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Immunoreactive Cycloimmunogen Design Based on Conformational Epitopes Derived from Human Immunodeficiency Virus Type 1 Coreceptors: Cyclic Dodecapeptides Mimic Undecapeptidyl Arches of Extracellular Loop-2 in Chemokine Receptor and Inhibit Human Immunodeficiency Virus Type 1 Infection

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Abstract: Human immunodeficiency virus type 1 (HIV-1) requires a chemokine receptor (CCR5 or CXCR4) as a coreceptor not only for initiate viral entry but also protecting highly conserved neutralization epitopes from the attack of neutralizing antibodies. Over the past decade, many studies have provided new insights into the HIV entry mechanism and have focused on developing an effective vaccine strategy. However, to date, no vaccine that can provide protection from HIV-1 infection has been developed. One reason for the disappointing results has been the inability of current vaccine candidates to elicit a broadly reactive immunity to viral proteins such as the envelope (env) protein. Here, we propose that chemokine receptors are attractive targets of vaccine development because their structures are highly conserved and that our synthetic cycloimmunogens can mimic conformational-specific epitopes of undecapeptidyl arches (UPAs: R_{168}-C_{178} in CCR5, N_{176}-C_{186} in CXCR4) and be useful for HIV-1 novel vaccine development.

Key Words: Cycloimmunogen, HIV-1, conformational epitope, CCR5, CXCR4.

INTRODUCTION

Two decades of intensive research has clarified the acquired immune deficiency syndrome (AIDS) causative agent, HIV-1, and many valuable details about the host-pathogen relationship. At the end of 1995, Cocchi *et al.* indicated that CC-chemokines, CCL3, CCL4, and CCL5, could inhibit HIV-1 replication [1]. Furthermore, at the beginning of 1996, Feng *et al.* showed that a second HIV-1 coreceptor beside CD4 was shown to be a chemokine receptor CXCR4 [2]. These independent discoveries opened a new frontier in HIV-1 research. Immediately after these discoveries it was shown that the counterpart of CXCR4 for strains associated with acute infection is the CC-chemokine receptor CCR5 [3-7]; a 32-bp deletion within the coding sequence of the CCR5 gene (CCR5-dela32) was found to be strongly protective against HIV-1 infection *in vitro* and *in vivo* [8-10]; and CXCL12 was identified as a specific CXCR4 ligand [11, 12]. These discoveries have greatly advanced our knowledge of the virus and the pathogenesis of HIV-1 infection for the development of novel therapeutic agents such as entry inhibitors and vaccines targeting the HIV-1 env protein. However, to date, there have been no antiretroviral therapies that can effectively eradicate HIV-1 infection *in vivo*.

Several studies have shown that a small number of HIV-exposed individuals do not become infected or can control HIV-1 infection at levels below the detection limit of standard assays, despite repeated and long-term exposures to the virus [13]. These individuals are defined as exposed seronegative (ESN). Although many different factors (e.g., CC-chemokine and neutralizing antibody (Ab) level) have been considered to explain the long-lasting protection in ESN individuals [13], we focused on the protective effects of anti-CCR5 autoantibodies in ESN individuals because these individuals possessing the delta32-CCR5 allele are healthy and highly resistant to HIV-1 infection [8-10, 14-17]. Therefore, to reproduce the ESN status with a candidate vaccine, chemokine receptors are attractive targets because they are cellularly highly conserved structures, unlike the HIV-1 env protein, that may rapidly mutate during the disease progression, and are the inescapable gateway for HIV-1 *in vivo*.

In this review article, we will introduce the findings on the immunogenicity of the cycloimmunogen mimicking the HIV-1 coreceptor CCR5 or CXCR4 and anti-HIV activities of induced Abs and antisera in mice and cynomolgus macaques.

DESIGN OF CHEMOKINE RECEPTOR-BASED HIV-1 VACCINE

Basic Mechanism of HIV-1 Entry

The HIV-1 mature envelope glycoprotein complex plays a pivotal role in the early events of viral attachment and entry into a target cell. The complex is arranged in a trimeric configuration of heterodimers, each consisting of a gp120 subunit noncovalently associated with a gp41 subunit after gp160 polypeptide chains are cleaved into two fragments, gp120 and gp41 [18-20]. The cleavage enables these proteins to undergo the first conformational changes before a virus interacts with CD4, and its coreceptor, CCR5 or CXCR4 [2, 21-23]. Furthermore, the second conformational changes are induced by CD4 binding and are required for the subsequent interaction between gp120 and its coreceptor. For binding to

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1871-5303/07 $50.00+.00

a coreceptor, the third variable (V3) domain of gp120 was identified as an important determinant, which makes direct interaction with a coreceptor [1, 4]. Generally, the structure and charge of the V3 loop change the specificity of coreceptor use. The bridging sheet was also identified as another important determinant, which mainly recognizes the chemokine receptor [24]. Finally, gp41 undergoes additional conformational changes resulting in the fusion of the viral and target cell membranes. [25, 26]. Therefore, the important extracellular domains of a coreceptor that the V3 domain and bridging sheet interact with are attractive targets for developing an HIV-1 entry inhibitor.

Functional Determinants of CCR5 for HIV-1 Entry

A lot of mutation and chimeric studies of HIV-1 coreceptors have provided important information on the extracellular domain involved in the interaction with gp120. Although the gp120-binding domain of an HIV-1 coreceptor is complex and varies according to the viral envelope examined and the various detection system have been used to test the role of the extracellular domain of CCR5, the general conclusions, to date, have been as follows. (A) The *N*-terminus (Nt) and second extracellular loop (ECL-2) play critical roles in gp120 binding and fusion [27, 28]. It has been shown that the negatively charged acidic amino acids and tyrosine residues in the CCR5 Nt (D_2, Y_3, Y_{10}, D_{11}, Y_{14}, Y_{15}, and E_{18}) are important for CD4-dependent gp120-CCR5 binding and viral entry [29-33]. Furthermore, residues in the CCR5 ECL-2 that were found to affect coreceptor function for nonclade B viruses include S_{169}, Q_{170}, K_{171}/E_{172}, Y_{176}, and T_{177} [34], although the dependence of nonclade B viruses on residues in ECL-2 for viral entry may be much higher than that of clade B [31, 33, 35]. (B) All the cysteine residues (C_{20}, C_{101}, C_{178}, and C_{269}) are important for the coreceptor function [34, 36]. In particular, the alanine mutation of cycteine 101 dramatically affected infection efficiency of clade B and non-clade B viruses. This cycteine residue is involved in the formation of a disulfide bond (C_{101}-C_{178}). (C) Posttranslational modifications of CCR5 modulate its function by affecting the affinity of gp120/coreceptor interaction. CCR5 Nt undergoes both *O*-glycosylation and tyrosine sulfation [37]. Serine 6 and serine 7 in Nt are modified by *O*-glycosylation in cell lines and in primary macrophages [38]. The absence of the *O*-glycosylation of CCR5 prevents the binding of CCL3 and CCL4, but has a minimal effect on the efficiency of HIV-1 infection [38]. On the other hand, the prevention of tyrosine sulfation substantially suppressed HIV-1 entry without affecting CCR5 expression [37, 38]. Furthermore, the interaction between soluble gp120-CD4 complexes and CCR5 Nt-based sulfopeptides involves residues located primarily in the V3 stem and the C4 region of gp120 [39, 40], suggesting that the sulfate moieties of tyrosine residues should facilitate electrostatic interactions with positively charged amino acids in the V3 base and the bridging sheet.

Functional Determinants of CXCR4 for HIV-1 Entry

A lot of mutation and chimeric studies of HIV-1 coreceptors have also provided important information on the extracellular domain of CXCR4 involved in the interaction with gp120, although, in contrast to the R5 virus, a region of CXCR4 that plays an important role in viral entry seems to be dispersed in an isolate-dependent manner and more complex. The general conclusions of these studies have been, to date, as follows: (A) Negatively charged acidic amino acids and tyrosine residues dispersed throughout the extracellular domains of CXCR4 are important for X4 viral entry. Multiple single mutations (Y_7, D_{10}, Y_{12}, E_{14}, E_{15}, D_{20}, Y_{21}, D_{22}, E_{26}, E_{32} in Nt and D_{182}, Y_{184}, D_{187}, Y_{190}, D_{193} in ECL-2) affect HIV-1 entry, albeite in an isolate-dependent manner [41-43]. These results show that X4 viral entry mainly depends on tyrosine, aspartate, and glutamate residues (YDE-rich cluster) in Nt and ECL-2. (B) All cysteine residues (C_{28}, C_{109}, C_{186} and C_{274}) are important for the coreceptor function although the alanine mutation of cysteine 274 alone in the third extracellular domain (ECL-3) yielded a CXCR4 molecule possessing an activity nearly 75% of that of the wild type [43]. Alanine mutants of cycteine residues (C_{109} and C_{186}) were essentially undetectable on the cell surface, suggesting that the disruption of disulfide bond formation between cysteine 109 and cysteine 186 results in misfolded CXCR4 molecules that are not well expressed on the cell surface and are important for maintaining coreceptor activity. (C) Like CCR5, CXCR4 is also posttranslationally modified by the sulfation of its amino-terminal tyrosines [44]. However, these finding suggest that the tyrosine sulfate groups at the amino terminus of CXCR4 play a much less important role in the entry of CXCR4-utilizing isolates (CF402.1, SG3, or HXBc2) than the tyrosine sulfate groups of CCR5 in the entry of all CCR5-dependent HIV-1 isolates (JRFL, YU2, and ADA) assayed previously.

Binding Mechanism of CC-Chemokines with CCR5

It is very important to determine which regions of CCR5 are responsible for the specificity of chemokine binding as well as determinants of coreceptor activity. CCR5 natural ligands (CCL3, CCL4, CCL5, and CCL8) are able to inhibit HIV-1 infection *in vivo* [1, 45-49]. Although they are unlikely to bind to identical sites on CCR5, a proposed model for chemokine binding to CCR5 shows a two-step mechanism of receptor activation. As the initial binding step, the Nt domain of CCR5 was shown to be required for chemokine binding, with several charged amino acids and aromatic residues playing a crucial role [29, 50]. In particular, the mutation (D_2, S_7 Y_{10}, D_{11}, and E_{18}) plays an important role in CCL4 binding and the mutation (I_{12}, C_{20}) severely suppressed CCL4 and CCR5 binding [29, 51]. Furthermore, as the second binding step, ECL-2 interacts with the CC-chemokines and confers ligand specificity [52]. Blanpain *et al.* suggested that the mutation (E_{172}, R_{168}, K_{191}) strongly affects CCL3 binding but has little effect on CCL5 binding [53].

Binding Mechanism OF CXC-Chemokine with CXCR4

Crump *et al.* proposed a two-site model for CXCL12-CXCR4 interaction derived from a three-dimensional structure [54]. As an initial docking step, the Nt of CXCR4 interacts with the R-F-F-E-S-H motif of CXCL12. This step leads to conformational changes that allow the subsequent interaction of CXCR4 with the K-P-V-S-L-S-Y-R-C-P-C motif of CXCL12 [55]. Brelot *et al.* [56] and Zhou *et al.* [57] suggested that according to this two-site model for CXCL12-CXCR4, site I involved in CXCL12 binding but not signaling is located in Nt. In particular, glutamamate 14 and/or

glutamate 15, aspartate 20 and tyrosine 21 have importance in the CXCL12 binding. Furthermore, residues required for both CXCL12 binding and signaling at site II were identified in ECL-2 (E_{187}) [56] and ECL-3 (E_{268}) [57]. On the other hand, aspartate 181, aspartate 182, arginine 183 and tyrosine 184 in UPA have no effect on CXCL12 binding as well as intracellular Ca^{2+} influx [56, 57]. Therefore, UPA may be an attractive target for developing peptide-based vaccines that induce anti-CXCR4 Abs with little effect on CXCR4 signaling.

Sequence Identity of Undecapeptidyl Arch Among CC and CXC Receptor Family Members and Among Various Species

Seven-transmembrane domain receptors, such as CCR5 and CXCR4, can exhibit conformational heterogeneity [58]. Therefore, the extracellular domain of those receptors that exists in antigenically same state may be suitable for an antigen based on the chemokine receptor. In our study, UPA was selected as the target because cysteine residues (C_{101} and C_{178}) in ECL-1 and ECL-2 form a rigid disulfide bond and UPA is one of the domains with which the HIV-1 env protein interacts [34, 41-43]. Furthermore, each amino acid sequence of UPA (UPAs: R_{168}-C_{178} in CCR5, N_{176}-C_{186} in CXCR4) is specific among each chemokine receptor family member and is highly conserved in the primates and nonprimates as shown in Fig. (**1**). These results suggest that each UPA in CCR5 or CXCR4 is a suitable target for eliciting autoantibodies against each receptor.

Benefits of Peptide Cycloimmunogen

The benefits of using a peptide immunogen are generally as follows: (i) its immunogenicity can be controlled by polymerization or conjugation with small carrier molecules such as a multiple-antigen peptide (MAP) [59], (ii) it can induce Abs against a very restricted region that includes biologically active epitope, and (iii) its chemical purity can be exactly defined and is cost-effective to produce [60, 61]. However, the mimicry of the conformation of native protein is the most important for inducing an Ab against the conformational epitope of an antigen. Hence, an Ab induced by a linear peptide could recognize a denatured protein but could not recognize the native protein. On the other hand, an Ab that recognizes the conformational epitope of an antigen could not recognize a denatured protein. Indeed, the anti-CXCR4 Ab 12G5 induced by the immunization of Sup-T1 cells that are chronically infected with SIVmac variant CP-MAC [62], could recognize cell surface CXCR4 as deter-

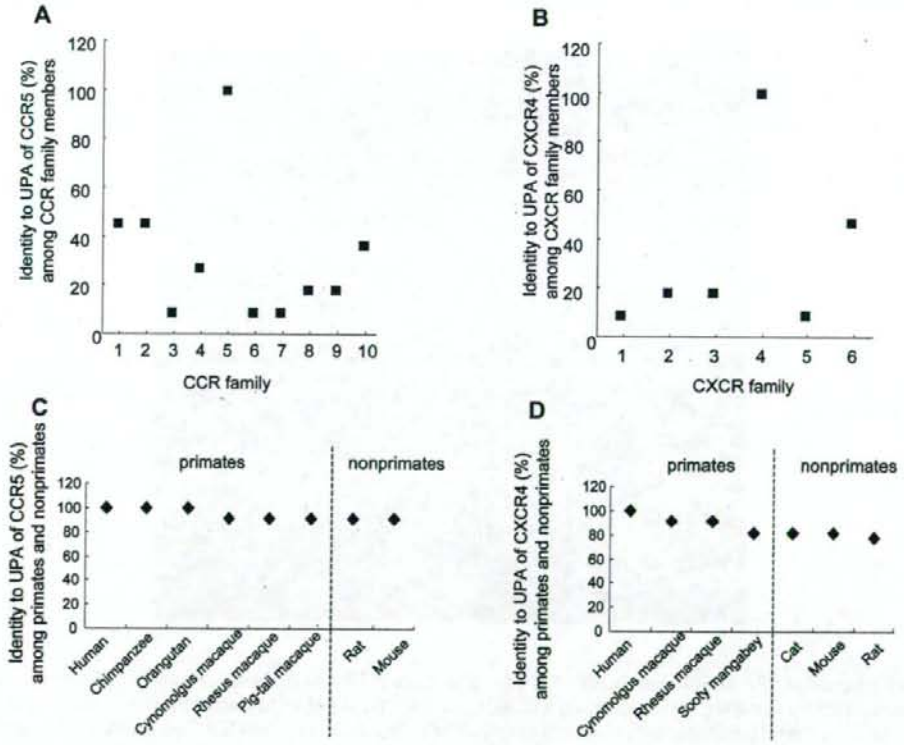

Fig. (1). (A) Sequence identity to CCR5 UPA among UPAs of CCR family. % identity is set to 100 when aligned sequences are 100% identical to the UPA of CCR5. (B) Sequence identity to CXCR4 UPA among UPAs of CXCR family. % identity is set to 100 when aligned sequences are 100% identical to the UPA of CXCR4. (C) Sequence identity to human CCR5 UPA among primates and nonprimates. % identity is set to 100 when aligned sequences are 100% identical to the UPA of CCR5. (D) Sequence identity to human CXCR4 UPA among primates and nonprimates. % identity is set to 100 when aligned sequences are 100% identical to the UPA of CXCR4.

mined by fluorescence-activated cell sorting analysis but could not detect denatured CXCR4 as determined by Western blot analysis. In our study, the peptide antigen was cyclized not by a disulfide bond but by a peptide bond because the conformational stability of a peptide antigen *in vivo* is also a key factor for generating Abs against the native protein. The peptide bond is more robust and stable than a disulfide bond for cyclization. Szewczuk *et al.* suggested that a cyclic peptide antigen is more resistant to proteolytic degradation as shown in their study of a thrombin-specific inhibitor [63]. Thus, the cyclopeptide is more suitable for a linear peptide to not only mimic the native conformational epitope of UPA in CCR5 and CXCR4, but also enhance immunogenicity itself by becoming resistant to proteolytic degradation.

Design and Synthesis of Cycloimmunogens

In our study, three cycloimmunogens were designed for eliciting autoantibodies against CCR5, CXCR4, or both. The hypothetical structural model of CCR5 and CXCR4 is based on its homology with rhodopsin [64], and energy-minimized using the molecular operating environment, (MOE, Chemical Computing Group Inc., Montreal, Quebec, Canada), Figs. (**2**) and (**3**) [65, 66]. The ECL-2 region of CCR5 has a unique arch consisting of 11 amino acid residues (UPA) formed on the basis of the cysteine 178 residue bound to a cysteine 101 residue of ECL-1 by a disulfide bond, Fig. (**2A**). The cDDR5 moiety designed to mimic the native conformational epitope of UPA was generated by the cyclization of a decapeptide (R_{168}SQKEGLHYT$_{177}$) derived from the UPA sequence by the insertion of a spacer-armed dipeptide (Gly-Glu) (shown in green in Fig. (**2B**)). The deduced structure of cDDR5 (shown in red) was adopted to the structural model of UPA in CCR5 using the MOE-Align tool (Chemical Computing Group Inc., Montreal, Quebec, Canada, shown in yellow in Fig. (**2B**)).

On the other hand, the ECL-2 region of CXCR4 also has a unique arch consisting of 11 amino acid residues (UPA) formed on the basis of the cysteine 186 residue bound to the cysteine 109 residue of ECL-1 by a disulfide bond, Fig. (**3A**). The cDDX4 moiety was also generated by the cyclization of a decapeptide (N_{176}VSEADDRYI$_{185}$) by the insertion of a spacer-armed dipeptide (Gly-Asp) (shown in green in Fig. (**3B**)). The deduced structure of cDDX4 (shown in purple) was adopted to the structural model of UPA in CXCR4 using the MOE-Align tool (Chemical Computing Group Inc., Montreal, Quebec, Canada, shown in yellow in Fig. (**3B**)). The cyclization of the linear peptide was confirmed by MALDI-TOF-MS [65, 66].

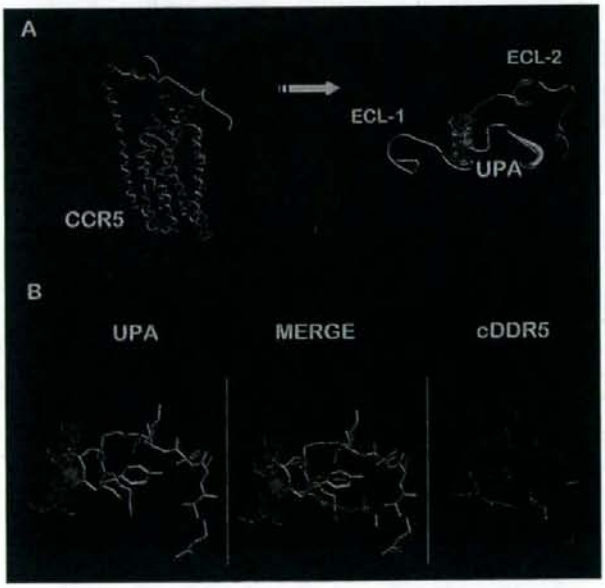

Fig. (2). Deduced structure of UPA in ECL-2 of CCR5. (A) Deduced structure of UPA in extracellular loop-2 of CCR5. The predicted model of CCR5 was constructed using the segmented approach, and MOE was used for actual calculation. The determined structure of rhodopsin was used as the template [64]. Transmembrane helixes are in cyan. The extracellular loops of CCR5 are color-coded: N terminus, purple; extracellular loop-1, green; extracellular loop-2, yellow-orange; UPA, yellow; and extracellular loop-3, magenta. The intercellular loops are in gray, and the C terminus is in red. Because cysteine residues in extracellular loop-1 and extracellular loop-2 form a disulfide bond, the extracellular loop-2 region of CCR5 has a unique arch structure consisting of 11 amino acid residues (yellow). In this study, UPA was selected as the target of peptide immunogens. (B) To mimic the deduced conformational epitope of UPA in CCR5, the decapeptide (R_{168}SQKEGLHYT$_{177}$) derived from the UPA sequence was cyclized by inserting the spacer-armed dipeptide (Gly-Glu in green), and the deduced structure of cDDR5 (in red) was adopted as the deduced structural model of UPA (in yellow) in CCR5 using the MOE-Align tool.

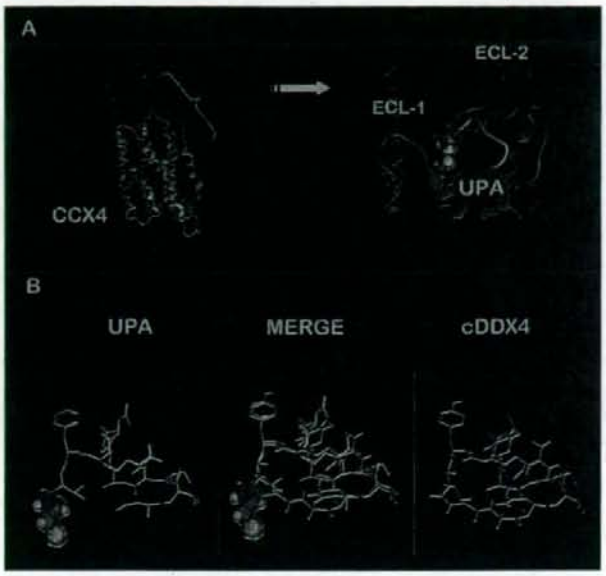

Fig. (3). Deduced structure of UPA in ECL-2 of CXCR4. (A) The predicted model of CXCR4 was constructed using the segmented approach, and MOE was used for actual calculation. The determined structure of rhodopsin was used as the template [64]. Transmembrane helixes are indicated in cyan. The extracellular loops of CXCR4 are color-coded: amino terminus, purple; ECL-1, green; ECL-2, yellow and orange; UPA, yellow; and ECL-3, magenta. The intercellular loops are indicated in white and the C terminus in red. Because cysteine residues in ECL-1 and ECL-2 form a disulfide bond, the ECL-2 region of CXCR4 has a unique arch structure consisting of 11 amino acid residues (UPA, undecapeptidyl arch, yellow). In this study, UPA was selected as the target of peptide immunogens. (B) To mimic the native conformational epitope of UPA in CXCR4, the decapeptide (N_{176}VSEADDRYI$_{185}$) derived from the UPA sequence was cyclized by the insertion of the spacer-armed dipeptide (Gly-Asp in green), and the deduced structure of cDDX4 (in purple) was adopted to the structural model of UPA (in yellow) in CXCR4 using the MOE-Align tool.

Furthermore, a cyclic chimeric dodecapeptide (cCD) mimicking the conformation-specific domains of CCR5 and CXCR4 was also prepared in which Gly-Asp links the amino and carboxyl termini of two combined pentapeptides (S_{169}-G_{173} of CCR5; E_{179}-R_{183} of CXCR4) to elicit the bifunctional Abs against CCR5 and CXCR4, Fig (**4**) [67, 68].

Finally, these cycloimmunogens were conjugated with MAP through the formation of the peptide bound between the beta- or gamma-carboxyl group of aspartate or glutamate within the spacer-armed dipeptide and the amino group within MAP for the immunization of BALB/c mice and cynomolgus macaques [65-67]. MAP, which is composed of a

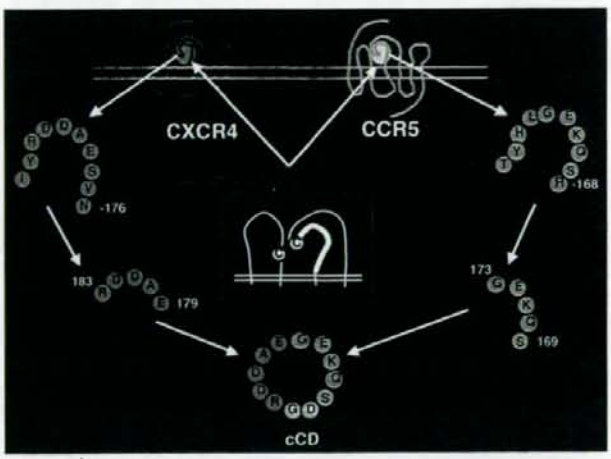

Fig. (4). Overview of preparation of cyclic chimeric peptide (cCD). cCD was constructed with a spacer-armed Gly-Asp dipeptide (in yellow) and two pentapeptides of UPA in ECL-2 derived from CCR5 (in light blue) and CXCR4 (in red).

twofold bifurcating polylysine core developed as a carrier of a peptide antigen, is capable of eliciting a strong Ab response in mice, monkeys, and humans [59].

ESTIMATION OF CHEMOKINE RECEPTOR-BASED HIV-1 VACCINE

Induction of Autoantibodies After Immunization with Chemokine Receptor-Based Cycloimmunogen in BALB/C Mice

One clone, KB8C12, producing the Ab to the UPA-based cycloimmunogen cDDR5-MAP, was effectively selected and purified [69]. To estimate the immunochemical specificity of KB8C12, a BIAcore biosensor bound with biotinylated cDDR5 was used. The result showed that the response of the biotinylated-cDDR5-bound biosensor was enhanced with increasin g concentration of the flowing Ab and that Ab-binding was competed by cDDR5 but not by linear DDR5 composed of the same amino acid residues, as shown in Table **1**. These results suggest that the cycloimmunogen is useful for mimicking the conformational epitope of the targeted region. Furthermore, the binding of KB8C12 to cells expressing CCR5 was determined using a flow cytometer [69]. These results taken together indicate that the Ab to cDDR5-MAP is conformation-specific and recognizes the conformation-specific domain of the UPA of ECL-2 in CCR5.

Moreover, the immunization of BALB/c mice with cDDX4-MAP or cCD-MAP also induces the autoantibodies production [65, 67]. As shown in Table **1**, the immunochemical specificities of the autoantibodies raised by cDDX4-MAP and cCD-MAP are summarized. Interestingly, the immunization of BALB/c mice with cCD-MAP induces the production of bifunctional Abs, CPMAb-I to CPMAb-VII.

Antiviral Activity of Monoclonal Antibodies Elicited by Chemokine Receptor-Based Cycloimmunogens

The anti-HIV-1 activity of KB8C12 was determined using MAGIC-5 cells [69, 70]. As expected, KB8C12 markedly suppressed infection by JRFL, but not by LAV-1, in a dose-dependent manner, and infection by R5 of clade A, C, and E (Table **1**).

Moreover, the anti-HIV activity of IA2-F9 or CPMAb-I to CPMAb-VII was also determined by an infection assay using MAGIC-5 cells [65, 67, 70]. The anti-HIV activities are summarized in Table **1**. Interestingly, CPMAb-I to CPMAb-VII inhibited HIV-1 infection in a clone-dependent manner. Therefore, these results indicate that Abs raised

Table 1. Induction of Autoantibodies After Immunization with Cycloimmunogen in Balb/c Mice and Antiviral Activities

Antigen	Clone	Ab-binding competition[a]		Flow cytometric analysis[b]		Anti-HIV activity[c]			
		Cycloimmunogen	Linear immunogen	CCR5	CXCR4	Clade B (Laboratory strain)		Non-clade B (Primary strain)	
						R5	X4	R5	X4
cDDR5-MAP	KB8C12	+	-	+	-	+	-	+ (A, C, E)	-
cDDX4-MAP	IA2-F9	+	-	-	+	-	+	ND[d]	ND[d]
cCD-MAP	CPMAb-I	ND[d]	ND[d]	+	+	+	+	+ (E)	-
cCD-MAP	CPMAb-II	ND[d]	ND[d]	+	+	+	+	-	-
cCD-MAP	CPMAb-III	ND[d]	ND[d]	+	+	+	-	-	+ (C)
cCD-MAP	CPMAb-IV	ND[d]	ND[d]	+	+	-	-	+ (A, C, E)	-
cCD-MAP	CPMAb-V	ND[d]	ND[d]	+	+	-	-	+ (A, E)	+ (E)
cCD-MAP	CPMAb-VI	ND[d]	ND[d]	+	+	+	-	+ (A, C)	+ (A, C, E)
cCD-MAP	CPMAb-VII	ND[d]	ND[d]	+	+	+	+	+ (A, E)	+ (A, C, E)

a) To estimate the immunochemical specificity of antoantibodies raised from each cycloimmunogen, a BIAcore biosensor bound with biotinylated cycloimmunogen was used as described in refs. [65, 69]. For Ab-binding competition assay, cycloimmunogens (cDDR5, cDDX4) or linear immunogens (linear DDR5, linear DDX4) were used as competitors. + indicates that the binding of KB8C12 or IA2-F9 is competed by only cDDR5 or cDDX4.

b) The binding of autoantibodies to cells expressing CCR5 or CXCR4 was determined using a flow cytometer as described in refs. 65, and 69. + indicates that the autoantibody recognizes native CCR5- or CXCR4-expressing cells.

c) The anti-HIV-1 activity of autoantibodies was determined using MAGIC-5 cells as described in refs. 65, 67, 69, and 70. These cells were separately inoculated with various strains of HIV-1 (clade B: R5 HIV-1 strain JRFL and X4 HIV-1 strain LAV-1; non-clade B: 93RW004 (R5 of clade A), MJ4 (R5 of clade C), 92TH009 (R5 of clade E), 92UG029 (X4 of clade A), 98IN017 (X4 of clade C) and CMU08 (X4 of clade E)) in the presence of the autoantibody. + indicates that the anti-HIV-1 activity is more than 50%. The clade of the primary isolate affected are shown in parentheses.

d) Not determined.

against cCD-MAP have a potent and broad-spectrum inhibitory activity against primary isolates.

Immunogenicity of Chemokine Receptor-Based Cycloimmunogens in Cynomolgus Macaques

To verify whether cDDR5-MAP can induce CCR5-specific Abs with anti-HIV-1 activity in nonhuman primates, an experiment was performed using cynomolgus macaques [66]. Three cynomolgus macaques were immunized with cDDR5-MAP in CFA or IFA by i.p. or s.c. injection according to our immunization schedule. Another three cynomolgus macaques were immunized with MAP as the control. cDDR5-specific Abs were significantly induced in the sera from cynomolgus macaques. The binding of the cDDR5-specific Abs was competed in the case of pretreatment with cDDR5, Fig. (**5A**). The sera showed the immunofluorescence staining of CEM-CCR5 cells, compared with preimmunization sera from these macaques and competed with 2D7 binding, Fig. (**5B**). In contrast, the immunization of

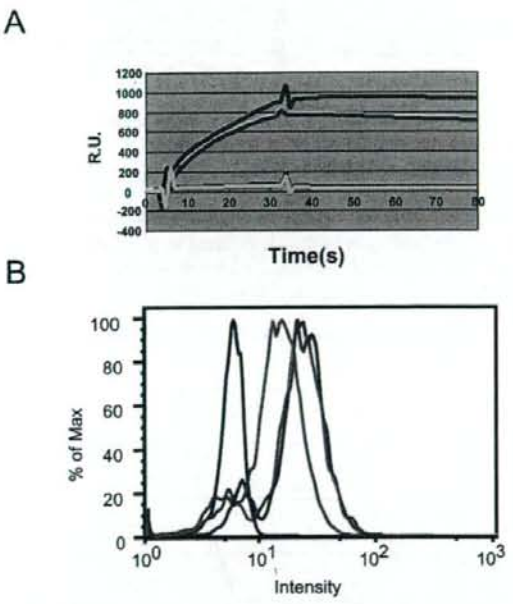

Fig. (5). (A) Immunochemical specificity of anti-cDDR5-specific Abs in sera from cynomolgus macaques. Binding specificity was determined by real-time biomolecular interaction analysis using surface plasmon resonance with a BIAcore biosensor coating biotinylated cDDR5. Antigen-Ab binding and competitive experiments were carried out with serum treated without (blue line) or with cDDR5 (0.1 (green line), 1 (red line), or 10 mmol (light blue)). (B) Anti-cDDR5 specific Abs elicited in cynomolgus macaques compete with 2D7. CEM-CCR5 cells were separately incubated with 2D7 (1 μg) in the absence (blue line) or presence of the preimmunization (green line) or 8 weeks postinitial immunization sera (orange line), and an isotype-matched IgG Ab (control; 1 μg; red line) at 4°C. Then the cells were washed with the washing buffer and resuspended in washing buffer containing FITC-conjugated anti-mouse IgG. The cells were analyzed using an EPICS XL flow cytometer.

cynomolgus macaques with MAP did not induce cDDR5-specific Abs. Furthermore, the anti-HIV-1 activity of sera from cDDR5-MAP immunized macaques was determined using MAGIC-5 cells expressing CCR5 [66]. The cells were separately inoculated with two laboratory strains of clade B HIV-1 (JRFL and LAV-1) or R5 nonclade B HIV-1 primary isolates (clade A: HIV93RW004 and clade C:HIVMJ4) in the presence or absence of immune sera. As expected, the sera from cDDR5-MAP-immunized cynomolgus macaques markedly suppressed infection by HIV-1 (JRFL) in a dose-dependent manner and also suppressed infection by R5 nonclade B HIV-1 primary isolates [66].

We also verified whether cDDX4-MAP can induce CXCR4-specific Abs with anti-HIV-1 activity in nonhuman primates as well as rodents [65]. As expected, cDDX4-bound Abs were detected in the sera from three monkeys 10 weeks postinitial immunization (wpim). However, the sera from two of three cDDX4-MAP-immunized monkeys significantly inhibited HIV-1 infection whereas the serum from one monkey did not, as shown by the comparison between monkeys 0 and 10 wpim. On the other hand, all sera from cDDX4-MAP-immunized monkeys at 27 wpim inhibited HIV-1 replication after boosting at 25 wpim.

Interestingly, the immunization of cynomolgus macaques with cCD-MAP induced cCD-specific Abs in the antisera within, at the most, 6 wpim, although some differences in cCD-specific Ab induction were observed among the macaques [67]. The antisera sampled after immunization specifically recognized CCR5- and CXCR4-expressing MAGIC-5 cells, and inhibited HIV-1 infection by the R5 and X4 laboratory strains of clade B and broadly inhibited infection by HIV-1 primary isolates of nonclade B (93RW004, MJ4, 92TH009, 92UG029, 98IN017 and CMU08, respectively). These results suggest that cCD-MAP induces Abs capable of protecting cross-clade HIV-1 infection.

Protection Against Simian/Human Immunodeficiency Virus Intravenous Challenge

The SHIV-$1_{SF162P3}$ bulk isolate is a pathogenic CCR5-specific simian/human immunodeficiency virus (SHIV) in rhesus macaques [71]. Because sera from cDDR5-MAP-immunized macaques significantly suppressed the infection of MAGIC-5 cells and CEM-CCR5 cells by the SHIV-$1_{SF162P3}$ bulk isolate, all of the MAP- and cDDR5-MAP-immunized macaques were i.v. challenged with 1 ml of 10 $TCID_{50}$/ml $SHIV_{SF162P3}$ five weeks after the third immunization [66]. The course of acute viral infection was monitored by measuring plasma viral RNA load in acutely infected macaques, Fig. (**6**). All of the three control macaques developed detectable plasma viremia, as demonstrated by viral peaks between 1 x 10^9 and 3 x 10^9 viral RNA copies/ml plasma, and sustained plasma viremia at >10^8 viral RNA copies/ml plasma for 3 weeks (1-4 weeks postchallenge). Furthermore, we compared the geometric mean plasma viral RNA loads of the vaccinated and control groups to monitor the effectiveness of vaccination. There were differences of ~19.95- to 217.10-fold in the geometric mean viral loads of the two groups between 1 and 4 weeks postchallenge, Fig. (**6**). However, previous study demonstrated that the immunization with cDDR5-MAP induced anti-cDDR5 serum pro-

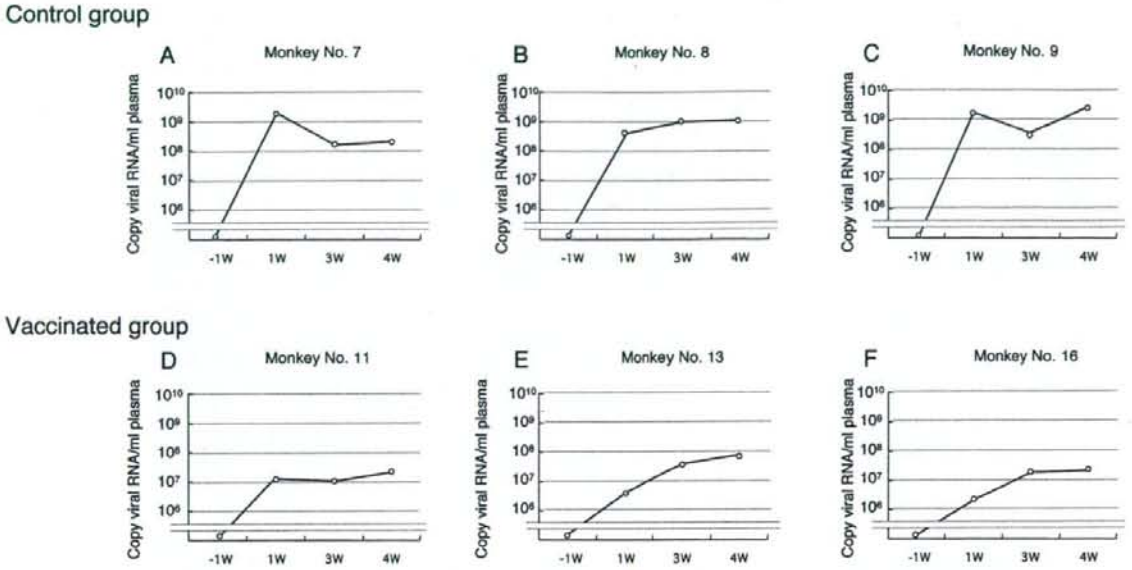

Fig. (6). (A) Intravenous challenge with $SHIV_{SF162P3}$. Whole-blood samples were collected at -1, 1, 3, and 4 week postchallenge, and the samples were examined for plasma viremia. Viral RNA was extracted from macaque plasma and then reverse-transcribed. The resulting cDNA duplicates were amplified by quantitative real-time PCR, as described in Materials and Methods [66].

duction for about 15 weeks after the third immunization although the titer of anti-cDDR5 sera declined over time until 21 wpim [66]. Taken together, these results suggest that the viral load in cDDR5-MAP-vaccinated macaques following a challenge with $SHIV_{SF162P3}$ can be controlled for a longer time when the anti-CCR5 Ab continues to be strongly induced by vaccination for a longer time.

We are currently investigating whether cDDX4- and cCD-MAP vaccination can protect against simian/human immunodeficiency virus challenge.

Effects of Boosting

We assessed whether revaccination is successful at boosting the anti-cDDR5 response. Unfortunately, the anti-cDDR5 Ab was not detected at 25 wpim, but revaccination at 25 weeks with cDDR5-MAP in complete Freund's adjuvant was successfully boosted the Ab response against cDDR5-MAP, Fig. (7). These results suggest that the UPA-based cycloimunogen strategy efficiently breaks B-cell tolerance and the titer of anti-cDDR5 sera was maintained by effective boostings.

On the other hand, cynomolgus macaques are immunized with cDDX4-MAP or cCD-MAP as well as cDDR5-MAP using the same vaccination protocol. The revaccination with cDDX4-MAP in complete Freund's adjuvant was also effectively boosted the Ab response against cDDX4-MAP. Interestingly, the immunization with cCD-MAP induced a longer-term production of anti-cCD serum, that is, for ~72 weeks after the third immunization and the revaccination with cCD-MAP in complete Freund's adjuvant was also successful at boosting the Ab response against cCD-MAP. These results suggest that cCD-MAP is one of the attractive mimotope peptide antigens for circumventing the tolerance mechanisms that the immune system has developed to normally block the maturation of B cells specific for self-antigens.

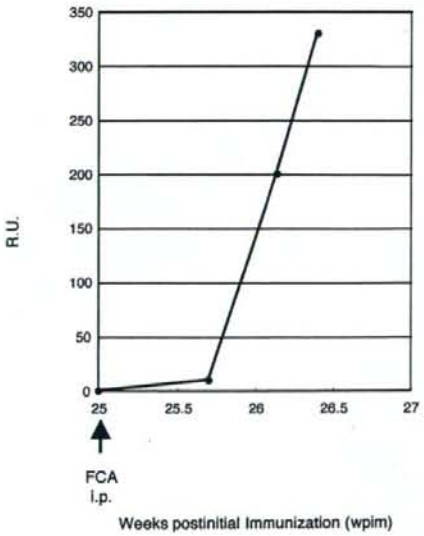

Fig. (7). Effects of boosting at 25 weeks postinitial immunization. The anti-cDDR5-specific Abs from serum were detected by real-time biomolecular interaction analysis using surface plasmon resonance with a biotinylated cDDR5-bound BIAcore biosensor, as shown in Fig. (5). The highest response units (R.Us.) are plotted.

Absence of Adverse Side Effects

Previous reports indicated that CCR5 is a redundant molecule in adults because CCR5-defective individuals have normal inflammatory and immune reactions [72]. On the other hand, studies with CXCR4 knockout mice revealed defects in B-cell lymphopoiesis and bone-marrow myelopoiesis. Moreover, mice lacking CXCR4 exhibit hematopoietic and cardiac defects identical to those observed in SDF-1-deficient mice, and die before birth [73-75]. However, all these deficits are developmental and it is not known whether SDF-1 and CXCR4 are essential for normal physiological processes after birth. Furthermore, although the safety of small-molecule CXCR4 inhibitors such as AMD3100 and ALX40-4C was investigated [76-78], it is still controversial. It is necessary to analyze the long-term safety of a novel approach targeting chemokine receptors under various conditions in human and nonhuman primates in more detail. However, no clinically significant adverse events were observed in all the monkeys immunized with cDDR5-MAP, cDDX4-MAP or cCD-MAP [65-67]. The previous pilot test demonstrated that the titer of anti-cDDR5 serum measured 4 weeks after the third immunization (10 wpim) was the highest in the vaccinated macaques. However, each macaque gained weight gradually during and after the immunization (Fig. (**8A**)). Furthermore, no significant changes in CD4+ cell/total PBMC (%) were observed in all the monkeys (monkeys nos. 11, 13, and 16 in Fig. (**6**)) immunized with cDDR5-MAP at 6, 10, and 12 wpim, as shown in Fig. (**8B**).

New Vaccine Strategy and Conclusions

We were interested in studying specific immune responses in HIV-1 infection in ESN individuals to reproduce the ESN immune status by a candidate vaccine. A small number of HIV-1-exposed individuals do not become infected, despite repeated and long-term exposures to the virus [79]. Interestingly, Lopalco *et al.* reported that anti-HLA class I, anti-CD4, and anti-CCR5 Abs are induced in ESN individuals [80-82]. In particular, the anti-CCR5 Ab seems to protect against R5 HIV-1 transmission as one of the "gatekeepers" as well as other restrictive barriers for HIV-1 transmission. Therefore, we created a cycloimmunogen (cDDR5-MAP) that mimics the deduced conformational epitope of UPA in CCR5 and immunized cynomolgus macaques. The results suggest that the high induction of the anti-CCR5 Ab can suppress viral propagation during acute HIV-1 transmission, but only a high induction of the anti-CCR5 Ab is not sufficient to clear detectable plasma-associated viruses because the anti-CCR5 Ab does not directly neutralize $SHIV_{SF162P3}$. It seems difficult to completely eliminate or inhibit HIV-1 acute infection *in vivo* when only the anti-CCR5 Ab delays viral propagation during the initial HIV-1 transmission. Lopalco *et al.* [83] have recently reported that anti-virus Abs such as IgA to gp41 are simultaneously induced with IgG to CCR5 and IgG to CD4 in some Italian ESN individuals. These humoral immune responses concomitantly seem to contribute to an extremely low level of viral replication below the detection limit of a standard assay in ESN individuals. Margolis and Shattok proposed that the field of vaccine development needs to establish strategies that target multiple arms of the immune system in the different stages of the viral life cycle [84]. Therefore, if the resistance to HIV-1 transmission is mediated by multiple mechanisms such as the simultaneous induction of the anti-CCR5 Ab and anti-HIV humoral responses (e.g., anti-gp41 Ab), the vaccine that targets multiple stages of mucosal transmission will most likely succeed in reproducing the ESN immune status, Fig. (**9**). We are developing a novel conjugate vaccine composed of cDDR5 and recombinant env protein.

Furthermore, a large fraction of patients progress to AIDS without the "phenotype switch", suggesting that in many patients. R5 viruses seem to be inherently more patho-

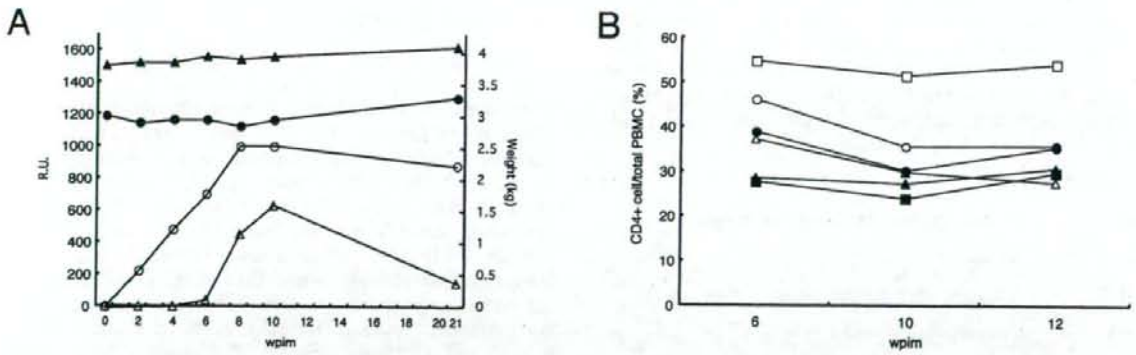

Fig. (8). Variations in weight and peripheral CD4+ T-cell number (%) after induction of cDDR5-specific Abs in cynomolgus macaques. (A) To determine whether the induction of cDDR5-specific Abs produces unwanted side effects, new serum samples obtained before and after immunization with cDDR5-MAP (at 0, 2, 4, 6, 8, 10, and 21 wpim) were collected. The antisera against the moiety of cDDR5 were detected by real-time biomolecular interaction analysis using surface plasmon resonance with a biotinylated-cDDR5-bound BIAcore biosensor (monkey No. 4: open circle; monkey No.5: open triangle). Simultaneously, the patterns of variation in body weight were investigated in two macaques (monkey No. 4: closed circle; monkey No.5: closed triangle). (B) Whole-blood samples derived from the cDDR5-MAP-vaccinated (monkey No. 11 (open circle), monkey No.13 (open triangle), monkey No.16 (open square) in Fig. (**6**)) and control (monkey No. 7 (closed circle), monkey No. 8 (closed triangle), monkey No. 9 (closed square) in Fig. (**6**)) groups were collected at 6, 10 and 12 wpim, and the samples were examined for peripheral CD4+ T-cell count (%).

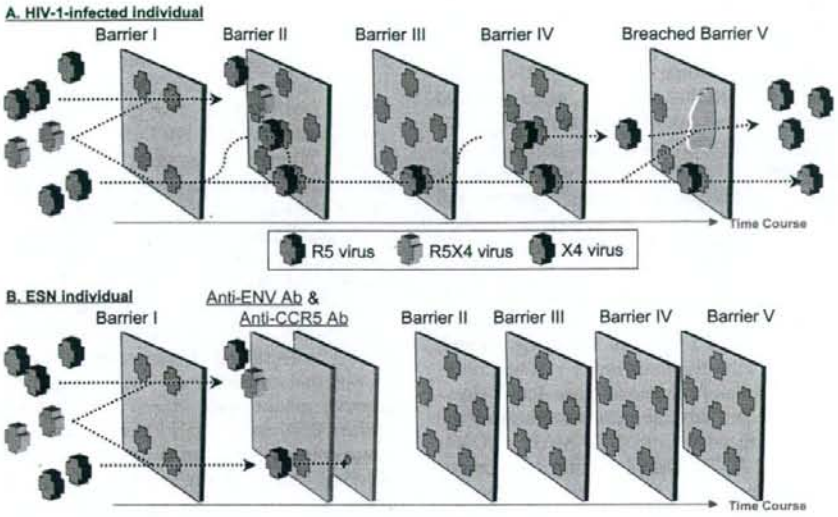

Fig. (9). Paradigm for the host's immune response to HIV and reproduction of ESN immuno status. (A) HIV-1-infected individual has a range of immune barriers (light gray squares edged with dark gray) that can potentially restrict the establishment of HIV-1 infection. Consequently, the HIV-1 strains that are most commonly responsible for transmission and dominate during the long asymptomatic phase are almost constantly restricted to CCR5 usage. A large fraction of patients progress to AIDS without the viral "phenotype switch" to CXCR4 usage. On the other hand, in some case, X4 virus emerges at the late phase of HIV-1 infection in the event that one of the barriers is breached (the barrier V in this figure), and its emergence is also associated with a rapid decline in the count of $CD4^+$ T cell and progression to AIDS. (B) In ESN individual, the resistance to HIV-1 transmission is mediated by multiple mechanisms such as the simultaneous induction of anti-CCR5 Ab (light gray square edged with green) and anti-HIV humoral responses (e.g. anti-gp41 Ab, light gray square edged with pink) in addition to potential immune barriers I-V. Therefore, we are developing a novel conjugate vaccine composed of cDDR5 and recombinant env protein to reproduce ESN immue status. Taken together, the vaccine that targets multiple stage of mucosal transmission could have the best of success.

genic at late phase of HIV-1 infection and X4 viruses appear only transiently on a background of sustained R5 dominance [85-87]. On the other hand, in some case, X4 viruses emerge during chronic infection, and its emergence is associated with a rapid decline in the count of $CD4^+$ T cell and progression to AIDS [88-90]. Therefore, the combination of cDDR5- and cDDX4-MAP, or cCD-MAP alone may provide a greater protection against HIV-1 infection than a single immunization of each cycloimmunogen.

ABBREVIATIONS

HIV-1 = Human immunodeficiency virus type 1

Env = Envelope

AIDS = Acquired immune deficiency syndrome

UPA = Undecapeptidyl arche

ESN = Exposed seronegative

Nt = *N*-terminus

ECL-2 = Second extracellular loop

MAP = Multiple-antigen peptide

MOE = Molecular Operating Environment

MAb = Monoclonal antibody

Ab = Antibody

SHIV = simian/human immunodeficiency virus

ACKNOWLEDGEMENTS

We thank Drs. Y. Maeda and S. Harada (Kumamoto University) for providing the CEM-CCR5 and NP2 cell lines. We also thank Dr. M. Tatsumi (National Institute of Infectious Diseases, Tokyo, Japan) for providing the MAGIC-5 cells. BIAcore analysis was supported by the Center for AIDS Research, Kumamoto University. We thank Dr. R. Mukai (National Institute of Infectious Diseases) for estimating the vaccine. We thank Drs. K. Tachibana, M. Umeda, T. Honda, and H. Shibata (Research Laboratory, Nissui Pharmaceutical) for excellent technical assistance. We are particularly grateful to Drs. Endo, and Nakayama, and collaborators, Messrs. S. Hayashi, Y. Ido, S. Kusaba, K. Tokunaga and T. Iiboshi, and Ms. R. Nakajima for excellent technical assistance. 93RW004, MJ4, 92TH009, 92UG029, 98IN017, and CMU08 and $SHIV_{SF162P3}$ were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a Health Science Research

Grant from the Ministry of Health, Labour, and Welfare of Japan. Back

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Received: 05 May, 2006 **Accepted: 25 August, 2006**

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Biochemical and Biophysical Research Communications 368 (2008) 501–507
BBRC
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Nonhuman primate intestinal villous M-like cells: An effective poliovirus entry site ☆

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Received 11 January 2008
Available online 7 February 2008

Abstract

Humans and some Old World monkeys, chimpanzees, and cynomolgus macaques, are susceptible to oral poliovirus (PV) infection. Interestingly, rhesus macaques, although sensitive to injected PV, are not susceptible to gut infection. Not much is known about the initial event of gut infection by PV in rhesus macaques so far. Here, we show that PV can efficiently enter the lamina propria (LP) by penetrating across intestinal villous M-like cells in rhesus macaques. We found by immunofluorescence analysis that PV effectively invades LP rather than germinal centers (GCs) in rhesus macaques despite expressing PV receptor CD155 on cells within GCs and LP. Furthermore, energy dispersive X-ray spectroscopy demonstrated that gold-labeled PV is spatiotemporally internalized into villous M-like cells and engulfed by macrophage-like cells in LP. These results suggest that rhesus macaques may be resistant to productive gut PV infection owing to a defective translocation of PV to GCs.

Keywords: Villous M-like cell; Mucosa; Old World monkeys; Poliovirus; Gut infection

Nonhuman primates are the closest evolutionary relatives of humans. Their underlying physiology and metabolism, as well as genomic structure, are more similar to those in humans than to those in other mammals. This makes nonhuman primates particularly important as models of human diseases, including viral infectious diseases. Although chimpanzees are the animals most similar to humans, they are unsuitable for preclinical studies on ethical grounds because they are an endangered or threatened species. For other primates, there are the requirements of research utility and availability. Because of this, the rhesus and cynomolgus macaques are the excellent choice for pathophysiological and preclinical studies.

Humans are the only known natural hosts of poliovirus (PV), which causes poliomyelitis. In humans, PV has been isolated from tonsillopharyngeal tissues, the wall of the ileum, and mesenteric lymph nodes [1]. Oral PV infection can be associated with extensive tissue destruction in lymphoid organs of the pharynx, including the tonsils, and the small intestine, including Peyer's patches (PPs) [2], suggesting that the virus replicates in these tissues. In contrast, nonhuman primates are highly susceptible to PV via all routes except the oral route, yet some species show a certain degree of oral susceptibility [3]. Cynomolgus macaques are susceptible to infection via the oral route but only when a large dose of PV is administered. On the other hand, rhesus macaques are rarely susceptible to PV administered

Abbreviations: $CCID_{50}$, 50% cell culture infective doses; EDS, energy dispersive X-ray spectroscopy; FAE, follicle-associated epithelium; GCs, germinal centers; IFNAR, alpha/beta interferon receptor; LP, lamina propria; PV, poliovirus.

☆ The Ministry of Education, Culture, Sports, Science and Technology of Japan (16017287), and a Health Science Research Grant from the Ministry of Health, Labour, and Welfare of Japan (18220501).

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0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.01.120

orally. Iwasaki et al. suggested that in rhesus macaques, CD155 expression levels are low in the follicle-associated epithelium (FAE) and CD155 is not present in GCs [4]. In contrast, in humans, CD155 was detected on the intestinal epithelium, on M cells in PPs, and in GCs within PPs [4]. These findings suggest that PV replication in the gut may depend on the presence of CD155 in FAE, including M cells, and on cells in PPs. However, the gut infection pattern of PV using rhesus macaque models has not been performed so far.

CD155 transgenic (Tg) mice are also not susceptible to oral infection by PV [5,6]. CD155 is present at very low levels in the intestinal epithelium of these mice and absent in PPs [4,7]. Overexpression of CD155 in the intestinal epithelium of Tg mice induced by a fatty acid binding protein promoter also does not lead to oral susceptibility to PV [7]. Recently, Ohka et al. [8] detected PV in the epithelia of the small intestine, which proliferated in the alimentary tract of CD155 Tg mice lacking the alpha/beta interferon receptor (IFNAR) gene. These results suggest that IFNAR plays an important role in determining permissivity in addition to the appropriate expression of CD155 in the alimentary tract of Tg mice. However, this still has not explained why healthy humans and limited Old World monkeys are highly susceptible to gut PV infection despite robust innate immune responses including interferon signaling.

Whether human epithelial or immune cells are the primary sites of PV replication in the intestinal mucosa has remained unclarified as well. Although PV may easily gain access to the surface of FAE because it remains largely free from secretions such as mucus, glycocalyx or IgA, enterocytes other than those in the FAE may not be sufficiently accessible to PV to initiate infection. Furthermore, Iwasaki et al. indicated that the distribution of CD155 on human FAE-enterocytes other than M cells may not be favorable for intestinal PV infection because the distribution is higher on the basal side [4]. This notion is supported by the finding that M cells in humans are the site of PV penetration of the intestinal epithelial barrier [9]. From these results, we speculate that PV may not be preferentially absorbed into GCs through rhesus FAE-M cells and replicates in rhesus lymphoid tissues.

Typical FAE-M cells, characterized by an irregular brush border and a reduced amount of glycocalyx, efficiently take up and transport a wide variety of macromolecules and microorganisms from the gut lumen into PPs [10–14]. A recent study demonstrated that intestinal villous M cells serve as another antigen gateway in mice for the sampling of gut bacteria and subsequent induction of Ag-specific immune responses in a PPs-independent manner [15]. Thus, it is possible that villous M cells also serve as the PV entry site in the mucosal epithelium and are involved in oral PV infection in human and some Old World monkeys.

In this study, we discovered that, in rhesus macaques, PV can penetrate into LP via intestinal villous M-like cells rather than FAE-M cells, suggesting that rhesus macaques may be resistant to gut PV infection due to a defective translocation of PV to GCs via FAE-M cells.

Materials and methods

Animals and tissue samples. Purpose-bred female rhesus macaques (*Macaca mulatta*) obtained from a supplier in China (10–12 years old, weighing 4.55–6.26 kg) were used for this study. PPs of cynomolgus macaques (weighing 5–6 kg) were obtained from 4 to 5 years old female monkeys. This study (the Permission No. 19–137) was approved and carried out according to the guidelines of the Animal Care and Use Committee of Kumamoto University.

Gold labeling of PV. PV was labeled with gold colloid solution (5 nm, British BioCell International, Ltd.) according to the instruction manual. Gold nanoparticles not anchored on PV were readily removed by centrifugation. Gold-labeled PV was subjected to negative staining electron microscopy.

Inoculation of PV. Rhesus macaques were fasted overnight. They were then inoculated with 1 ml of PV solution (type I, $10^{5.5}$–$10^{6.5}$ 50% cell culture infective doses ($CCID_{50}$); type II, $10^{4.5}$–$10^{5.5}$ $CCID_{50}$; type III, $10^{5.0}$–$10^{6.0}$ $CCID_{50}$) or 1 ml of gold-labeled PV at a site in the ileum (15 cm from the cecum) after celiotomy under anesthesia induced by a subcutaneous injection of urethane (ethyl carbamate, 800 mg/mL; 1.5 mL/kg body weight; Wako Pure Chemical Industries, Ltd.) solution and an intravenous injection of alpha-chloralose (Wako Pure Chemical Industries, Ltd.; 20 mg/mL; 5.5 mL/kg body weight) into the cephalic vein.

Collection of PPs. The rhesus monkeys were euthanized by exsanguination under anesthesia, and the part of the ileum (15 cm from cecum) including the inoculation site was collected. After washing the collected part of the ileum, 2 cm^2 blocks of PPs were fixed in ice-cold 3% glutaraldehyde/0.1 M sucrose/phosphate-buffered saline (pH 7.4). After 30 min of fixing, PPs were kept at 4 °C.

Histopathological study. Tissue samples were fixed in 10% neutral buffered formalin and were trimmed, embedded in paraffin, sectioned, stained with hematoxylin–eosin and examined by light microscopy.

Immunofluorescence staining. To examine the distribution of CD155 expression, the frozen sections of PPs were stained with various antibodies. In brief, 5 μm frozen sections were fixed in cold acetone and blocked with 1% nonfat skim milk in PBS(−). CD155 was detected using mAb D171 (Abcam Inc.) and FITC-conjugated anti-mouse IgG Ab, or FITC-labeled mouse mAb TX21 (MBL International). At the end of the staining, silides were wased and incubated with 4′,6-diamidino-2 phenylindole (DAPI) for nuclear staining (molecular Probes).

To examine how PV is incorporated into the lymphoid organ, the sections were stained with an anti-poliovirus antibody (II-MAP-01, Japan Poliomyelitis Research Institute) for 15 min. The sections were then incubated with TRITC-labeled goat anti-mouse IgG (Jackson Immunoresearch Laboratories) for 15 min. Finally, the sections were stained with FITC-labeled mouse mAb TX21. After the staining, slides were washed and analyzed with a Keyence Biozero BZ-8000.

Scanning electron microscopy (SEM). The tissue samples were rinsed in phosphate-buffered saline with 0.1 M sucrose (pH 7.4) and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer at 4 °C for 2 h. All the samples were dehydrated with 50:50, 70:30, 80:20, 90:10, and 95:5 ethanol/water mixtures and 100% ethanol for 10 min each and rinsed three times with 100% ethanol for further dehydration. The samples were critical-point dried by flooding with liquid carbon dioxide at 5 °C for 20 min and then raising the temperature to the critical-point (JCPD-5, JEOL). For SEM, samples were sputter-coated with gold (JFC-1100E, JEOL) and examined with a JEOL JSM-5200 scanning electron microscope at an accelerating voltage of 15 kV.

Transmission electron microscopy (TEM). The tissue samples were rinsed in phosphate-buffered saline with 0.1 M sucrose (pH 7.4) and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer at 4 °C for 2 h. All the samples were rinsed briefly with 50:50, 70:30, 80:20, 90:10, and 95:5 ethanol/water mixtures and 100% ethanol for 10 min each and three

times with 100% ethanol for dehydration, and then embedded in epoxy resin (Quatol 812). One micrometer sections were cut using a glass knife and then stained with toluidine blue. Suitable areas for ultrastructural study were chosen after examining 1 μm sections under a light microscope. Sections of 60–90 nm were cut on a Leica EM UC6 ultramicrotome using a diamond knife and sections were mounted on a copper grid and stained with 1% uranyl acetate and Reynolds lead citrate. The grids were examined under a JEOL JEM 1200-EX electron microscope.

Energy dispersive X-ray spectroscopy (EDS). EDS analysis, which was consigned to JEOL Datum Ltd., was performed to quantify PV by measuring gold concentration within a specimen.

Results

Intraintestinal inoculation site of PV in rhesus macaques

To elucidate the details of PV gut infection in rhesus macaques, an attenuated PV Sabin strain was carefully inoculated into the lumen of the ileum at 15 cm from the ileocecal valve (Fig. 1A). After inoculation, the terminal ileum tissue was subjected to light microscopy, immunofluorescence microscopy, SEM, and TEM. As shown in Fig. 1B, the largest Peyer's patch is found in the lumen of the terminal ileum in rhesus macaques. Light microscopy revealed the typical structure of a mucosal lymphoid follicle, composed of GCs and a dome area bulging into the lumen (Fig. 1C). Furthermore, SEM revealed that hemispherical domes were distributed between intestinal villi (Fig. 1D).

Comparison of CD155 expression in PPs between cynomolgus and rhesus macaques

To clarify the basis for the difference in susceptibility to oral PV infection between humans and rhesus macaques, Iwasaki et al. assessed the expression pattern of CD155 in PPs of these species. The results suggested that the suboptimal expression of CD155 in the rhesus macaque FAE and the lack of expression in GCs in PPs may explain why rhesus macaques are not susceptible to oral infection. Therefore, it is intriguing to speculate that in cynomolgus macaques, which are susceptible to oral PV infection, CD155 is most likely optimally expressed in FAE and GCs in PPs. To determine CD155 expression in cynomolgus macaques, we performed immunofluorescence staining of PPs using anti-CD155 Abs. The expression of CD155 in GCs in PPs was prominent in the case of staining with mAb D171 (Supplementary Fig. S1A) and mAb TX21 (Supplementary Fig. S1B) but that in FAE was not prominent. These results indicate that the mAbs used in this experiment can specifically stain CD155 on cells in cynomolgus GCs.

Furthermore, we assessed whether CD155 is not expressed in rhesus macaque PPs. Unexpectedly, in rhesus macaques, the expression level of CD155 on cells within FAE and GCs is not significantly different from that observed in cynomolgus macaques (Fig. 1E and F; see the less intensely stained GCs depicted by the DAPI nuclear staining in Fig. 1F). Furthermore, anti-CD155 mAb (TX21) can also stain cells in GCs in different sections of rhesus macaque PPs (Supplementary Fig. S1C).

Inoculated PV was efficiently incorporated into rhesus macaque villi

To examine how PV is incorporated into the lymphoid organ, attenuated PV was inoculated into the rhesus macaque ileum. One hour after the injection, the tissue was subjected to immunofluorescence analysis. As shown in Fig. 1H, the virus was detected inside the villi but was hardly detected in GCs that were strongly stained by anti-CD155 mAb (Fig. 1G). A merged image of panels 1G and 1H is shown in panel 1I. As shown in Fig. 1I, the colocalization of PV and CD155 was prominent in LP (Fig. 1I). When PPs from a different section were further observed, the virus was clearly detected in the LP of villi (Fig. 1J). On the other hand, weak fluorescence was detected in the subepithelial dome region in PPs (Fig. 1J) although fluorescence was hardly detected in GCs. These results indicate that PV efficiently enters into the villi rather than GCs within PPs in rhesus macaques. It is highly possible that a cofactor involved in PV accumulation in GCs is lacking in rhesus macaques.

SEM and TEM of FAE-M Cells

To examine why PV is not efficiently transported into cells in GCs underneath FAE, we investigated whether there are no typical M cells in rhesus macaque PPs because it has been suggested that PV is transported into PPs via human M cells [9]. Microscopy at a low magnification revealed hemispherical domes that were distributed between intestinal villi (Fig. 1K). Among cells in FAE, there were a few M cells showing a typical depressed surface with short and irregular microvilli (Fig. 1L). Rhesus macaque M-cell microvilli were shorter, thicker and fewer in number than the microvilli of adjacent absorptive enterocytes (Fig. 1M). Furthermore, the M-cell cytoplasm was invaginated by migrating lymphoid cells (Fig. 1M). These results demonstrated that there are typical M cells in rhesus macaque FAE, although we cannot exclude the possibility that rhesus macaque M cells in PPs cannot efficiently take up PV in comparison to human M cells.

SEM and TEM of villous M-like cells

To examine why PV is efficiently transported into LP, we investigated whether there are M-like cells in rhesus macaque villi because it has been demonstrated that intestinal villous M cells serve as a gateway for the antigen sampling of gut in mice [15]. SEM of rhesus macaque villous M-like cells revealed the hallmark feature of M cells, which is a typical depressed surface with short and irregular microvilli (Fig. 1N and O). TEM also showed villous M-like cells (Fig. 1P). Furthermore, infiltrating lymphocytes

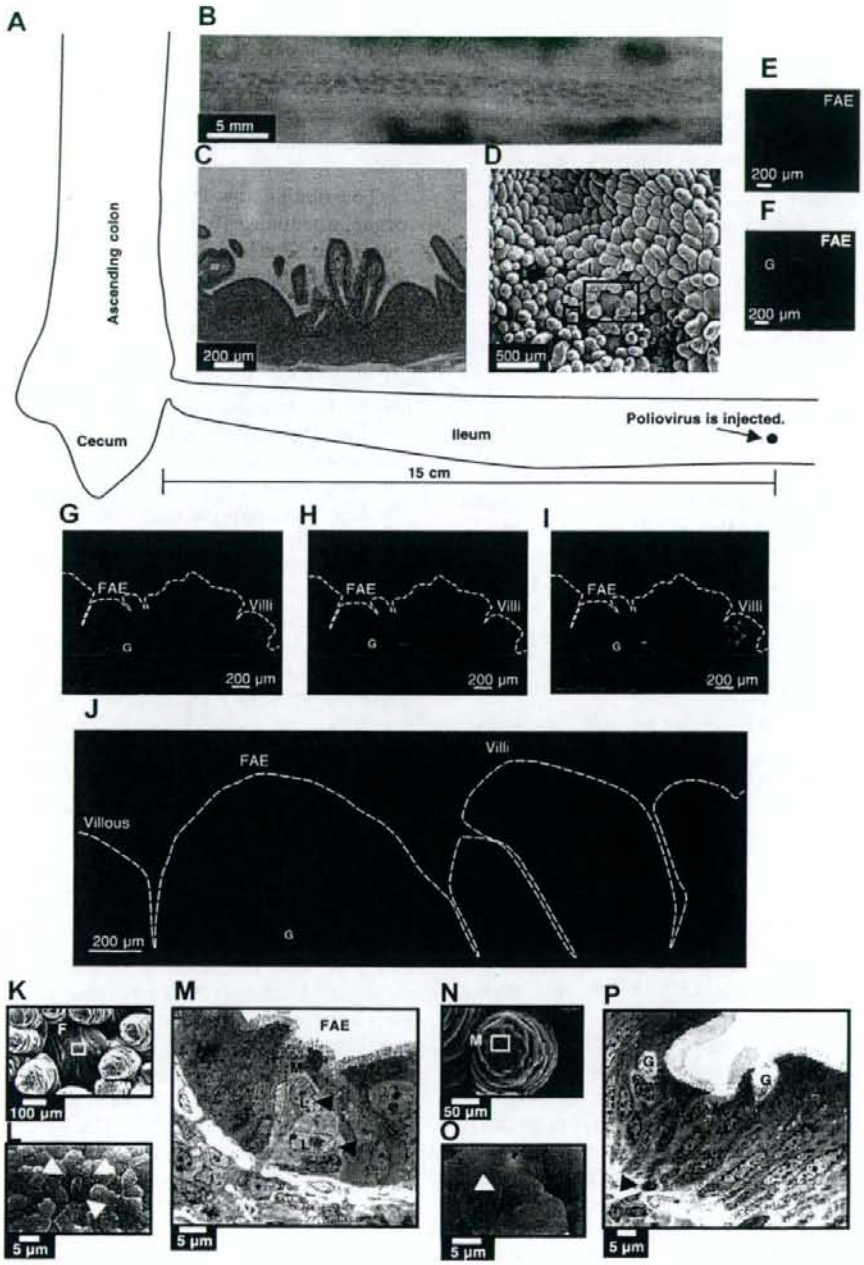

Fig. 1. PV is clearly detected in the lamina propria (LP) of villi. Attenuated PV was inoculated into the lumen of the ileum at 15 cm from the ileocecal valve (A). After inoculation, the parts of the ileum including the inoculation site and Peyer's patchs (PPs) were collected (B). Hematoxylin-eosin staining of rhesus PPs (C). Scanning-electron micrograph showing the ileum dome bulging into the gut lumen between intestinal villi (D). One hour after PV inoculation, the portion between the inoculation site of PV and the ileocecal valve was excised and subjected to immunofluorescence analysis. Frozen sections of rhesus macaque PPs were labeled with mAbs (D171 (E) and TX21 (G)) specific for CD155 (green) and nucleus was stained with 4', 6-diamidino-2 phenyindole (blue) (F). CD155 staining (G), PV staining (H), and merged (I) images are shown. (J) Different sections of rhesus macaque PPs were also stained by anti-PV mAb. PV can efficiently penetrate into the LP rather than into the subepithelial dome region. G, germinal center. SEM demonstrates that the M cells (K and L) in PPs and the villous M-like cells (N and O) in the villus epithelium are distinguishable from enterocytes by their relatively depressed and dark brush border (white arrowhead). TEM images of M cells in PP (M) and villous M-like cells in villi (P) show short stublike microvilli and the presence of infiltrating lymphocytes (L) in their pockets (black arrowhead). M, M cell or villous M-like cell.

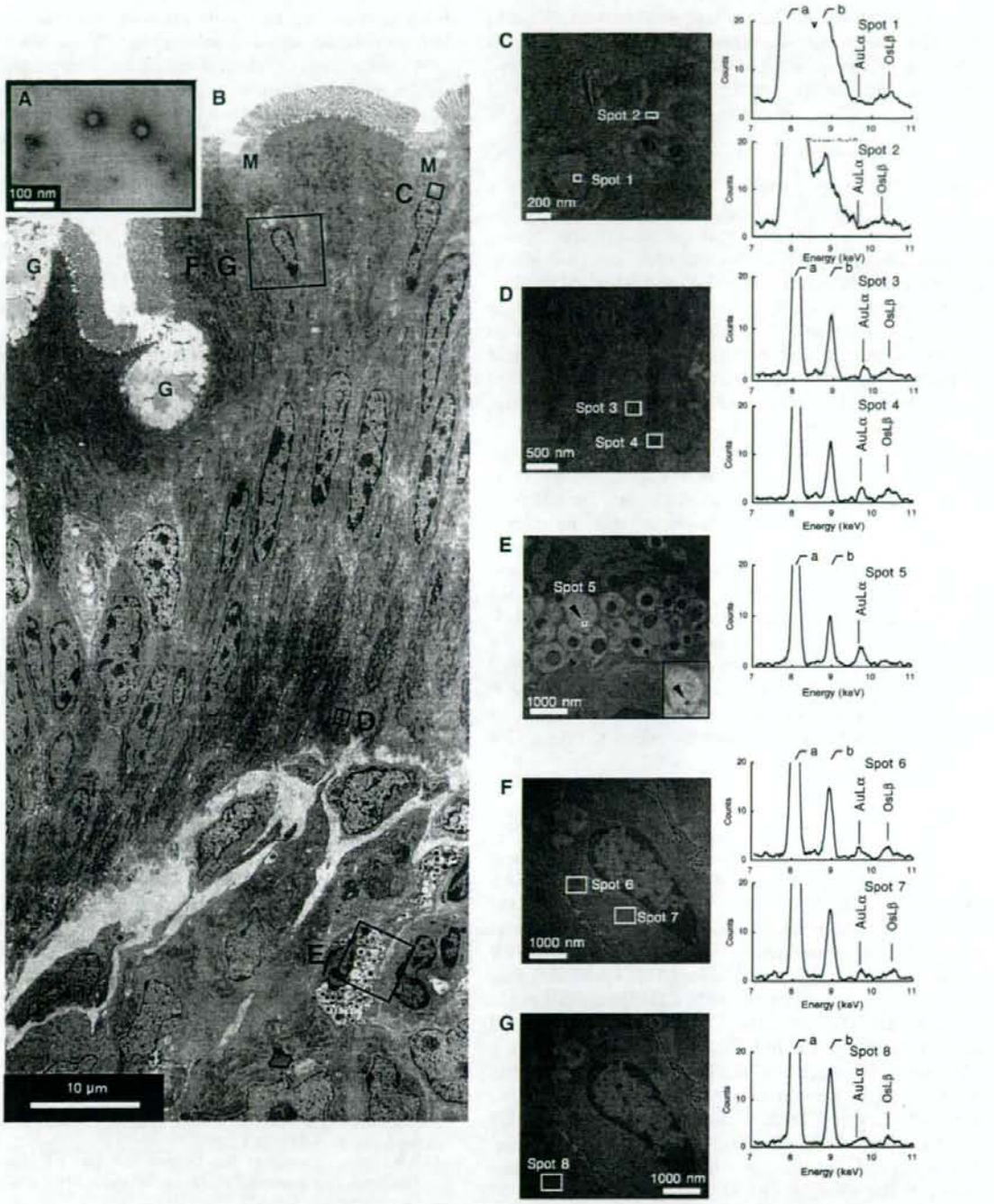

Fig. 2. PV can efficiently penetrate into villous M-like cells. PV was labeled with 5 nm gold particles and subjected to electron microscopies (A). A TEM view of villous epithelium (B) G, goblet cells; M, villous M-like cells. Panels (C–G) depict higher-magnification images of panel B for EDS. Graphs show EDS of spots (1–8) confirming the presence of gold particles (C–G). The a and b signals come from Cu (8.040 and 8.904 keV) that is attributed to the sample holder, and the b signal also contains the signal of OsLα (8.910 keV). The intensities of background signals from spots 1 and 2 in panel C were higher than those from other spots from panels D–G because spots 1 and 2 were on the TEM Cu-grid.

were also observed in the pockets of villous M-like cells (Fig. 1P). These results suggest that villous M-like cells may form an alternative gateway for PV and provide evidence that nonhuman primate M-like cells develop and localize in the villous epithelium as well as in the FAE of PPs.

Internalization of PV by villous M-like cells

To investigate whether rhesus macaque villous M-like cells can take up PV, the rhesus macaque ileum was inoculated with gold-labeled PV (Fig. 2A) and subjected to EDS. The advantage of EDS is that gold-labeled PV can be directly detected when PV is completely embedded in an ultrathin section. The characteristic X-ray peak from gold (AuL-alpha: approximately 9.712 keV) is used to confirm the presence of Nanogold within a section. Forty-five minutes after inoculation, EDS demonstrated the presence of gold-labeled PV in the basolateral cytoplasm (spots 3 and 4; Fig. 2B and D) but not in the apical cytoplasm (spots 1 and 2; Fig. 2B and C) of villous M-like cells. In addition, gold-labeled PV was also specifically engulfed by macrophage-like cells in LP (spots 5; Fig. 2B and E). As shown in the insert of Fig. 2E, a PV particle was clearly and directly detected. Furthermore, we examined whether the adjacent M-like cells also take up PV (spots 6 and 7; Fig. 2B and F). Results revealed the localization of PV beside the nucleus (Fig. 2F). In contrast, there is no signal corresponding to PV in an adjacent epithelial cell (spot 8; Fig. 2G). Taken together, these results indicate that rhesus macaque villous M-like cells have the ability to take up PV from the lumen.

Discussion

The premise of oral PV infection in humans postulates that CD155 is expressed not only on enterocytes in FAE but also on cells in GCs [4]. If so, cynomolgus epithelial cells in FAE and cells in GCs should express CD155 because cynomolgus macaques are susceptible to infection by PV administered orally. We searched for CD155 in PPs in cynomolgus macaques. Staining with CD155-specific mAbs (D171 and TX21) revealed that CD155 was highly expressed not only on enterocytes in FAE but also on cells in GCs.

However, not much has been known about the PV infection patterns in the gut of rhesus macaques so far. We examined whether CD155 is indeed not expressed within rhesus macaque GCs and whether PV is indeed not incorporated into rhesus macaque intestinal lymphoid tissue. Unexpectedly, staining with CD155-specific mAbs (D171 and TX21) revealed that rhesus macaque CD155 was highly expressed not only on cells in GCs but also on those in LP. Furthermore, our immunofluorescence analysis indicated that PV is efficiently incorporated into LP in the villi rather than into GCs. These findings suggest that in rhesus macaques, an as yet unidentified factor is required for the translocation of PV into GCs after PV penetrates into FAE-M cells, and that there are cells that function similarly to FAE-M cells as a gateway for PV on villi.

A recent study [15] has directly demonstrated the function of mouse villous M cells as a gateway for bacteria. The morphological characteristics of the rhesus macaque FAE-M cells in ileal PPs were previously reported [16], but to our knowledge, our present study is the first to show the function of nonhuman primate villous M-like cells as a gateway for PV. Rhesus macaque villous M-like cells were distinguishable from intestinal villous epithelial cells on the basis of the criteria including (i) short and irregular microvilli, (ii) endocytic activity and ability to take up PV as well as macromolecules, and (iii) an intraepithelial pocket that allows a cluster of lymphocytes to be located in the FAE of PPs; currently, there is no reliable identified antigen marker that can be used to positively identify primate villous M cells. Indeed, EDS analysis demonstrated that PV was internalized within villous M-like cells and transported into LP.

Acknowledgment

Both attenuated PV (Sabin Strain) and anti-PV Ab were obtained from the Japan Poliomyelitis Research Institute.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.120.

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