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Human monoclonal antibodies to neutralize all dengue virus serotypes using lymphocytes from patients at acute phase of the secondary infection

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ABSTRACT

The global spread of the four dengue virus serotypes (DENV-1 to -4) has made this virus a major and growing public health concern. Generally, pre-existing neutralizing antibodies derived from primary infection play a significant role in protecting against subsequent infection with the same serotype. By contrast, these pre-existing antibodies are believed to mediate a non-protective response to subsequent heterotypic DENV infections, leading to the onset of dengue illness. In this study, we prepared hybridomas producing human monoclonal antibodies (HuMAbs) against DENV using peripheral blood mononuclear cells (PBMCs) from patients in the acute phase (around 1 week after the onset of illness) or the convalescent phase (around 2 weeks after the onset of illness) of secondary infection. Interestingly, a larger number of hybridoma clones was obtained from patients in the acute phase than from those in the convalescent phase. Most HuMAbs from acute-phase infections were cross-reactive with all four DENV serotypes and showed significant neutralization activity to all four DENV serotypes. Thus, secondary DENV infection plays a significant role in stimulating memory cells to transiently increase the number of antibody-secreting plasma cells in patients in the early phase after the secondary infection. These HuMAbs will enable us to better understand the protective and pathogenic effects of DENV infection, which could vary greatly among secondarily-infected individuals.

1. Introduction

Mosquito-borne dengue virus (DENV) infection occurs in tropical and subtropical regions around the world. The spread of this virus, combined with its severe clinical outcome, has made dengue a major and increasing global public health concern.

DENV has a positive-sense, single-stranded RNA genome of approximately 11 kb that encodes a capsid protein (C), a pre-membrane protein (prM), and an envelope glycoprotein (E), in addition to seven nonstructural proteins (NS) such as NS1 [1].

When humans are repeatedly infected with the same virus, pre-existing memory immune cells quickly produce neutralizing antibodies to protect against the current infection [2]. In DENV, pre-existing neutralizing antibodies raised by the primary infection are protective against subsequent infections with the

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same DENV serotype [3]. Severe dengue cases mostly occur among patients secondarily infected with different DENV serotypes [3]. This may be due to antibody-dependent enhancement (ADE), by which the current infecting virus can use pre-existing anti-DENV antibodies raised during the primary infection to gain entry to Fc receptor-positive macrophages [4–6]. However, it is thought-provoking that most DENV infections are asymptomatic [7], even among individuals secondarily infected with heterotypic DENV [8], and some of these cases show a wide spectrum of clinical symptoms, from a mild illness such as dengue fever (DF) to severe illness such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [9]. In this study, we comparatively prepared hybridomas producing human monoclonal antibodies (HuMAbs) using peripheral blood mononuclear cells (PBMCs) from dengue patients at the acute and convalescent phases of secondary infection.

2. Materials and methods

2.1. Patients

Patient participants were selected based on clinical diagnosis and the results of a rapid test with immunochromatography (SD BIOLINE Dengue Duo kit, SD, Kyonggi-do, Korea). A total of 9 blood specimens for cell fusion were collected from eight Thai dengue patients at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University: three acute-phase patients (around 1 week after the onset of fever) and four convalescent-phase patients (around 2 weeks after the onset of fever), with one patient for both of the acute (D23) and convalescent phases (D26) (Table 1). PBMCs isolated from peripheral blood as described below were used for cell fusion.

2.2. Cell lines and viruses

SPYMEG cells used as fusion partner cells to develop hybridomas producing HuMAbs, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) [10]. Vero cells were maintained in a 5% CO_2 incubator at 37 °C in minimum essential medium (MEM) with 10% FBS. The DENVs used in this study were the Mochizuki strain of DENV-1, the 16681 and New Guinea C (NGC) strains of DENV-2, the H87 strain of DENV-3, and the H241 strain of DENV-4. Culture supernatants from C6/36 cells infected with individual strains were used as viral stocks. Infectivity titers were estimated according to the number of focus-forming units (FFU) as described previously [11].

2.3. Reverse transcriptase (RT)-polymerase chain reaction (PCR) for DENV serotyping

Total RNA was extracted from patient plasma using a QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. This RNA was used as the template for reverse transcription using the Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). Oligonucleotide primer pairs previously reported for serotyping were used for the amplification of the DENV E gene [12].

2.4. Hybridoma preparation

Approximately 10 ml of blood was obtained from individual patients and the PBMCs were isolated by centrifugation through Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). The PBMCs were fused with SPYMEG cells at a ratio of 10:1 as described previously [10].

2.5. Indirect immunofluorescence (IF) assay

Vero cells in a 96-well microplate were mock-infected or infected with DENV. After incubation for 16–24 h, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 1% Triton X-100 in PBS. Undiluted hybridoma culture fluids were used for the HuMAbs. As a positive control, cells were incubated with 4G2, anti-flavivirus E mouse MAb [13]. The plate was stained with 4G2 at 4 °C overnight. The bound antibody was visualized by further reaction with an AlexaFluor 488-conjugated anti-mouse antibody (1:1,000; Invitrogen).

2.6. Neutralization assay

The virus neutralization assay was conducted on culture media of individual hybridoma clones, as described previously [14]. Twenty-five microliters of hybridoma culture supernatant or DMEM supplemented with 15% FBS (as a negative control) was mixed with 100 FFU of individual DENV serotypes (25 µl). After incubation for 15 min, the mixture was used to infect Vero cells in a 96-well microplate. After inoculation at 37 °C for 2 h, 100 µl of MEM with 3% FBS was added. After incubation at 37 °C overnight, the cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. The plate was stained with 4G2 at 4 °C overnight, as for the IF assay. The assays were performed in duplicate and the results expressed as averages. Neutralization activity of HuMAbs in the culture medium from hybridoma clones was expressed as "–" (<50%) and "+" (50–<90%), or "++" (⩾90% reduction in FFU), compared with the negative control.

Table 1
Summary of patients' background and HuMAbs obtained in this study.

Patient	Gender	Age	Diagnosis	Blood collection[a]		Rapid test[b]		PCR serotyping	Hybridoma clone obtained	Isotyping of HuMAb			
				Days	Phase	IgG	IgM			IgG	IgA	IgM	None[c]
D23[d]	Female	33	DF	5	Acute	+	+	DENV-2	75	70	3	0	2
D30	Female	23	DHF grade 1	8	Acute	+	+	DENV-2	25	22	3	0	0
D32	Male	19	DF	6	Acute	+	+	DENV-2	5	5	0	0	0
D33	Male	31	DF	8	Acute	+	+	DENV-2	16	14	2	0	0
D22	Female	25	DHF grade 3	12	Convalescent	+	+	NT[e]	4	3	0	0	1
D25	Male	27	DF	14	Convalescent	+	+	NT	5	5	0	0	0
D26[d]	Female	33	DF	19	Convalescent	+	+	NT	2	2	0	0	0
D27	Male	21	DHF grade 2	13	Convalescent	+	+	NT	2	2	0	0	0
D28	Female	23	DF	15	Convalescent	+	+	NT	2	1	0	0	1

[a] Blood were collected at days after the onset of fever: 5–8 days for acute and 12–19 days for convalescent phase.
[b] Rapid test for D22, D25–D28 was performed with the plasma from these patients at their acute phase.
[c] HuMAbs not reacted with any of IgG, IgA, nor IgM.
[d] D23 and D26 were derived from the same patient at acute and convalescent phases, respectively.
[e] Not tested, because enough amounts of the plasma from the patients at acute phase for RT-PCR were not available.

2.7. Expression vectors for DENV proteins

The CMV4-HA vector was used for the molecular cloning of a fusion form of the E and prM (prM-E) and E DENV genes. On the other hand, the pcDNA3-C-Flag vector was used for the molecular cloning of the prM and C DENV genes. The individual coding regions for these viral proteins derived from DENV-2 NGC strain were amplified and cloned in the above vectors. The NS1 gene was cloned as reported previously [15]. 293T cells transfected with individual plasmids were used as viral antigens for the identification of viral proteins recognized by HuMAbs by IF.

2.8. Isotyping of HuMAbs

HuMAbs were isotyped using the Human IgG ELISA Quantitation set, Human IgM ELISA Quantitation set, and Human IgA ELISA Quantitation set (Bethyl Laboratories, Inc., Montgomery, TX). Fluids from individual hybridoma clone cultures were used for this isotyping.

2.9. Ethics

The research protocols for human samples were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University and informed consent was obtained from all patients before enrollment.

3. Results

3.1. Patients demographics

In this study, the preparation of hybridomas producing HuMAbs against DENV was examined using specimens from Thai patients. PBMC samples were obtained from patients in the acute and convalescent phases. A total of nine samples from eight Thai patients were used: three patients (D30, D32, and D33) in the acute phase, ranging between 6 and 8 days after the onset of fever; and four patients (D22, D25, D27, and D28) in the convalescent phase, 12–15 days after onset of fever. Samples were collected from one patient during both the acute phase and the convalescent phase of infection [D23 (5 days after the onset of fever) and D26 (19 days after the onset of fever)] (Table 1). All acute-phase four patients, based on the results for both anti-dengue IgG and IgM, as well as RT-PCR for DENV serotyping, were the cases of secondary infection with DENV-2. For patients from whom blood samples were available for hybridoma preparation at the convalescent phase, acute-phase plasma samples were used for rapid tests. These tests were all positive for both anti-dengue IgG and IgM, indicating that these patients were also secondarily infected.

3.2. Hybridoma preparation

PBMCs from four acute-phase patients and five convalescent-phase patients were used to prepare hybridomas by fusion with SPYMEG cells, as described [10]. As summarized in Table 1 (see Supplementary Table S1 for the data on individual HuMAbs), 121 acute-phase and 15 convalescent-phase hybridomas showing stable proliferation and production of anti-DENV MAbs were obtained. Isotyping showed IgG-type in 91.7% (111/121) of HuMAbs from acute-phase cells and 86.7% (13/15) of HuMAbs from convalescent-phase cells. IgA-type was detected only in 6.6% (8/121) of HuMAbs from acute-phase cells. There were no positive cases for IgM-type. Culture fluids of four hybridoma clones did not react for IgG, IgA, or IgM.

3.3. Cross-reactivity of HuMAbs with four DENV serotypes

The HuMAbs obtained as described above were characterized for their serological reactivity to all four DENV serotypes by IF and neutralization assays. HuMAbs in the fluids of individual hybridoma cell cultures were used for these assays. As shown in Fig. 1A, the HuMAbs were classified into groups 1–10 and groups A–X based on their cross-reactivity with the four serotypes of DENV in IF and neutralization assays, respectively: group A showed no neutralization activity to any of four serotypes; groups 1–2 and groups B–E showed specific reactions with a single serotype; groups 3–6 and groups F–H showed cross-reactions with two serotypes; groups 7–9 and groups I–O showed cross-reactions with three serotypes; and group 10 and groups P–X showed cross-reactions with all four serotypes.

The IF assay revealed that 109 of 121 clones (90.1%) derived from acute-phase patients were cross-reactive with all four serotypes (Fig. 1A): 65 of 75 clones (86.7%) from D23, 23 of 25 clones (92.0%) from D30, five of five clones (100%) from D32, and 16 of 16 clones (100%) from D33 (Supplementary Table S1). By contrast, only seven of 15 clones (46.7%) derived from convalescent-phase patients were shown to be cross-reactive with all four serotypes (Fig. 1A): three of four clones (75.0%) from D22, two of five clones (40.0%) from D25, one of two clones (50.0%) from D26, one of two clones (50.0%) from D27, and neither of the two clones from D28 (0%) (Supplementary Table S1). Thus, obtaining HuMAbs cross-reactive with all four serotypes was significantly more efficient using PBMCs from acute-phase patients, as compared to convalescent-phase patients ($P = 0.008$). The IF profiles of several representative HuMAbs by IF are shown in Fig. 1B.

Next, we examined the neutralization activity of HuMAbs. The culture fluids from individual hybridoma clones were reacted with DENV-1 to -4. Under these conditions, the control 4G2 showed a $\geqslant$90% reduction in FFU compared with the negative control (DMEM with 15% FBS) in all four serotypes of DENV and, therefore, this MAb was classified into group X. On the other hand, 103 of 121 acute-phase clones (85.1%) and four of 15 convalescent-phase clones (26.7%) showed a $\geqslant$50% reduction in viral replication (Fig. 1A). A $\geqslant$90% reduction in viral replication was detected in 62 of 121 acute-phase clones (51.2%) and one of 15 convalescent-phase clones (6.7%) (Fig. 1A). A total of 70 acute-phase clones (57.9%) and one convalescent-phase clone (6.7%) showed neutralization activity (a $\geqslant$50% reduction in viral replication) against all four serotypes, while only 11 acute-phase (9.1%) and no convalescent-phase clones (0%) showed neutralization activity (a $\geqslant$90% reduction in viral replication) against all four serotypes (Fig. 1A and Supplementary Table S1).

There were inconsistencies between the IF and neutralization data regarding the four HuMAbs: one from patient D23 belonging to group 4-C (in the IF and neutralization assays, respectively), one from patient D23 belonging to group 5-K, one from patient D23 belonging to group 8-U, and one from patient D30 belonging to group 7-N (Fig. 1A).

3.4. Viral protein recognized by HuMAbs

293T cells transfected with expression vectors for the DENV-2 prM, E, NS1, and C proteins, or for the prM-E fusion protein, were used as targets for the identification of viral proteins recognized by individual HuMAbs by IF. Summarized data on viral proteins recognized by individual HuMAbs classified in groups 1–10 by IF assay and in groups A–X by neutralization assay are shown in Tables 2 and 3, respectively (the results from individual HuMAbs are shown in Supplementary Table S1). Of the acute-phase HuMAbs, 99 were reactive with E, eight with prM, four with NS1, and none with C. Culture fluid from the remaining 10 hybridoma clones

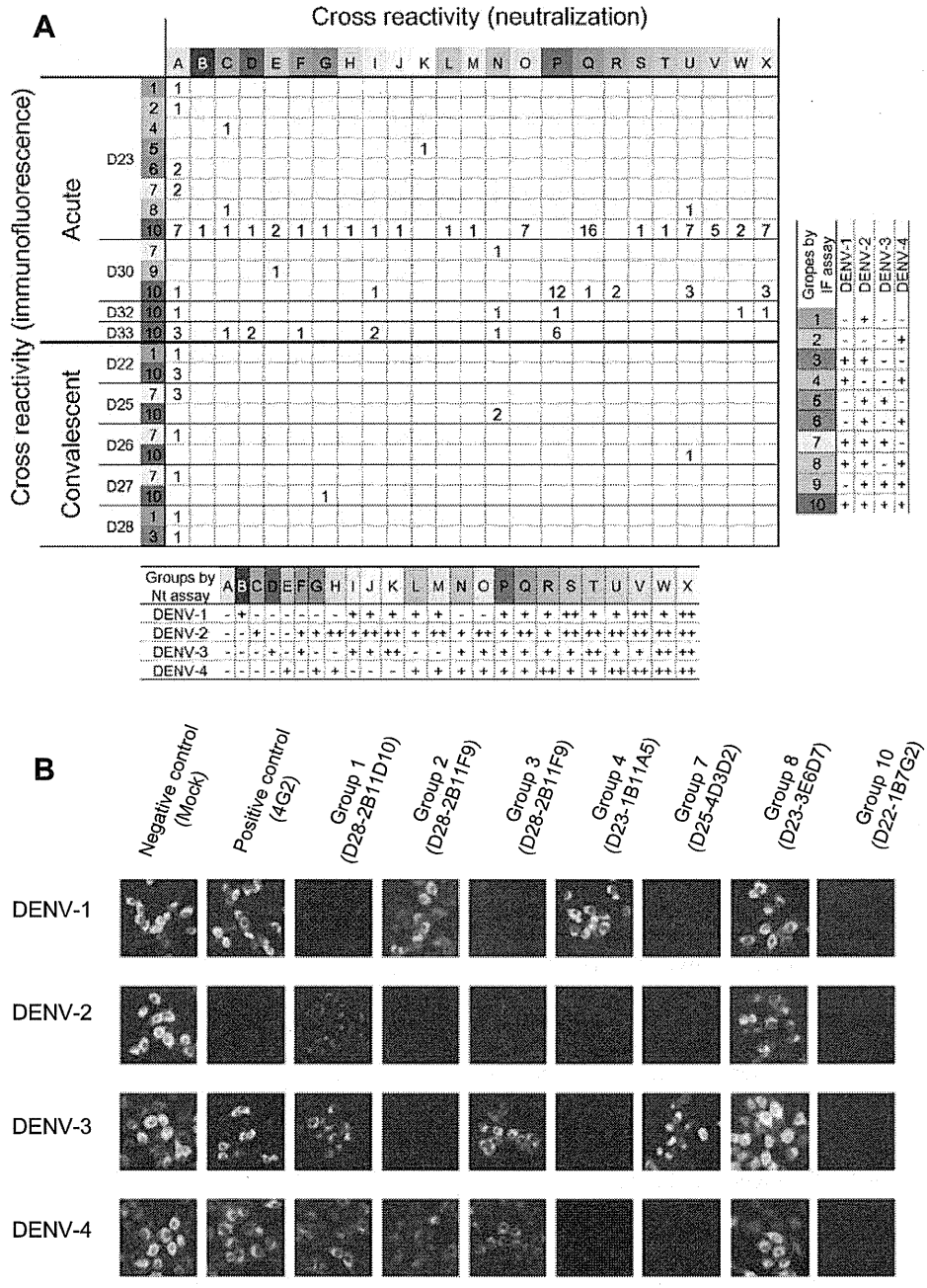

Fig. 1. Correlation between IF and neutralization assay results. (A), A total of 121 acute-phase HuMAbs and 15 convalescent-phase HuMAbs are shown separately to highlight the correlation between IF and neutralization assay ("-",<50%; "+", 50–<90%; and "++", ⩾90% neutralization) according to their cross-reactivity with different DENV serotypes (groups 1–10 according to the IF assay and groups A–X according to the neutralization assay). Culture fluids of HuMAb-producing hybridoma clones were used. Individual groups are shown by different colors. Vero cells individually infected with DENV-1–4 were used as target cells in these assays. (B), The HuMAbs in the culture fluids of hybridoma clones producing DENV serotype-specific (D28–2B11D10 in group 1 and D23–4A7D6 in group 2), cross-reactive with two serotypes (D28–2B11F9 in group 3 and D23–1B11A5 in group 4), and cross-reactive with three serotypes (D25–4D3D2 in group 7 and D23–3E6D7 in group 8), and cross-reactive with all four serotypes (D22–1B7G2 in group 10) antibodies were used for IF. Vero cells mock-infected with PBS or individually infected with DENV-1–4 were used as target cells. As a positive control, 4G2 anti-flavivirus E mouse MAb [13] was used.

was not reactive with the E, prM, NS1, or C proteins ("Other"). Of the convalescent-phase HuMAbs, two were reactive with E, two with prM, eight with NS1, and none with C, and the remaining three were not reactive with any of the proteins assayed. Interestingly, five HuMAbs obtained from D25 in the convalescent phase were all reactive against NS1 (Supplementary Table S1).

Tables 2 and 3 summarize the viral proteins recognized by HuMAbs broken down according to reactivity group. The 98 HuMAbs recognizing E (96 of 99 HuMAbs from the acute-phase and two of two HuMAbs from the convalescent-phase) were all in group 10 (cross-reactive with all four serotypes) according to the IF assay (Table 2). Of these, 70 acute-phase and one convalescent-phase HuMAbs showed ⩾50% neutralization activity against all four DENV serotypes (groups P to X). Of the 70 acute-phase HuMAbs, 11 also showed ⩾90% neutralization activity against all four DENV serotypes (group X).

Table 2
Target viral proteins of HuMAbs categorized by immunofluorescence assay results.

	DENV serotype				Immunofluorescence assay							
					HuMAb from acute phase				HuMAb from convalescent phase			
	1	2	3	4	E	prM	NS1	Other[a]	E	prM	NS1	Other[a]
1	-	+	-	-				1				2
2	-	-	-	+				1				
3	+	+	-	-							1	
4	+	-	-	+				1				
5	-	+	+	-	1							
6	-	+	-	+				2				
7	+	+	+	-			3[b]				5	
8	+	+	-	+	2							
9	-	+	+	+				1				
10	+	+	+	+	96	8	1	4	2	2	2[c]	1

[a] No reaction at least with prM-E, E, prM, and NS1.
[b] The HuMAb (D30–2B1G5) is also reactive with E weakly.
[c] The HuMAb (D25–2B11G11) is also reactive with E and prM weakly, while the HuMAb (D25–4D4F10) is also reactive with prM weakly.

Table 3
Target viral proteins categorized by neutralization assay results.

	DENV serotype				Neutralization assay							
					HuMAb from acute phase				HuMAb from convalescent phase			
	1	2	3	4	E	prM	NS1	Other[a]	E	prM	NS1	Other[a]
A[b]	-	-	-	-	5	5	3	5	1	1	6	3
B	+	-	-	-		1						
C	-	+	-	-	3			1				
D	-	-	+	-	1			2				
E	-	-	-	+		2		1				
F	-	+	+	-	2							
G	-	+	-	+	1					1		
H	-	++	-	+	1							
I	+	+	+	-	4							
J	+	++	+	-	1							
K	+	++	++	-	1							
L	+	+	-	+				1				
M	+	++	-	+	1							
N	-	+	+	+	2		1[c]				2[d]	
O	-	++	+	+	7							
P	+	+	+	+	19							
Q	+	++	+	+	17							
R	+	+	+	++	2							
S	++	++	+	+	1							
T	+	++	++	+	1							
U	+	++	+	++	11				1			
V	++	++	+	++	5							
W	+	++	++	++	3							
X	++	++	++	++	11							

[a] No reaction at least with prM-E, E, prM, and NS1.
[b] HuMAbs showing positive reactions with DENV by immunofluorescence assay, but no neutralization activity to any serotypes of DENV.
[c] The HuMAb (D30–2B1G5) is also reactive with E weakly.
[d] The HuMAb (D25–2B11G11) is also reactive with E and prM weakly, while the HuMAb (D25-4D4F10) is also reactive with prM weakly.

4. Discussion

A total of 136 hybridoma clones producing specific HuMAbs against DENV were obtained using PBMCs from nine blood samples from eight patients. The samples from the four acute-phase patients secondarily infected with DENV-2 efficiently generated hybridomas producing specific and robust HuMAbs, compared with those from the five convalescent-phase patients. In addition, most of the acute-phase HuMAb clones were cross-reactive with all four serotypes of DENV by IF. Further, most of these cross-reactive HuMAb clones recognized the viral E protein and were able to neutralize all four serotypes of DENV. Thus, humoral immune status in patients seems to be dynamically changing between the acute and convalescent phases of secondary DENV infection. Antibodies at the acute phase showed complex cross-reactivity with all four DENV serotypes, with much stronger neutralization activity not only against DENV-2, which was replicating in the patient, but also against the other serotypes of DENV.

PBMC samples in this study were collected from patients at the acute phase (5–8 days after the onset of fever) or at the convalescent phase (12–19 days for convalescent phase) of secondary infection. This study enabled us to compare the efficiency of obtaining HuMAbs at each stage. From the acute-phase PBMCs, 81.8% anti-E, 6.6% anti-prM, and 3.3% anti-NS1 HuMAbs were obtained, while 13.3% anti-E, 13.3% anti-prM, and 53.3% anti-NS1 HuMAbs were obtained from convalescent-phase PBMCs. Several groups have used PBMCs from convalescent-phase, but not acute-phase, patients to prepare HuMAbs by immortalizing patient-derived B cells with EB virus. Dejnirattisai et al. [16] observed that 89% of anti-E HuMAbs were cross-reactive with all four serotypes. Surprisingly,

their studies resulted in the preparation of more anti-prM than anti-E HuMAbs. Beltramello et al. [17] performed a large screen to gain insights into the domain specificity and cross-reactivity of E domain III-specific antibodies. A study by de Alwis et al. [18] showed that the efficiency of preparation of DENV complex cross-reactive neutralizing HuMAbs was significantly higher in secondary infection cases. Indeed, Beltramello et al. [17] differentiated their HuMAbs into two categories: those that recognized the DENV E domain III and showed complex cross-reactive neutralization activity, and those that recognized domain I/domain II and were more broadly cross-reactive but showed lower neutralization activity. Furthermore, our data in this study is the first to report the efficient preparation of HuMAbs with strong neutralization activity against all four DENV serotypes, using PBMCs from acute-phase patients secondarily infected with DENV.

It was an unexpected finding that acute-phase PBMCs were more efficient in the production of DENV-specific HuMAbs than convalescent-phase PBMCs, as neutralizing antibody titers tended to be slightly higher in convalescent-phase patients. This finding is similar to the findings of Wrammert et al. [19], who demonstrated a similar phenomenon for HuMAbs against the influenza virus in vaccinated donors. That study found a rapid and robust induction of influenza-specific IgG^+ antibody-secreting plasma cells, which accounted for up to 6% of the peripheral blood B cells at the peak of the response, approximately 7 days after vaccination. However, the influenza-specific IgG^+ memory B cells fell to an average of 1% of all B cells by 14–21 days after vaccination. Generally, reports show a difference in the B cell phenotype between acute- and convalescent-phase patients with infectious diseases [20]. Consequently, many HuMAbs showing neutralizing activity could be obtained in the acute phase. In addition, neutralization assay of the HuMAbs obtained in this study classified them into heterogeneous groups: serotype-specific HuMAbs and cross-reactive HuMAbs with two, three, and all four serotypes of DENV. These HuMAbs will also be highly useful as probes to understand the complex mechanisms through which the same antibodies mediate neutralization and ADE of heterologous DENV serotypes. Further epitope mapping studies of these HuMAbs would help shed light on this important issue.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.057.

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Poly (I:C), an agonist of toll-like receptor-3, inhibits replication of the Chikungunya virus in BEAS-2B cells

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Abstract

Background: Double-stranded RNA (dsRNA) and its mimic, polyinosinic acid: polycytidylic acid [Poly (I:C)], are recognized by toll-like receptor 3 (TLR3) and induce interferon (IFN)-β in many cell types. Poly (I:C) is the most potent IFN inducer. In *in vivo* mouse studies, intraperitoneal injection of Poly (I:C) elicited IFN-α/β production and natural killer (NK) cells activation. The TLR3 pathway is suggested to contribute to innate immune responses against many viruses, including influenza virus, respiratory syncytial virus, herpes simplex virus 2, and murine cytomegalovirus. In Chikungunya virus (CHIKV) infection, the viruses are cleared within 7–10 days postinfection before adaptive immune responses emerge. The innate immune response is important for CHIKV clearance.

Results: The effects of Poly (I:C) on the replication of CHIKV in human bronchial epithelial cells, BEAS-2B, were studied. Poly (I:C) suppressed cytopathic effects (CPE) induced by CHIKV infection in BEAS-2B cells in the presence of Poly (I:C) and inhibited the replication of CHIKV in the cells. The virus titers of Poly (I:C)-treated cells were much lower compared with those of untreated cells. CHIKV infection and Poly (I:C) treatment of BEAS-2B cells induced the production of IFN-β and increased the expression of anti-viral genes, including IFN-α, IFN-β, MxA, and OAS. Both Poly (I:C) and CHIKV infection upregulate the expression of TLR3 in BEAS-2B cells.

Conclusions: CHIKV is sensitive to innate immune response induced by Poly (I:C). The inhibition of CHIKV replication by Poly (I:C) may be through the induction of TLR3, which triggers the production of IFNs and other anti-viral genes. The innate immune response is important to clear CHIKV in infected cells.

Keywords: Chikungunya virus, Poly (I:C), BEAS-2B cells, TLR3

Introduction

Chikungunya virus (CHIKV), the causative agent for Chikungunya fever, was first described in 1952 during an epidemic in Tanzania, East Africa [1,2]. CHIKV is a positive-sense single-strand RNA virus belonging to the genus *Alphavirus* of the family *Togaviridae*, and it is maintained in two distinct transmission cycles, a sylvatic cycle and a human-mosquito-human cycle. The scale of epidemics of the former is smaller and is mainly confined within African countries, involving primates such as monkeys and forest-dwelling Aedes mosquitoes [3]. CHIKV is mainly transmitted by *Aedes aegypti* and *Aedes albopictus*. CHIKV epidemics have often been characterized by long interepidemic (more than 10 years) periods in many parts of Southern and Southeast Asia [4-7]. During the past 8 years, major outbreaks have occurred among islands in the Indian Ocean, with Reunion Island being one of the most severely hit islands. One-third of its population were infected, and more than 240 people died [8-12]. The symptoms of Chikungunya generally start 4–7 d after the bite. Acute infection lasts 1–10 days and is characterized by a painful polyarthralgia, high fever, asthenia, headache, vomiting, rash, and myalgia [13,14]. CHIKV infection has affected as many as

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3–4 million people in the Indian Ocean zone, and it spread to Europe in 2005–2007. This disease has recently received considerable attention in Thailand [15-19].

CHIKV transmission is rapid and extensive; however, humans are not defenseless, and in fact, CHIKV is efficiently cleared within 4–7 days after infection *in vivo* [20-22]. As a typical adaptive immune response, such as CHIKV-specific B-cell and T-cell activation, requires at least 1 week for development, the innate immune system seems to control CHIKV infection [23]. CHIKV is known to infect many different cell types, including fibroblasts and epithelial and endothelial cells *in vitro* [24] and fibroblast cells *in vivo* [25]; however, epithelial cells are armed with various mechanisms that are able to sense viral components and initiate intracellular signal transduction to respond rapidly to viral infections [26]. Polyinosinic: polycytidylic acid [Poly (I:C)], a synthetic double-stranded RNA (dsRNA) analog, is an immunostimulant that acts as the most potent interferon (IFN) inducer [27]. In *in vivo* mouse studies, intraperitoneal injection of Poly (I:C) elicited IFN-α/β production and natural killer (NK) cells activation [28,29]. Poly (I:C) is known to interact with toll-like receptor 3 (TLR3), which is expressed in the membrane of B-cells, macrophages, and dendritic cells.

TLRs are a member of the family of host innate immune receptors, and they are essential for detecting pathogen-associated molecular patterns. TLRs are transmembrane signaling proteins designed to specifically recognize various proteins, carbohydrates, lipids, and nucleic acids of invading microorganisms. When a TLR is activated, it triggers immune and inflammatory responses to infectious agents [30]. The TLR3 pathway contributes to an innate immune response against many viruses, including influenza virus [31], respiratory syncytial virus [32], herpes simplex virus 2 [33], and murine cytomegalovirus [34]. The detection of viral dsRNA and Poly(I:C) in the cytosol is mediated through the helicase family members retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5), thus allowing the host to sense directly an intracellular viral infection in a TLR3-independent way [35,36]. *In vitro* studies have shown that RIG-I and MDA-5 are both capable of responding to Poly(I:C) and RNA viruses [37].

In this study, Poly (I:C) was used to examine the innate immune response *in vitro*. We found that Poly (I:C) suppressed the cytopathic effect (CPE) induced by CHIKV infection and inhibited the replication of CHIKV in human bronchial epithelial-derived cells, BEAS-2B, by inducing the expression of IFNs and interferon-inducible intracellular antiviral factor genes, including OAS and MxA. Based on our results, we concluded that the CHIKV was sensitive to IFNs and that the innate immune response plays an important role in the clearance of CHIKV.

Results

Poly (I:C) suppressed CPE induced by CHIKV infection

BEAS-2B cells were seeded in 6-well plates ($1x10^6$ cells/well) one day before Poly (I:C) treatment. One hour before infection at multiplicity of infection (MOI) 0.01, 1, or 5, the cells were pre-treated with 4 μg/ml of Poly (I:C) or left untreated. After adsorption, the cells were maintained in the medium with or without Poly (I: C) (4 μg/ml). The CPE was observed at 24, 48, and 72 h postinfection (p.i.) under a microscope. No CPE was found in Poly (I:C)-treated cells at 24 or 48 h p.i., even when MOI 5 was used. Although CPE was found in the Poly (I:C)-treated cells at 72 h p.i., it was less significant compared with that of untreated cells (Figure 1), demonstrating that Poly (I:C) treatment appeared to decrease CPE induced by CHIKV infection. Because the protection of CPE was decreased at 72 h p.i., we conclude that the protection is important in the early phase of infection.

Poly (I:C) inhibited replication of CHIKV in BEAS-2B cells

Since the Poly(I:C) decreased CPE in BEAS-2B cells induced by CHIKV infection, we supposed that Poly(I:C) may inhibit the replication of CHIKV. To clarify the effect of Poly (I:C) treatment, we measured the virus titers produced by Poly (I:C)-treated and mock-treated cells by plaque assay (Figure 2). The supernatant was collected at 24, 48, and 72 h p.i. at each MOI. The virus titers from mock-treated cells were $1.5x10^6$, $5.5x10^5$, and $4.5x10^3$ pfu/ml at MOI 0.01; $4.3x10^6$, $1x10^6$, and $5x10^4$ pfu/ml at MOI 1; $3.5x10^8$, $6.9x10^7$, and $3x10^5$ pfu/ml at MOI 5 at 24, 48, and 72 h p.i., respectively. The virus titers of the supernatant from Poly (I:C)-treated cells were $2.5x10^2$, $1.5x10^2$, and $1x10^2$ pfu/ml at MOI 0.01; $2.5x10^3$, $2x10^3$, and $6.3x10^2$ pfu/ml at MOI 1; $2x10^6$, $6x10^5$, and $5.5x10^3$ pfu/ml at MOI 5 at 24, 48, and 72 h p.i., respectively, indicating that Poly (I:C) treatment significantly lowers the virus titers. With either Poly (I:C) treatment or non-treatment, the virus titers showed a high peak at 24 h p.i. in the infections with the same MOIs and a trend to decrease at 48 and 72 h p.i. These results indicated that Poly (I:C) inhibited the replication of CHIKV in BEAS-2B cells. This is probably because IFN-β induced by Poly (I:C) treatment plays a role, as described previously [31].

Induction of IFN-β and stimulation of TLR3 expression in BEAS-2B cells by poly (I:C) treatment or CHIKV infection

Poly (I:C) is a strong IFN inducer. The effects of Poly (I:C) treatment on CHIKV infection in BEAS-2B cells may be due to the production of IFNs. To elucidate

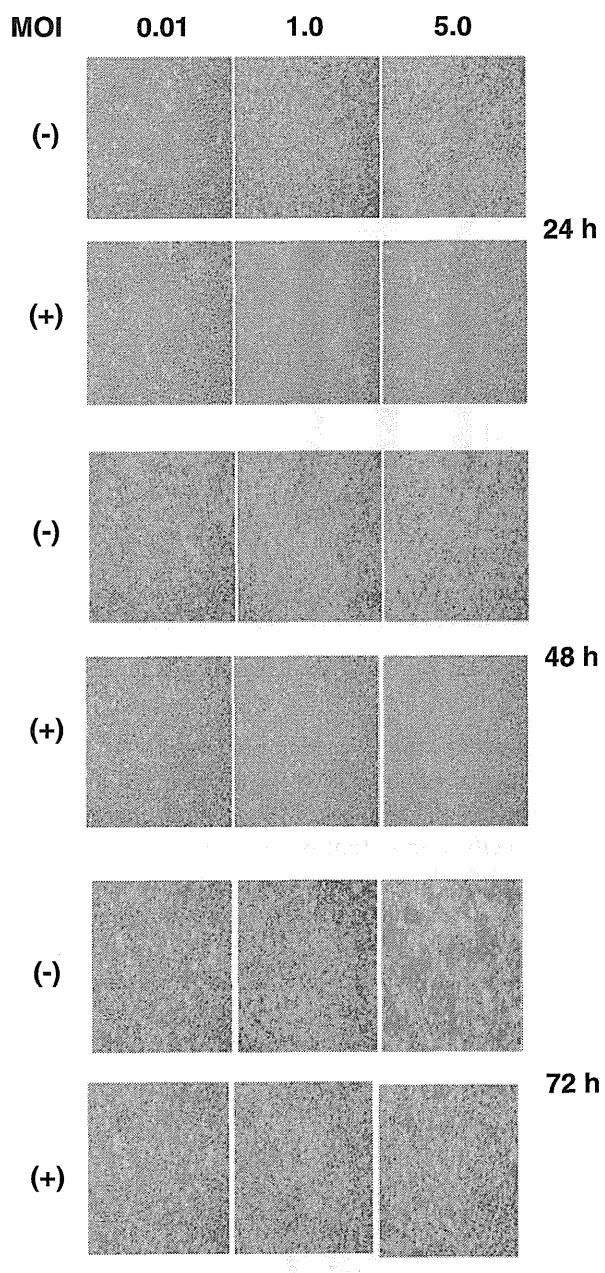

Figure 1 Effect of Poly (I:C) treatment on CPE. BEAS-2B cells were treated with 4 μg/ml of Poly (I:C) **(+)** or mock-treated **(–)** for 1 h, and then infected with CHIKV at MOI 0.01, 1, and 5. At 24, 48, and 72 h p.i., CPE was observed under a microscope.

the level of IFN-β, we treated BEAS-2B cells with 4 μg/ml of Poly (I:C) and measured the amount of IFN-β by ELISA. The concentration of IFN-β in the supernatant at 0, 2, 4, 8, 16, and 24 h p.i. was 490.60, 681.69, 984.61, 947.82, 736.91, and 710.54 pg/ml, respectively, indicating that Poly (I:C) treatment induced the secretion of IFN-β. The IFN-β level reached a peak at 4 h during the treatment (Figure 3A). The IFN-β was also induced by CHIKV infection (MOI 0.8) and reached a peak at 24 hours p.i. The concentration at 0, 2, 4, 8, 16, and 24 h p.i. was 501.54, 526.67, 547.23, 907.43, 1585.95, and 2614.92 pg/ml, respectively, (Figure 3B). TLR3 was known as a receptor for dsRNA [38,39], and upon recognition of dsRNA, TLR3 transmits signals that activate the transcript factors IFR-3, NF-⊠B, and AP-1, leading to the induction of type I IFN [40] [41]. The level of TLR3 expression examined by PCR is shown in Figure 3C. The expression of TLR3 mRNA was upregulated by both Poly (I:C) treatment and CHIKV infection after 24 hours. The induction of IFN-β may be triggered through the upregulated expression of TLR3.

Induction of IFN-α, IFN-β, MxA, and OAS genes

One unique feature of TLR3 is to trigger the induction of the type I IFNs (IFN-α/β). In addition TLR3 is known to induce the expression of interferon-inducible intracellular antiviral factors including OAS and MxA [42,43]. We examined the expression of mRNA of these genes using RT-PCR. As shown in Figure 4, Poly (I:C) treatment stimulated the induction of IFN-β mRNA, and a significant upregulation was observed at 2 h post treatment. The expression level was still apparent, albeit at a lower level, at 4 and 8 h post treatment. A significant upregulation of IFN-α was observed at 16 h poststimulation. Exposure of BEAS-2B cells to Poly (I:C) induced time-dependent expression of MxA and OAS mRNA; however, unlike IFN-α/β, the levels of these two transcripts remained elevated 4, 8, 16, and 24 h poststimulation (Figure 4.) These results indicated that Poly (I:C) induced the anti-viral genes that may contribute to the inhibition of CHIKV replication.

Discussion

The innate immune response is the first barrier against the viruses [44], initiated within hours after the viruses bind to the receptor, and it plays a central role in the detection of invading pathogens. The innate immune system responds through activating inflammatory and antiviral defense mechanisms against the infectious agents [45]. Innate immunity involves the induction of many factors, including IFNs-α/β, which induce a range of antiviral processes. In infected cells, it is believed that the proximal inducer of IFNs-α/β is intracellular dsRNA generated as an intermediate during viral replication [38].

During virus replication, not only dsRNA but also single-stranded RNA (ssRNA) molecules are recognized as intermediate by TLRs expressed in dendritic cells, natural killer cells, and macrophages, as well as in epithelium [46]. The dsRNA triggers a series of events culminating in the activation of PKR and other kinases. Phosphorylation of the substrates of these

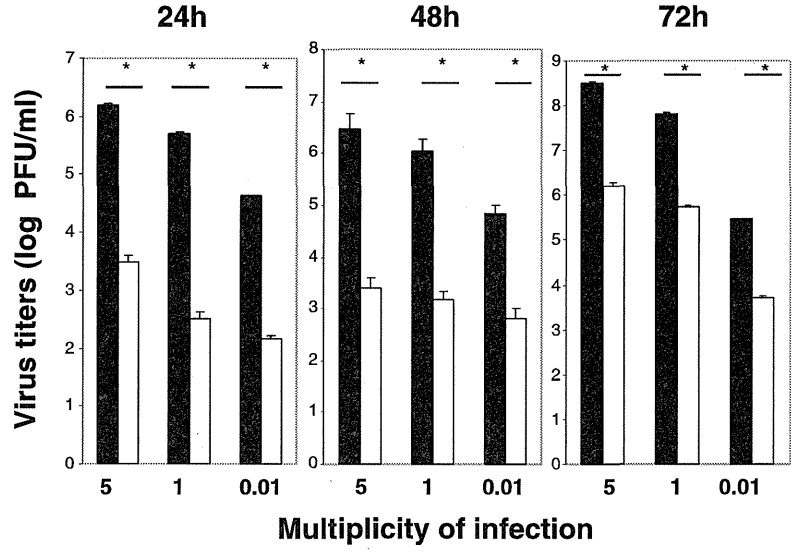

Figure 2 Effect of Poly (I:C) treatment on CHIKV growth. BEAS-2B cells were treated with 4 μg/ml of Poly (I:C) (□) or mock-treated (■) for 1 h, and then infected with CHIKV at MOI 0.01, 1, and 5. At 24, 48, and 72 h p.i., the virus titer in the supernatant was measured by a plaque assay. *P < 0.01 by Student's unpaired *t*-test.

enzymes results in the translocation of transcription factors, NF-⊠B and IRF-3, from the cytoplasm to the nucleus, where they bind to the IFN-β promoter to form a transcription complex that ultimately drives IFN-β production [47,48] [49,50]. Several *in vitro* studies have demonstrated that Poly (I:C), a TLR3 agonist, induces antiviral responses through the induction of IFN-β [51].

In the present study, we demonstrated that following Poly (I:C) treatment, BEAS-2B cells produced antimicrobial factors IFN-β, OAS, and MxA, which may constitute a highly specific and potent barrier against CHIKV infection. Poly (I:C) is known to markedly upregulate the IFN-β mRNA level in a dose-dependent manner in mouse osteoblastic MC3T3-E1 cells [52]. Similarly, trophoblast cells are known to express and secrete

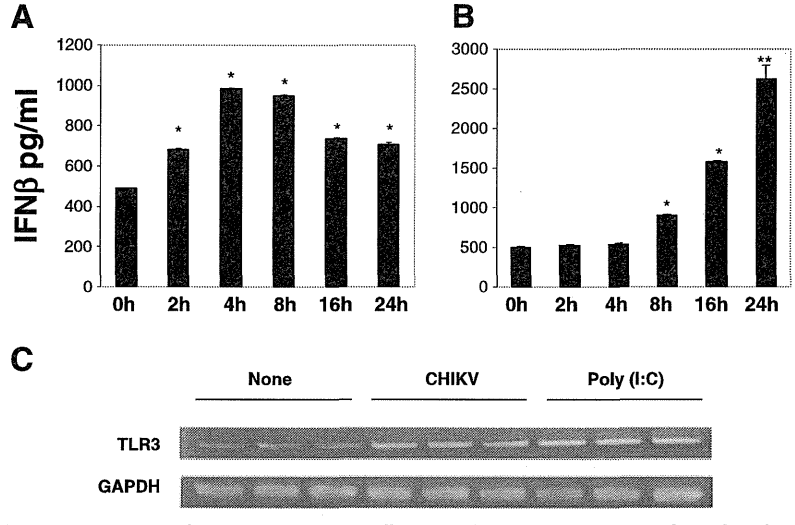

Figure 3 Induction of IFN-β and expression of TLR3 in BEAS-2B cells treated with Poly (I:C) or infected with CHIKV. (**A**) BEAS-2B cells were incubated in the presence of 4 μg/ml of Poly (I:C), and IFN-β secreted in the medium was measured by an ELISA. The samples were collected at 0, 2, 4, 8, 16, and 24 p.i. (**B**) BEAS-2B cells were infected with CHIKV at MOI 0.8. IFN-β in the medium was measured by an ELISA at 0, 2, 4, 8, 16, and 24 h p.i.. * P < 0.05: ** P < 0.01 relative to the 0 h time point. (**C**) Expression of TLR3 in BEAS-2B cells was detected by RT-PCR. The cells were incubated with 4 μg/ml of Poly (I:C) or infected with CHIKV at MOI 0.8. Total RNA was extracted from the cells at 24 h p.i., and TLR3 mRNA was amplified by RT-PCR. The products were analyzed by agarose gel electrophoresis. A representative result of the experiment performed in triplicate is shown.

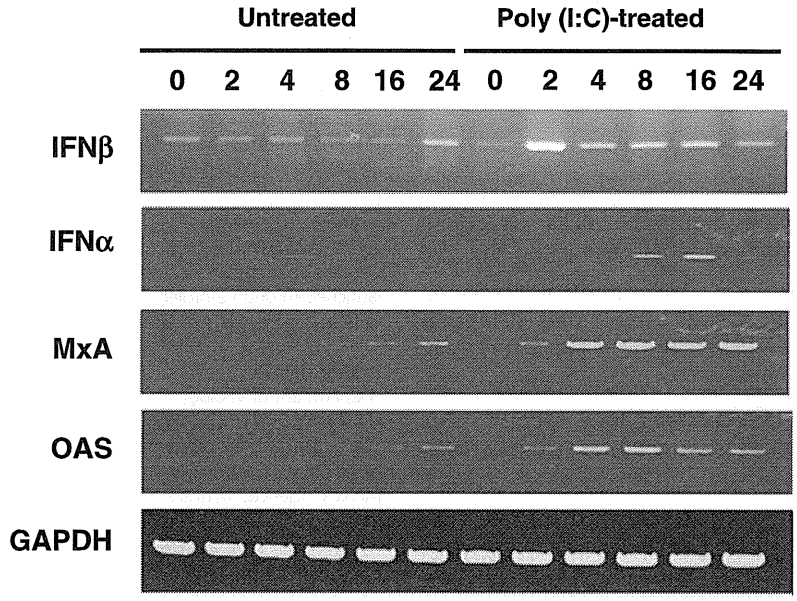

Figure 4 Expression of anti-viral genes after Poly (I:C) treatment. BEAS-2B cells were treated or not treated with Poly (I:C), and a total RNA was extracted from the cells at 0, 2, 4, 8, 16, and 24 h p.i. The IFN-β, IFN-α, MxA, and OAS genes were amplified by RT-PCR, and the products were analyzed by agarose gel electrophoresis.

antiviral factors, such as OAS, MxA, and APOBEC3G, by Poly (I:C) [53]. Poly (I:C) treatment also inhibited the multiplication of xenotropic baboon type C endogenous retrovirus M7 in chronically infected human AV3-M7 cells [54] and human immunodeficiency virus amplification in dendritic cells via type I IFN-mediated activation of APOBEC3G [55].

Based on the results of the present study, we conclude that both Poly (I:C) and CHIKV infection enhanced the expression of TLR3. The stimulation of TLR3 by dsRNA transduces signals to activate the transcription factors NF-ĸB and IRF/interferon-sensitive response element (ISRE) via myeloid differentiation factor 88 (MyD88)-independent signaling pathways, which involve a distinct adaptor molecule, namely the Toll-interleukin (IL)-1 receptor (TIR) domain containing adaptor-inducing IFN-β (TRIF), also called the TIR domain containing adaptor molecule 1 (TICAM-1) [56,40]. This molecule elicits an antiviral response, especially through the production of IFNs-α/β [57]. Therefore, IFNs could contribute to decrease the CPE and inhibit the replication of CHIKV through TLR3 stimulation. Similar phenomena were reported in influenza virus in BEAS-2B cells. Both Poly (I:C) and influenza virus infection induced IFN- β [31]. The replication of CHIKV is controlled by IFNs-α/β [24], which is critically dependent on the action of non-hematopoietic cells through the induction of one or more IFN-stimulated genes (ISGs) [58]. Therefore, induction of IFNs and antiviral genes observed in this study could contribute to the Poly (I:C)-mediated suppression of CPE and inhibition of the replication of CHIKV in BEAS-2B cells.

Poly (I:C) was widely used as an adjuvant for vaccine research. Poly (I:C)-combined intranasal vaccine protected mice against influenza virus infection, including that due to highly pathogenic H5N1 [59-61]. Synthetic dsRNA is adjuvant for the induction of T helper 1 and humoral immune response to human papillomavirus in rhesus macaques [62]. Therefore, Poly (I:C) could be an adjuvant for CHIKV vaccine, which can increase the immune response in humans to clear the CHIKV.

Conclusions

CHIKV is sensitive to innate immune response induced by Poly (I:C). Poly (I:C) decreased CPE and inhibited the CHIKV replication in BEAS-2B cells. The Poly (I:C) inhibition of CHIKV replication may be through the induction of TLR3, which triggers the production of IFNs and other anti-viral genes, such as MxA and OAS. The innate immune response is important to clear CHIKV in infected cells.

Materials and methods

Viruses, cells, and reagents

Chikungunya viruses (Ross Strain) were propagated in Vero-E6 (Vero) cells. The virus titer was measured by a plaque assay. BEAS-2B, a SV-40-transformed airway bronchial epithelial cell line, was purchased from American Type Culture Collection (Manassas, VA). Cells were

maintained in RPMI-1640 supplemented with 10% FCS. All experiments were performed in a biosafety level 3 containment laboratory. Poly (I:C) was purchased from Sigma-Aldrich (St. Louis, MO).

RT-PCR

Total RNA was extracted from the cells by using TRIzoL (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RT was performed using 3.5 μg of total RNA. PCR was performed using an INF-α forward primer (5'-TTTCTCCTGCCTGAAG GACAG-3') and an INF-α reverse primer (5'-TCTCATGATTTCTGCTCTGACA-3'), a IFN-β forward primer (5'-CTGTGGCAATTGAATGGGAGGC -3') and a IFN-β reverse primer (5'-CAGGCACAGT GACTGTCCTCCTT-3'), a MxA forward primer (5'-CATACTGCGAGGAGATCCTCCTT-3') and a MxA reverse primer (5'-AGCATCCGAAATCTCAATCTCGTA -3'), a OAS forward primer (5'-AGAATGTCAGACACT GATCGACGA-3') and a OAS reverse primer (5'-TGTTCCCAGGCATACACCGTA-3'), a TLR3 forward primer (5'-AAATTGGGCAAGAACTCACAGG-3') and a TLR3 reverse primer (5'-GTGTTTCCAGAGCC GTGCTAA-3'), and a GAPDH forward primer (5'-CAC-CACCAACTGCTTAGCAC-3') and a GAPDH reverse primer (5'-CCCTGTTGCTGTAGCCAAAT-3'). Amplification products were resolved on 1.5% agarose gel containing ethidium bromide.

Plaque assay

Vero cells were seeded at $2.5x10^5$ cells per well in 24-wells plates, incubated at 37°C overnight, and washed once with phosphate buffered saline (PBS). Ten-fold serial dilutions of the virus mixture were prepared in Hanks buffer (Sigma-Aldrich), and then 0.1 ml of the mixture was inoculated into each well and incubated for 1 h at 37°C, during which we agitated the plate every 15 minutes. After adsorption for 1 h, the plate was washed with PBS three times, and 1 ml of DMEM containing 2% carboxymethyl cellulose (W/V) (Sigma-Aldrich) and 5% FBS was layered onto the cells. The plates were incubated in a humidified incubator at 37°C with 5% CO_2 for 3 days. The overlay was removed and washed with PBS. Plaques were visualized by staining the monolayer with 1 ml 0.5% crystal violet containing 10% formaldehyde (Sigma-Aldrich) for 2 h at room temperature. The virus plaques were counted after thorough washing with tap water.

Cytokine measurements

The concentration of human IFN-β in cell culture supernatants was determined by using DuoSet Elisa kits (R&D Systems, Minneapolis, MN).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YG. Li and SA conceived and designed the experiment. US, UT, NN, AA, YP. MK, KT. Performed the experiments. KI, NT. YG. LI and SA analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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A technique for capturing broad subtypes and circulating recombinant forms of HIV-1 based on anionic polymer-coated magnetic beads

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Abstract. Magnetic beads coated with an anionic polymer, poly(methyl vinyl ether-maleic anhydrate) [poly(MVE-MA)], were used in a method to capture human immunodeficiency virus type-1 (HIV-1). The beads were incubated with either HIV-1-infected cell culture medium or plasma from HIV-1 infected individuals and separated from the supernatant by applying a magnetic field. After thorough washing, adsorption of HIV-1 by the beads was confirmed by reverse transcription (RT)-polymerase chain reaction (PCR), real-time PCR, enzyme-linked immunosorbent assay and western blotting. The results confirmed the presence of envelope, polymerase, Nef and the viral genome of HIV-1. Furthermore, various subtypes and circulating recombinant forms (CRFs) of HIV-1 including subtype B, C and CRF01_AE and the immature form of subtype B HIV-1 could be captured. Preincubation with neutralizing antibody against HIV-1 envelope gp41 decreased the capture efficiently, suggesting that poly(MVE-MA) binds HIV-1 *via* gp41. We believe that this capture procedure will be a valuable tool for detecting various types of HIV-1 in both clinical and experimental samples.

Introduction

Rapid and sensitive detection of viruses in blood is critically important in order to reduce the spread of disease as a result of transfusion (1). Human immunodeficiency virus type-1 (HIV-1) is a major virus linked to transfusion-associated transmission of disease. Although serological detection of antibodies against HIV-1 can reduce the risk of disease transmission, a better procedure is urgently required (2-8). For example, during the pre-seroconversion window period the quantity of antibody against the virus is low despite the a high load of HIV-1 present in the blood. Infection with immunovariant viruses and immunosilent carriage cause a similar condition. Recent developments in enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and immunochromatography facilitate the detection of HIV-1 in biological samples (9-12). Nonetheless, the sensitivity of these procedures is insufficient to eliminate the risk of viral transmission.

There are two major limiting factors in the development of a protocol to concentrate HIV-1: i) compatibility with current methods of detection and ii) requirement for a straightforward procedure. Several approaches have been used to increase the concentration of viruses in order to enhance the sensitivity of detection (13-15). For example, ultracentrifugation and polyethylene glycol (PEG) mediated precipitation have been used to concentrate a number of different viruses including HIV-1. Ultracentrifugation is a well-known procedure, but is time-consuming and can increase the false-positive rate when combined with PCR (12,16). Although PEG precipitation is simple and easy to perform, the PEG sometimes interferes with the subsequent PCR (17). One alternative approach is to use magnetic beads coated with molecules that efficiently bind viral particles. Indeed, we and other groups have reported that an anionic polymer, poly(methyl vinyl ether-maleic anhydrate) [poly(MVE-MA)] can be used to capture different viruses.

Here, we report that magnetic beads coated with poly(MVE-MA) are useful for the capture of various subtypes and circulating recombinant form (CRF) of HIV-1. The potential of this method and the mechanisms by which the beads bind HIV-1 are being discussed.

Materials and methods

Reagents. Unless otherwise specified, chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 300-nm-diameter magnetic particles (reducing sedimentation and offering a broad binding surface) with a high ferrite content (allowing

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separation under a magnetic field) were prepared by grafting of poly(MVE-MA) in dimethyl sulfoxide/phosphate buffer 5/95 solution for 3 h at 37°C (Flavigny *et al*, American Society for Microbiology 104th General Meeting, 166, 2004). The anionic magnetic beads, Viro-adembeads, were obtained from Ademtech (Pessac, France).

Samples. For analysis, we used either cell culture medium of HIV-1 (LAI or L2)-infected MT4 cells (NIH AIDS Research and Reagent Program) or 293T cells (American Type Culture Collection CRL-11268) transfected with HIV-1 molecular clones (pNL4-3, pBal, pIndie-C1, pL2 and 95TNIH022). In addition, plasma from 4 HIV-1-infected individuals was also used. The plasma from HIV-1-infected individuals at a very early stage of infection was purchased from Alpha Therapeutic Corporation (Calexico, CA). The plasma samples were tested for the following: human hepatitis B virus (HBV) surface antigen (-), anti-HIV-1/HIV-2 (-), HIV-1 by PCR (+), anti-human hepatitis virus (HCV) (-), HCV by PCR (-), HBV by PCR (-), human hepatitis A virus by PCR (-) and parvovirus by PCR (-). These results were reported in the Final Viral Marker Report (Repeat Donors) of Alpha Therapeutic Corporation Consolidated Test Results from Memphis Lab and PCR pooling Lab (Finalized date: 14-Apr-2003).

HIV-1 capture. Viral capture was performed according to the manufacturer's instructions (Ademtech). Briefly, after 2 washes with binding buffer, anionic magnetic beads (50 μl) were further washed twice with phosphate-buffered saline (PBS). Then, 50 μl of cell culture medium or plasma diluted with 450 μl of PBS was added to the washed beads and incubated for 20 min at room temperature. A magnetic field was then applied to the tubes containing the magnetic beads. The supernatant was discarded and the beads were thoroughly washed 3 times with PBS. The washed beads were resuspended with PBS and subjected to viral RNA extraction, western blotting or ELISA. After separation, 4 fractions were obtained as follows: i) bead fraction (BD), ii) sample before incubation with the beads (BF), iii) supernatant after incubation (SP) and iv) total sample containing the same quantity (50 μl) of cell culture medium or plasma as BD (TL). The viral capture procedure was typically completed within 30 min.

Capture inhibition by anti-HIV antibody. In order to verify the mechanism of viral capture, HIV-1-infected cell culture media were incubated with anti-HIV-1 Env gp41 antibody, 4E10 (Polymun Scientific Immunbiologische Forschung GmbH) or anti-α-tubulin, B-5-1-2 (Sigma-Aldrich) for 30 min at 37°C prior to addition of the magnetic beads. The samples were then subjected to bead incubation and magnetic separation as described before.

Western blotting. Each fraction was solubilized in an equal volume of 2X sodium dodecyl sulfate (SDS) gel-loading buffer [90 mM Tris-HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenol blue and 20% glycerol], boiled for 5 min and separated on an SDS-12% polyacrylamide gel electrophoresis (PAGE) before being electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ) for 60 min at

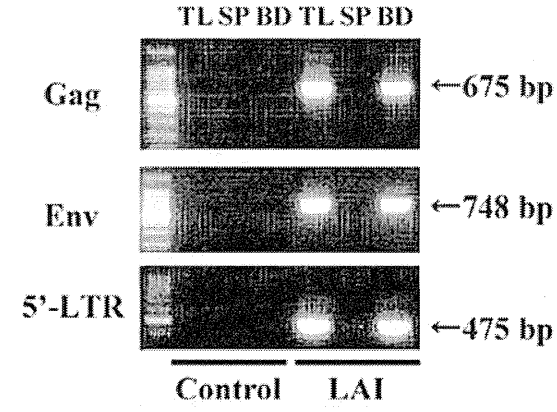

Figure 1. Detection of the RNA genome of HIV-1 adsorbed onto anionic magnetic beads. Culture medium of HIV-1 (LAI)-infected and uninfected (control)-MT4 cells were mixed with anionic magnetic beads. After incubation, the following fractions were obtained: bead fraction (BD), supernatant after incubation (SP) and sample containing the same quantity of culture medium as BD (TL). Viral genomic RNA was extracted from the fractions using a QIAamp viral RNA mini kit and subjected to reverse transcription (RT)-reaction. The resultant cDNAs of HIV-1 Gag protein gene (675 bp), Env protein gene (748 bp) and 5'-LTR (475 bp) were amplified by PCR as described in the Materials and methods. The left lane is the size marker (100 bp ladder).

15 V. Blots were treated with 5% skimmed milk for 1 h at room temperature and then incubated with anti-HIV-1 p24 antibody (03-HIV-18; Biomarket, Ltd.), Nef antibody (clone 2A3, 03-HIV-3; Biomarket, Ltd.), envelope (Env) antibody (SF2 gp120#387, NIH AIDS Research and Reference Program) and acquired immunodeficiency syndrome (AIDS) patient serum in PBS containing 0.1% Tween-20 (PBS-T) and 0.5% skimmed milk for 1 h at room temperature. After 3 washes with PBS-T, the membrane was incubated in horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS-T and 0.5% skimmed milk for 1 h at room temperature. After 3 washes with PBS-T, the probed proteins were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

ELISA. ELISA for HIV-1 p24 was performed using HIV-1 p24 ELISA kit (BioAcademia, Osaka, Japan). Absorbance at 450 nm was measured to quantify the level of HIV-1 by microplate reader (Labsystems Multiskan MS; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan).

Reverse transcription (RT)-PCR. Viral RNA from beads or an aliquot of each sample was extracted with the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was extracted from the magnetic beads by adding lysis buffer prior to removing the beads. RNA was then eluted in 60 μl of nuclease-free water. For the RT-reaction, random primers were added and after incubation at 25°C for 10 min, the RNA was reverse-transcribed at 65°C for 50 min followed by denaturation of the enzyme at 85°C for 5 min. The diluted cDNA was amplified in a reaction mixture containing primers, Ex Taq (Takara Bio, Inc., Otsu, Japan) and Ex Taq buffer under conditions of 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. PCR was carried out using the following primers for the HIV-1 Gag

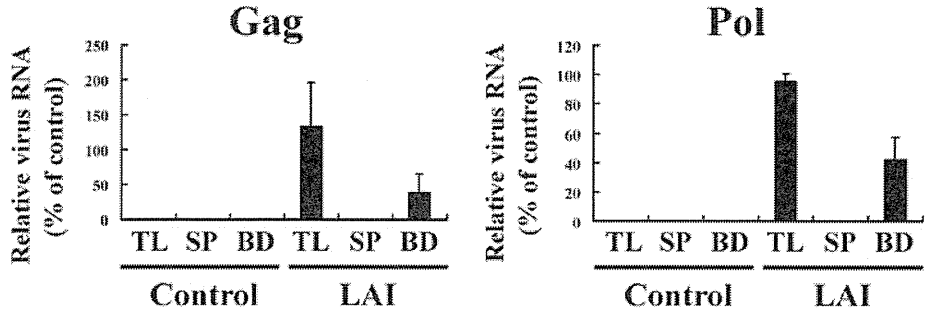

Figure 2. Quantitative analysis of viral protein (Gag and Pol) genes of HIV-1 adsorbed onto anionic magnetic beads. (A) Anionic magnetic beads were used to capture HIV-1 from 500 μl of culture medium of cells infected with HIV-1 (LAI) and then analyzed by real-time PCR. Uninfected cells were used as a negative control. Samples were divided into 3 categories: i) bead fraction (BD), ii) supernatant after the incubation (SP) and iii) sample containing the same quantity of culture medium as BD (TL). RNA was purified using a QIAamp viral RNA mini kit, reverse-transcribed and then analyzed by real-time PCR with HIV-1 Gag protein gene (Gag) and Pol protein gene (Pol).

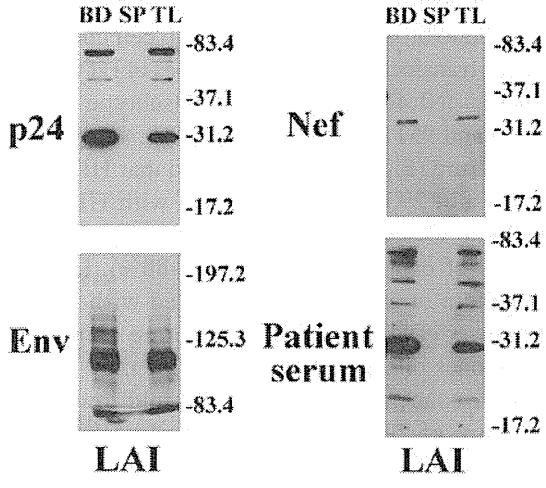

Figure 3. Detection of viral proteins of HIV-1 adsorbed onto anionic magnetic beads. Culture medium of HIV-1 (LAI)-infected MT4 cells was diluted with PBS and subjected to incubation with anionic magnetic beads. Western blotting was performed with anti-HIV-1 p24, Nef and Env antibody and AIDS patient serum. Samples were divided into 3 categories: i) bead fraction (BD), ii) supernatant after the incubation (SP) and iii) total sample containing the same quantity of nasal aspirate as BD (TL). The samples were solubilized with SDS loading buffer and then subjected to western blotting. Molecular weight marker (KDa) is shown in the right lane.

gene: HIV Gag RTPCR AE B common F, 5'-ggggaagtgacatagcagga-3' and R, 5'-ctgttggctctggtctgctc-3'; for the HIV-1 Env gene: HIV Env RTPCR AE B common F: 5'-gacggtacaggccagacaat-3' and R, 5'-tcccagaagttccacaatcc-3'; for the HIV-1 5'long terminal repeat (LTR): HIV 5'LTR RTPCR AE B common F, 5'-ccctgattggcagaactacac-3' and R, 5'-agcactcaaggcaagcttta-3'. The amplified products were purified and cloned in pT7Blue T-vector (Novagen, Madison, WI). DNA sequencing (ABI PRISM3100 Genetic Analyzer; Applied Biosystems, Foster City, CA) with the R-20mer primer and U-19mer primer (Novagen) was used to verify the product sequence.

Real-time RT-PCR. The cDNAs produced in the RT reactions above were also analyzed by real-time PCR (Q-PCR, quantitative PCR). For real-time PCR, a Brilliant SYBR-Green Q-PCR mastermix was used according to the manufacturer's instructions (Stratagene, La Jolla, CA). Briefly, the Q-PCR components included Brilliant Q-PCR mastermix, reverse-transcribed cDNA, and the forward and reverse target gene primers: realHIVgag F, 5'-caagcagggagctagaacga-3' and R, 5'-ttgtctacagccttctgatgtctc-3'; realHIVpol F, 5'-aaattcaaaatttttcgggtttattac-3' and R, 5'-aggagctttgctggtccttt-3'. The Q-PCR program used in a Mx3000P™ Real-time Q-PCR System (Stratagene) was: denaturation (at 95°C for 10 min) and then 40 cycles of denaturation (95°C for 30 sec), annealing (58°C for 60 sec) and extension (72°C for 30 sec). Each reaction was done in triplicate. The results were analyzed using the Mx3000P™ system software. The relative expression ratio of each sample was calculated using a mathematical model based on the amplification efficiency. PCR specificity was verified by dissociation curve analysis of the amplified DNA fragments.

Results

To investigate whether Viro-Adembeads could be used to capture HIV-1, cell culture medium from HIV-1 (LAI)-infected MT4 cells was mixed with the anionic polymer-coated magnetic beads. The mixture was then magnetically separated and bead (BD), supernatant (SP) and total (TL) fractions were prepared. Cell culture medium from mock-infected MT4 cells was also used to prepare control fractions. The fractions were then analyzed by RT-PCR, real-time PCR, western blotting and ELISA to determine the extent of HIV-1 capture by the beads.

Firstly, RT-PCR was performed to detect HIV-1 genomic RNA in order to examine the capacity of the beads to capture HIV-1 (Fig. 1). RT-PCR analysis gave a single band of 675 bp for Gag, 748 bp for Env and 475 bp for 5'-LTR in the bead fraction (BD) and samples containing the same quantity of cell culture medium as BD (TL) using cell culture medium from HIV-1 (LAI)-infected MT-4 cells. No signal was detected in the supernatant after incubation (SP). In contrast, RT-PCR analysis of BD, SP and TL using cell culture medium obtained from Mock-infected MT4 cells (control) did not give any signal. The 675-, 748- and 475-bp bands were confirmed to be Gag, Env and 5'-LTR gene of HIV-1, respectively by DNA sequencing (identity to Genebank accession number AF324493 was 96, 98 and 99%, respectively). Therefore, these results confirm that the bead fraction includes the HIV-1 genomic RNA.

The amount of HIV-1 genomic RNA in the BD, SP and TL fractions was measured by real-time RT-PCR, relative to that in a control (a TL sample with the highest value was taken as

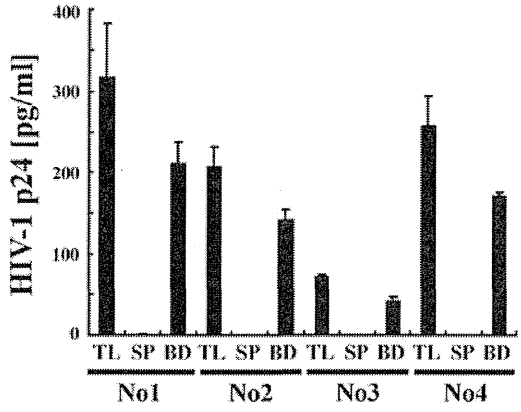

Figure 4. Quantitative analysis of HIV-1 adsorbed onto anionic beads. Plasma from 4 HIV-1-infected individuals (No. 1-4) were diluted with PBS and subjected to incubation with anionic beads. HIV-1 p24 was adsorbed onto the beads using HIV-1 p24 ELISA kit. Samples were divided into 3 categories: i) bead fraction (BD), ii) supernatant after incubation (SP) and iii) sample containing the same quantity of culture medium as BD (TL). The concentration of HIV-1 in each fraction was calculated as an index of absorbance at 450 nm by comparison with HIV-1 p24 standard.

100 [%]). For HIV-1 (LAI)-infected cell culture medium, the amount of viral RNA in the BD fraction was ~33 and 50% that in the TL from real-time PCR of Gag and Pol genes, respectively (Fig. 2). In contrast, cell culture medium from mock-infected MT4 cells (control) showed no amplification of genomic RNA in BD, SP and TL. The specificity of these PCR reactions was confirmed by dissociation curve analysis of the reaction products. These results showed that HIV-1 in cell culture medium could be captured by the magnetic beads, but that a fraction of HIV-1 was lost during the procedure. A significant fraction of HIV-1 in the culture medium of virus-infected cells was lost during the capture procedure using anionic polymer-coated magnetic beads. The loss of HIV-1 may have been due to serum components in the cell culture medium, such as albumin, binding to the magnetic beads and thereby hindering viral capture (18).

Western blotting demonstrated that the total sample fraction (TL) and bead fraction (BD), but not the supernatant fraction (SP), in cell culture medium of HIV-1 (LAI)-infected MT4 cells had a major band of 30, 34 and 110 kDa for p24, Nef and Env proteins, respectively (Fig. 3). These bands correspond to the respective deduced mass of HIV-1 p24, Nef and Env protein based on their amino acid sequences. Thus, HIV-1 was detected at similar levels in the total sample fraction (TL) and in the bead fraction (BD), but not at all in the supernatant fraction (SP). In addition, the corresponding bands were detected using serum from an AIDS patient. These results support the idea that HIV-1 is efficiently captured by anionic magnetic beads.

Next, we examined the efficiency with which HIV-1 was captured from plasma by conducting a quantitative analysis using ELISA (Fig. 4). HIV-1 in plasma from 4 HIV-1-infected individuals (No. 1-4) was recovered using anionic magnetic beads (BD) at a level of 65-80% that from samples containing the same quantity of plasma as BD (TL). In contrast, HIV-1 was below the detection limit in the supernatant after incubation (SP). These findings suggest that most of the HIV-1 was efficiently captured from plasma by the magnetic beads.

To further examine whether this magnetic capture method can be applied to broad subtypes of HIV-1, cell culture medium of 293T cells transfected with various types of HIV-1 molecular clones, such as pNL4-3 (subtype B), pBal (subtype B), pIndie-C1 (subtype C) and 95TNIH022 (CRF01_AE), were subjected to magnetic capture (Fig. 5). ELISA showed that HIV-1 from cell culture media of 293T cells transfected with HIV-1 pNL4-3, pBal or pIndie-C1 could be captured by magnetic beads at a similar level of capture efficiently (60-80%). However, cell culture medium of 293T cells transfected with 95TNIH022, showed a lower efficiency of HIV-1 capture compared to the molecular clones of subtype B and C. This may be due to the overall lower concentration (about 100-fold lower) of HIV-1 in cell culture of 293T cells transfected with 95TNIH022 compared to the other molecular clones.

Next, we used gp120-containing, protease-deficient clone (L2), which is derived from LAI and generate immature and defective doughnut-shaped particles (19) (Fig. 6 and 7). ELISA (Fig. 6) and western blotting (Fig. 7) showed that HIV-1 produced by transfection of pL2 into 293T cells could be efficiently captured by magnetic beads. Although L2 expresses an immature form of polymerase and decreased levels of Env compared to wild type LAI, the L2 polymerase and Env were efficiently captured by anionic beads. Finally, we investigated the mechanisms by which poly(MVE-MA) binds HIV-1. HIV-1 LAI in cell culture medium was preincubated with anti-HIV-1 Env gp41 neutralizing antibody 4E10 before incubation

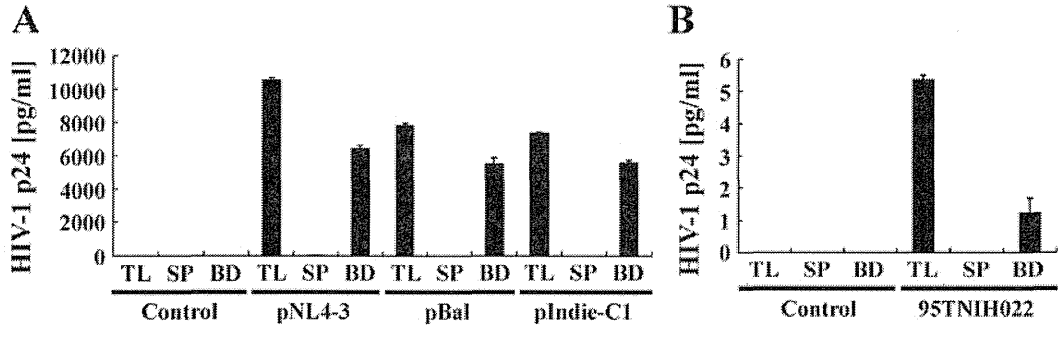

Figure 5. Adsorption of HIV-1 with subtype B, C and circulating recombinant form CRF_A E onto anionic magnetic beads. (A) HIV-1 in cell culture medium of 293T cells transfected with HIV-1 molecular clone of pNL4-3, pBal and pIndie-C1 or (B) MT-4 cells infected with HIV-1 95TNIH022 before and after adsorption onto anionic magnetic beads were quantitatively analyzed by ELISA using HIV-1 p24 ELISA kit. Samples were divided into 3 categories: i) bead fraction (BD), ii) supernatant after the incubation (SP) and iii) sample containing the same quantity of culture medium as BD (TL).