005 virus challenge in the nasal infection model. In addition, mycelial extracts induced proinflammatory cytokines and CD40 expression in bone marrow-derived dendritic cells. These results suggest that mycelial extract-adjuvanted vaccines can confer cross-protection against variant H5N1 influenza viruses. The use of extracts of mycelia derived from edible mushrooms is proposed as a new safe and effective mucosal adjuvant for use for nasal vaccination against influenza virus infection. ***J. Med. Virol. 82:128–137, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: avian influenza; adjuvant; immunoglobulin A; hetero-subtypic immunity

INTRODUCTION

When developing a vaccine, both prophylactic effectiveness and safety must be considered. The mucosal immune system of the respiratory tract, which is a primary site of influenza infection, is usually the first immunological barrier against influenza virus infection. The influenza virus is able to cause annual epidemics of influenza by altering the antigenic properties of its surface hemagglutinin (HA), the antigenic glycoprotein that is responsible for binding of the virus to sialic acids

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on the surface of susceptible cells. Inactivated vaccines against the influenza virus are administered parenterally to induce the production of anti-HA IgG antibodies that are highly protective against homologous virus infection, but less effective against heterologous virus infection [Ichinohe et al., 2008]. In contrast, a number of studies have shown that the mucosal immunity acquired through natural infection, which is mainly due to the secreted form of IgA in the respiratory tract, is more effective and provides greater cross-protection against virus infections than systemic immunity induced by parenteral vaccination in humans and mice [Asahi et al., 2002]. In this regard, induction of secreted IgA in the respiratory tract has a stronger potential to confer protection against unpredictable epidemics of influenza.

In the effort to develop effective intranasal vaccines, cholera toxin and *Escherichia coli* heat-labile toxin have been used as adjuvants to enhance the mucosal immune response [Tamura et al., 2005]. Although these toxins effectively provoke mucosal immune responses, they elicit adverse clinical side effects, such as nasal discharge and the facial paralysis of Bell's palsy [Mutsch et al., 2004]. Therefore, other adjuvants that are both effective and safe for human use have been developed for clinical application with intranasal influenza vaccine [Coulter et al., 2003; Hasegawa et al., 2005; Ichinohe et al., 2005, 2006, 2007a,b; Asahi-Ozaki et al., 2006].

It has been reported that extracts derived from certain mushrooms can elicit an innate immune response, resulting in activation of NF-κB, and strongly stimulate cellular and humoral immunity [Kim et al., 2003; Kuo et al., 2006]. These mushroom extracts induce phenotypic and functional maturation of dendritic cells, tumoricidal activity in macrophages, and augmentation of natural killer cell activity [Sorimachi et al., 2001; Kodama et al., 2005; Kim et al., 2006]. It has also been shown that oral administration of mushroom extracts has an anti-inflammatory effect [Bernardshaw et al., 2006] and decreases IgE levels through modulation of the Th1/Th2 balance [Inagaki et al., 2005; Lim et al., 2005]. In an experimental peritonitis model, mice that were treated orally with edible mushroom (*Agaricus blazei*) extracts prior to bacterial challenge showed significantly lower levels of septicemia and improved survival rates [Bernardshaw et al., 2006]. Extracts from these mushrooms also have been used in immunotherapy to prevent tumor growth and metastasis [Ukawa et al., 2000; Sanzen et al., 2001]. These findings prompted an investigation into whether the administration of intranasal influenza vaccine in combination with mushroom extracts would induce a protective immune response against a lethal and heterologous virus challenge. To accomplish this, the effectiveness of 12 mycelial extracts as an immune-enhancing adjuvant was assessed by comparison with the effects of the adjuvant, poly(I:C). The results of the present study demonstrate for the first time that intranasal administration of inactivated influenza virus vaccine in combination with mycelial extracts as a mucosal adjuvant induces cross-protective immune responses against homologous and heterologous variant influenza viruses, including highly pathogenic influenza A H5N1 virus isolates.

MATERIALS AND METHODS

Mice

Six- to 8-week-old female BALB/c mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). MyD88-deficient mice were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) [Adachi et al., 1998]. Mice were kept under specific pathogen-free conditions approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases.

Vaccines and Influenza Viruses

HA vaccine (split-product virus vaccine) was prepared at the Kitasato Institute (Saitama, Japan) from members of the family Orthomyxoviridae, genus *Influenzavirus A, B*, species *Influenzavirus A*, including influenza A/PuertoRico/8/34 (A/PR8; H1N1). The virus was grown in allantoic cavities of 10- to 11-day-old fertile chicken eggs, purified and disintegrated with ethyl ether. The vaccines contained all proteins from the virus particle; however, the major component of the vaccine was HA (about 30% of the total protein). The A/PR8 virus used for the challenge experiments was adapted for use in mice by subculturing 148 times in ferret, 596 times in mouse, and 73 times in 10-day-old fertile chicken eggs.

The strains of influenza A virus H5N1 used in this study were A/Vietnam/1194/2004 and A/Indonesia/6/2005 [Gao et al., 1999]. The influenza A/Vietnam/1194/2004 virus and influenza A/Indonesia/6/2005 virus obtained from patients with H5N1 disease were propagated in 10-day-old embryonated chicken eggs for 2 days at 37°C. These viruses were stored at −80°C and viral titers were quantified by plaque assay using MDCK cells. The H5N1 vaccine used in these studies was NIBRG14, a formalin-inactivated whole virus vaccine derived from a recombinant avirulent avian virus containing modified HA and neuraminidase from the highly pathogenic avian influenza A/Vietnam/1194/2004 virus and other viral proteins from influenza A/PR/8/34 (H1N1) [Nicolson et al., 2005]. Modified HA lacks the multibasic amino acids at the cleavage site.

Preparation of Adjuvants

The mycelia extracts of *Phellinus linteus*, *Cordyceps militaris*, *Lyophyllum decastes*, *Macrolepiota gracilenta*, *Naematoloma sublateritium*, *A. blazei*, *Grifola frondosa*, *Ganoderma lucidum*, *Hericium erinaceum*, *Inonotus obliquus*, *Lentinula edodes*, and *Pleurotus nebrodensis* were kindly provided by Intelligence Biological Institute Co., Ltd (Nirasaki, Yamanashi, Japan). The extracts of mycelia were prepared as described previously [Inagaki et al., 2005]. Synthetic double-stranded RNA poly(I:C) was kindly provided by Toray