gene, is also associated with a delay in HIV-1 disease progression (Smith *et al.*, 1997; Kostrikis *et al.*, 1998; Mummidi *et al.*, 1998).

With respect to the promoter region, Martin *et al.* (1998) identified 10 polymorphic nucleotide positions in the 517 bp region of Caucasian *CCR5* promoters, and described 10 haplotypes in this region. Of these, the *P1* haplotype has G, C, and A at the 208th, 627th, and 676th positions, respectively, from the transcription start site of the *CCR5* gene. Although both *CCR2 64I* and *CCR5Δ32* were consistently found in the *CCR5 P1* haplotype, the homozygotes for (*CCR2 64V/CCR5 P1*/lacking *CCR5Δ32*) haplotype exhibited an epidemiological association with rapid progression to AIDS (Martin *et al.*, 1998). McDermott *et al.* (1998) reported that the presence of homozygous for *CCR5 59029G* lacking *CCR5Δ32* and *CCR2 64I* caused a delay by 3.8 years in the progression to AIDS compared to that seen in patients homozygous for *59029 A*. Position 59029 in McDermott's report corresponds to position 303 from the transcription start site. Since different reports have adopted different numbering systems for the *CCR5* gene (Moriuchi *et al.*, 1997; Guignard *et al.*, 1998; Kostrikis *et al.*, 1998; Martin *et al.*, 1998; McDermott *et al.*, 1998; Mummidi *et al.*, 1998), Carrington *et al.* (1999) proposed a new numbering system starting from the translation start codon. Using this numbering system, Mummidi *et al.* (2000) reported six human haplogroups (*HHA, -B, -C, -D, -E, -F* and -G) according to *CCR2 64, CCR5Δ32,* and eight polymorphic positions (–2733, –2554, –2459, –2135, –2132, –2086, and –1835) in the 926 bp region of Caucasian and African-American *CCR5* promoters.

It is well known that genetic polymorphisms and haplotype structures can vary among ethnic groups. *CCR5Δ32* is extremely rare in Asian populations (Martinson *et al.*, 1997). To explore the *CCR2-CCR5* haplotype structure in an Asian population, we performed a systematic survey of natural sequence variations in an 8.1-kb region of the entire *CCR5* gene as well as of *CCR2V64I* in 50 Japanese individuals and evaluated effects of those variations on *CCR5* promoter activity. Our results show several novel characteristics of the *CCR2-CCR5* haplotype structure that were not reported from studies on Caucasians and African-Americans.

Materials and methods

Clinical samples

Blood samples were collected with anticoagulant from randomly selected, non-related 80 non-HIV-1-infected and 50 HIV-1-infected Japanese at the outpatient clinic of the Institute of Medical Science, the University of Tokyo, Tokyo, Japan, after their written informed consent had been obtained. Blood samples were also collected with anticoagulant from randomly selected, non-related 97 non-HIV-1-infected and 89 HIV-1-infected Thai subjects at the Bamrasnaradura Institute, Nonthaburi, Thailand, after their written informed consent had been obtained. Peripheral blood mononuclear cells (PBMC) were obtained from blood with the Ficoll–Histopaque method, and DNA was extracted from the PBMC with a previously described method (Shioda *et al.*, 1994). The six French samples with *CCR5Δ32* (one homozygote and five heterozygotes) used for this study were described previously (Meyer *et al.*, 1997; Magierowska *et al.*, 1999).

Genotyping of CCR5 and CCR2

The 8.1-kb *CCR5* target region was amplified from each of the genomic DNA samples in seven (R5-1 to R5-7) overlapping segments using the amplification primers shown in Table 1. Polymerase chain reaction (PCR) was performed in a 50-μL reaction mixture containing 1 μg of DNA. Thermal cycling was performed with an initial 94 °C for 3 min followed by 40 cycles at 94 °C for 30 s, primer annealing for 30 s and primer extension at 72 °C and a final extension at 72 °C for 7 min. The PCR conditions of annealing temperature and extension time were shown in Table 2. The positions specified in *CCR5* were based on the numbering system proposed by Carrington *et al.* (1999). We defined this system as numbering system A and used this system in the present study. The positions in *CCR2* were based on the sequence with GenBank accession number U95626. McDermott *et al.* (1998) used this system, and we defined this system as numbering system B. The numbering system starting from the transcription start site (Martin *et al.*, 1998) was defined as numbering system C in the present study. Table 3 shows polymorphic positions in those three different numbering systems. Sequencing reactions were performed according to the dideoxy-chain-termination method using the ABI PRISM 377 (Applied Biosystems, Foster City, CA, USA) automated DNA sequencer. The sequences of primers used for sequencing reaction are shown in Table 1. The ABI sequence software (version 2.1.2) was used for lane tracking and first pass base-calling (PerkinElmer, Wellesley, MA, USA). Variant sites identified by both software and visual inspection were scored and entered into a database for subsequent analysis. Each variant site was confirmed by reamplifying and resequencing the variant site from the opposite strand. The linkage disequilibrium coefficient was calculated by using a software (ARLEQUIN version 2.01, Gennetica and Biometry Laboratory, Geneva, Switzerland).

Haplotype determination

Haplotypes were inferred from the samples by using a heuristic algorithm based on population genetic principles (Clark, 1990). Direct molecular haplotyping was used to confirm linkage relationships in compound heterozygotes using allele-specific PCR combined with direct sequencing and limited cloning of the specific products. All inferred haplotypes were confirmed with these molecular haplotyping techniques.

Allele-specific PCR

For this procedure, the amplification refractory mutation system (ARMS) was used (Newton *et al.*, 1989). An

Table 1. Primer sequences

Sense primer	5′ to 3′ sequence	Position[a]	Position[b]	Fragment	Antisense primer	5′ to 3′ sequence	Position[a]		Position[b]	Fragment
1. Primers used for PCR amplification and sequencing										
CCR2F	ATGCTGTCCACATCTCGTTC		46106 to 46125	R2-1	CCR2R	GGAAATTATTCCATCCTCGTG			46709 to 46689	R2-1
P61	AGCAAGGAGACAGCAAAGCA	−4796 to −4777	56696 to 56715	R5-1	P63	CCTTGTATTCACTGGTAATCC	−3924 to −3944		57568 to 57548	R5-1
P66	AGAACCAGCAATTGCCACAAACAGA	−4012 to −3988	57480 to 57504	R5-2	P68	GCTTCCGTGACCTTGGCTCTAG	−2941 to −2962		58547 to 58526	R5-2
LK81	AATTTTGCTGTTTGGGGTCTC	−3022 to −3002	58466 to 58486	R5-3	LK83	CTGATTATCTTAAGAGTTGC	−2369 to −2388		59119 to 59100	R5-3
LK84	AAGTCCAGGATCCCCCTCTA	−2443 to −2424	59045 to 59064	R5-4	LK87	CATTCCAAACTGTGACCCTTTCC	−1756 to −1778		59732 to 59710	R5-4
CR53F	TCCAGTGAGAAAAGCCCGTAAATA	−2162 to −2139	59326 to 59349	R5-5	CR53R	TGCCCACAAAACCAAAGATGAACA	142 to 119		61624 to 61601	R5-5
CKR5a +	CAGTTTGCATTCATGGAGGG	−84 to −65	61399 to 61418	R5-6	CKR5a−	CTAAGCCATGTGCACAACTC	1110 to 1091		62592 to 62573	R5-6
CR52F	GGAAATATCTGTGGGCTTGT	1038 to 1057	62520 to 62539	R5-7, IIIa, IIIb	CR52R	CTGCTTATAAAATGCTCTGG	3383 to 3364		64865 to 64846	R5-7
2. Primers used for sequencing										
P62F	TGAACCCCCCTGTCCTGAG	−4425 to −4407	57067 to 57085	R5-1, Ia, Ib	P62R	GCTAGATGGGAAACAAGGTGAGGA	−4295 to −4318		57197 to 57174	R5-1
P67F	GATTCGCTGTCCTTGTTATG	−3533 to −3514	57955 to 57974	R5-2, Ia, Ib	P67R	CCCAGCGATCAAGACACCC	−3436 to −3454		58052 to 58034	R5-2, Ia, Ib
LK82F	AGAACCTGAACTTGACCAT	−2811 to −2793	58677 to 58695	R5-3, Ia, Ib	LK82R	TTTTAACTATGGGCTCACG	−2573 to −2591		58915 to 58897	R5-3, Ia, Ib
LK85	GTGTAGTGGGATGAGCAGAGA	−2198 to −2178	59290 to 59310	R5-4, II a, II b	LK85R	TTTGAAGGAGGGTGGAGTT	−2019 to −2037		59469 to 59451	R5-4
433	ACACCAAGTGCTCATACAAT	−1825 to −1806	59663 to 59682	R5-5	1552R	TAGAGTTAGCCCACAAAGAA	−685 to −704		60801 to 60782	R5-5, IIa, IIb
CR53S1	TTTTCTAGGGGCTTCTCTCA	−1367 to −1348	60121 to 60140	R5-5, IIa, IIb	2046 R	CTGCTAGCTTCCCTGTCCACT	−187 to −207		61296 to 61276	R5-5, IIIa, IIIb
CR53S2	GCAGGCTTCCCGCATTCAAA	−938 to −919	60548 to 60567	R5-5, IIa, IIb	CKR5e−	GCTGCGATTTGCTTCAC	89 to 73		61571 to 61555	R5-5
1770	ACTTGGAGGGGTGAGGTGAG	−486 to −467	61000 to 61019	R5-5, IIa, IIb	CKR5d−	GAAGATTCCAGAGAAGAAGCC	351 to 331		61833 to 61813	R5-6
CKR5b +	GAGCATGACTGACATCTACC	186 to 205	61668 to 61687	R5-6	322	GTGAAGATAAGCCTCACAGCC	716 to 696		62198 to 62178	R5-6
CKR5c +	CTGTGTTTGCGTCTCTCC	467 to 484	61949 to 61966	R5-6, IIIa, IIIb	CR5R1	TGTCTTTTCTCCCCATAGCA	1527 to 1508		63009 to 62990	R5-7
CKR5d +	CAGTAGCTCTAACAGGTTGG	807 to 824	62289 to 62308	R5-6	CR5R4	TCACCACTATAGGGACCCCTT	2578 to 2559		64060 to 64041	R5-7
CR5S1	GCTGATTCTTGAGTTTAGTG	1387 to 1406	62869 to 62888	R5-7						
CR5S2	CATGGGGAGGAAGGACAAGG	1768 to 1787	63250 to 63269	R5-7, IIIa, IIIb, IVa						
CR5S3	GGAGGAGGAGGTTTAGGTCA	2168 to 2188	63650 to 63670	R5-7						
CR5S4	AAGGGGTCCCATAGAGGTGA	2559 to 2578	64041 to 64060	R5-7						
CR5S5	TGAATTTGGGGGATGGCTAA	2954 to 2973	64436 to 64455	R5-7						
3. Primers used for allele-specific PCR amplification										
627UA	TCCTATGGGGTGTCCGAATGT	−4638 to −4618	56854 to 56874	Ia, Ib	627N	GAATAGATCTCTGGTCTGtAA	−2115	−2135	59373 to 59353	Ia
303 N	GAGAGTGGAGAAAAAGGtGG	−2478 to −2459	59010 to 59029	IIa	627M	GAATAGATCTCTGGTCTGtAG	−2115	−2135	59373 to 59353	Ib
303 M	GAGAGTGGAGAAAAAGGtGA	−2478 to −2459	59010 to 59029	IIb	303DA	TAAGAAACTGGGTCAAGCAT	−316	−335	61167 to 61148	IIa, IIb
2398 N	CTGTCTCACAACAACAtCAG	−381 to −362	61105 to 61124	IIIa	2398DA	AACCAGACCCATCCTTTTAC	2226	2207	63708 to 63689	IIIa, IIIb
2398 M	CTGTCTCACAACAACAtCAA	−381 to −362	61105 to 61124	IIIb	5765M	ATGTGCACAATCATATGtGAC	2939	2919	64421 to 64401	IVa
5765UA	GACTTAGAACCAGGCGAGAG	1602 to 1621	63084 to 63103	IVa						

Underline; allele-specific nucleotide.

Lower case t; additional internal (position −3) mismatch that increases specificity of allele-specific PCR.

[a]Position according to Mummidi *et al.* (2000) (numbering system A).

[b]Position in the baseline sequence (GenBank accession number U95626, numbering system B).

Table 2. Amplification protocol

Fragment	Sense primer	Antisense primer	Annealing temp (C)	Extension time (s)	Length (bp)	Position[a]	Position[b]
R2-1	CCR2F	CCR2R	60	30	604		46106 to 46709
R5-1	P61	P63	60	30	873	−4796 to −3924	56696 to 57568
R5-2	P66	P68	60	30	1072	−4012 to −2941	57480 to 58547
R5-3	LK81	LK83	60	30	654	−3022 to −2369	58466 to 59119
R5-4	LK84	LK87	60	30	688	−2443 to −1756	59045 to 59732
R5-5	CR53F	CR53R	58	120	2303	−2162 to 142	59326 to 61624
R5-6	CKR5a +	CKR5a −	58	60	1194	−84 to 1110	61399 to 62592
R5-7	CR52F	CR52R	56	120	2346	1038 to 3383	62520 to 64865
Ia	627UA	627N	55	120	2520	−4637 to −2115	56854 to 59373
Ib	627UA	627M	55	120	2520	−4637 to −2115	56854 to 59373
IIa	303N	303DA	61	120	2158	−2478 to −318	59010 to 61167
IIb	303M	303DA	61	120	2158	−2478 to −318	59010 to 61167
IIIa	2398N	2398DA	63	120	2607	−382 to 2226	61105 to 63708
IIIb	2398M	2398DA	63	120	2607	−382 to 2226	61105 to 63708
IVa	5765UA	5765M	60	70	1338	1602 to 2939	63084 to 64421

[a]Position according to Mummidi *et al.* (2000) (numbering system A).
[b]Position in the baseline sequence (GenBank accession number U95626, numbering system B).

ARMS primer contained allele-specific nucleotide at its 3′ end. Therefore, we could specifically amplify DNA sequence on the chromosome carrying the allele. Allele-specific products ranged in size from 1.3 to 2.6 kb. Primers used for allele-specific PCR are shown in Table 1. PCR and sequencing were performed in the same manner as described above (Table 1 and Table 2).

PCR-restriction fragment length polymorphism analysis

The region spanning CCR5 −2852 was amplified by PCR with primer pair P67F and LK83 (Table 1). PCR was performed for 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Digestion of the 1164-bp amplified products with Aci I yielded 680- and 464-bp fragments when position −2852 was G.

Promoter assay

Luciferase reporter gene assays were performed as described previously (Liu *et al.*, 1999). Briefly, the promoter region of each haplotype was PCR-amplified and cloned into a pGL3-Basic vector carrying the firefly luciferase gene (Promega, Madison, WI, USA). All constructs were verified for sequence authenticity by sequencing the entire insert, and 5 μg of the resultant constructs was transfected with DMRIE-C (Invitrogen, Carlsbad, CA, USA) into monocytic U937 cells. Transfection efficiency was normalized by cotransfecting 0.2 μg of pRL-TK, which expresses *Renilla* luciferase under the control of the herpes simplex virus thymidine kinase promoter. Cells were harvested 40 h after transfection, and firefly and *Renilla* luciferase activities were determined with the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. Relative luciferase expression was derived with the following equation: (firefly luciferase activity of CCR5 promoter construct/*Renilla* luciferase activity)/(firefly luciferase activity of promoterless vector pGL3-Basic/*Renilla* luciferase activity). Data points are means derived from measurements of two independent clones of each of the constructs.

Results

Sequence polymorphisms in human *CCR5* gene

The human *CCR5* gene, located on chromosome 3, has four exons distributed across 6 kb of genomic DNA (Mummidi *et al.*, 1997). We amplified seven overlapping DNA fragments covering an 8.1-kb genomic region spanning 2031 bp of the upstream non-coding region, the exons and introns in their entirety, and 81 bp of the 3′ untranscribed region of *CCR5* gene. The fragments were obtained from 50 randomly selected Japanese subjects comprising 38 non-HIV-1-infected and 12 HIV-1-infected individuals. Direct sequencing of the PCR fragments allowed us to identify 25 polymorphic positions in this region (Table 3). Among the 25 variable positions, four were insertions or deletions, and 21 were single-nucleotide substitutions. There was one additional C-to-T substitution in the 4-base insertion at position −3887 (Table 3). Except for this tri-allelic site, all the other variations in these samples were di-allelic. No *CCR5Δ32* or *CCR5 m303* was found in these Japanese samples, which confirms previously reported observations (Martinson *et al.*, 1997; Quillent *et al.*, 1998).The genotype frequencies in 50 individuals analysed in this study were consistent with the Hardy–Weinberg equilibrium, suggesting the absence of any tendency towards an excess or deficiency of any particular genotype. Overall, nucleotide diversity was 0.00108, which is the equivalent of approximately one variant in every 926 bp. The sequence diversity in coding-region was lower (0.00012) than in non-coding region (0.00122). In addition to the *CCR5* gene, we analysed the *CCR2* gene of the same 50 individuals for the *CCR2 64I* allele, which has been reported to be associated with delayed HIV-1

Table 3. Sequence variants identified in *CCR2* and *CCR5* genes and used to define haplotypes

Site[a]	Position[b]	Position[c]	Position[d]	Variant	NCBI SNP ID	Allele frequency			
						Japanese[j]	Thais[k]	Caucasian	African-American
1		46295		G, A (CCR2 V64I)	rs1799864	0.67, 0.33	0.83, 0.17	0.90, 0.10[l]	0.84, 0.16[l]
2	−4358	57134		G, A	rs7637813	0.20, 0.80			
3	−3900	57592		A, C	rs2856757	0.57, 0.43			
4[e]	−3888 ~ −3884	57604		GCTAT, G, GTTAT	rs10577983	0.56, 0.43, 0.01			
5	−3458	58030		G, T	rs2734225	0.57, 0.43			
6	−2852	58636		G, A	rs2227010	0.21, 0.79	0.18, 0.82		
7	−2554	58934	208	G, T	rs2734648	0.57, 0.43			
8	−2459	59029	303	A, G	rs1799987	0.56, 0.44		0.57, 0.43[m]	0.43, 0.57[m]
9	−2135	59353	627	C, T	rs1799988	0.55, 0.45	0.35, 0.65		
10	−2086	59402	676	A, G	rs1800023	0.60, 0.40	0.39, 0.61		
11	−1835	59653	927	C, T	rs3181036	0.66, 0.34	0.83, 0.17	0.90, 0.10[n]	0.80, 0.20[n]
12[f]	−1132 ~ −1130	60356		CAG, C	rs3054375	0.57, 0.43			
13	−1060	60426		C, T	rs2856762	0.99, 0.01			
14	−976	60510		C, T	rs2254089	0.57, 0.43			
15	−651	60835		C, T	rs2856764	0.57, 0.43			
16	−451	61035		C, T		0.97, 0.03			
17	−444	61042		G, A	rs2856765	0.57, 0.43			
18	−362	61124		A, G		0.57, 0.43			
19[g]	−361 ~ −359	61125		CAAC, C		0.57, 0.43			
20[h]	668	62150		G, A	rs1800452	0.95, 0.05			
21[h,i]	893	62375		C, -		0.99, 0.01			
22	1171	62653		C, G		0.98, 0.02			
23	1823	63305		C, T	rs17765882	0.99, 0.01			
24	2077	63559		G, T	rs1800874	0.57, 0.43			
25	2150	63632		G, C		0.98, 0.02			
26	2919	64401		G, T	rs746492	0.56, 0.44			

[a]Site number assigned to a variable character used to define haplotypes in order 5′ to 3′.
[b]Position according to Mummidi *et al.* (2000) (numbering system A).
[c]Position in the baseline sequence (GenBank accession number U95626, numbering system B).
[d]Position according to Martin *et al.* (1998) (numbering system C).
[e]There was one additional C-to-T substitution within the duplication of CTAT at position −3887.
Except for this tri-allelic site, all the other variations were di-allelic in these samples.
[f]Insertion of AG.
[g]Duplication of CAA.
[h]Variant in coding region.
[i]Deletion of C.
[j]Allele frequencies of site1,6,9,10 and 11 in 130 Japanese. Allele frequencies of other sites in 50 Japanese.
[k]Allele frequencies of site1,6,9,10 and 11 in 186 Thais.
[l]Allele frequencies reported by Martin *et al.* (1998).
[m]Allele frequencies reported by McDermott *et al.* (1998).
[n]Allele frequencies reported by Mummidi *et al.* (1998).

disease progression (Kostrikis *et al.*, 1998; Smith *et al.*, 1997). The *CCR2* gene was found to be located 14-kb upstream of the *CCR5* gene. The *CCR2* and *CCR5* genotypes of the 50 individuals analysed were sorted and renumbered according to their genotypes and are shown in Fig. 1. Using the method described by Clark *et al.* (1990) we were able to infer all the haplotypes that were subsequently confirmed by allele-specific PCR as well as limited cloning and sequencing of the PCR-amplified fragments. As shown in Fig. 2, we were able to identify 12 independent haplotypes that showed a high degree of linkage disequilibrium of multiple variable sites. *CCR2 64I* was found in *H6*, *H7*, and *H8*, and *CCR5* −1835T in *H6*, *H7*, *H8*, *H9*, and *H10*. As reported previously, *CCR2 64I* is in strong linkage disequilibrium with *CCR5 −1835T* (D′ = 1). *CCR5 P1* (*−2554G*, *−2135C* and *−2086A*, which correspond to 208G, 627C, and 676 A, respectively, in numbering system C) was found in *H6*, *H7*, *H8*, *H9*, *H10* and *H11*. The *−2459A*, which corresponds to 59029 A in numbering system B, was in complete linkage disequilibrium with *CCR5 P1* and found in *H6*, *H7*, *H8*, *H9*, *H10* and *H11*. All the samples showed C in nucleotide position −2132, where T is located in approximately 11.3% of African-American chromosomes (Bamshad *et al.*, 2002).

Twelve haplotypes were subsequently categorized into four major haplotype groups based on the polymorphisms at positions −2135, −2086, and −1835 (Fig. 2). Haplotype group I, comprising *H1*, *H2*, *H3*, *H4*, and *H5*, seems to

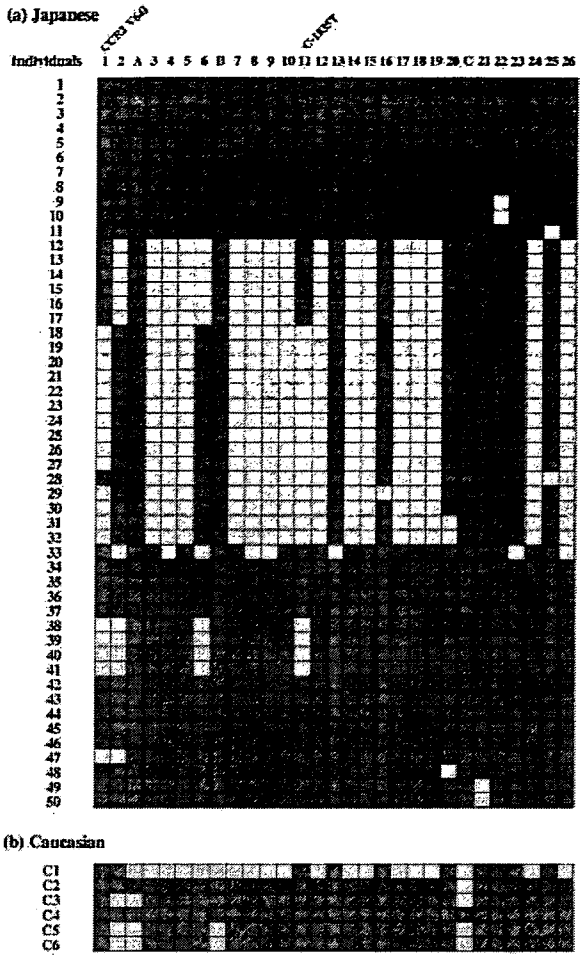

Figure 1. *CCR2* and *CCR5* genotypes of the 50 Japanese (a) and six French (b) subjects at each of the polymorphic sites. Individual samples were sorted and re-numbered according to their genotype, and sample identifiers are shown on the left side of the array. The 26 polymorphic sites observed in Japanese are numbered consecutively across the top. A, B, and C denote polymorphisms specifically observed in the French samples. Positions of *CCR2 V64I* and *CCR5 –1835T* are indicated. Genotypes for each individual were assigned directly based on the fluorescence sequencing trace at each position. At every site, individuals homozygous for the baseline allele (U95626) are shown in red, heterozygotes in yellow and those homozygous for the variant in blue. The heterozygote for the four-base insertion and the additional substitution at position –3887 is shown in white.

correspond to *HHC* according to Mummidi *et al.* (2000). *CCR5 –1835T* was found only in haplotype group II (*H6*, *H7*, *H8*, *H9*, and *H10*), which corresponds to *HHF*. *CCR2 64I* and the Asian-specific disrupting mutation in the *CCR5* open reading frame, *CCR5 893 (–)* (Shioda *et al.*, 2001), was found in haplotype group II. Haplotype group III (*H11*) lacked the *CCR2 64I* but showed sequences identical to the consensus sequence of haplotype group II except for nucleotides at positions *–2852G* and *–1835C*. Haplotype groups III and IV (*H12*) correspond to *HHE* and *HHA*, respectively. None of the single mutations was associated with *CCR2 64I*, except for *CCR5 –1835T*. No *HHB*, *HHD*, *HHG*1* or *HHG*2* was observed in the 100 Japanese chromosome 3. The frequencies of *HHA*, *HHC*, *HHE*, and *HHF* in these chromosomes were similar to those reported for 27 Asians (Bamshad *et al.*, 2002). Mummidi *et al.* (2000) previously pointed out that disease-accelerating genotypes (*CCR5 P1* or *59029 A* lacking *CCR2 64I* and *CCR5Δ32*) are a mixture of haplotypes *HHE*, *HHF*1*, and *HHG*1*. In our study, those disease-accelerating alleles were found in *H9* (*HHF*1*), *H10* (*HHF*1*), and *H11* (*HHE*). On the other hand, the –2852 G allele was found in *H11* (*HHE*), but not in *H9* (*HHF*1*) or *H10* (*HHF*1*). Therefore, the *–2852G* was found to be associated with 90% (19/21) of the disease-accelerating alleles in those Japanese subjects (Fig. 2).

To determine the sequence polymorphisms associated with Caucasian-specific *CCR5Δ32*, we also analysed six French individuals carrying *CCR5Δ32*. The results showed that the nucleotide sequence of the haplotype with *CCR5Δ32* (*H13* in Fig. 2) was very similar to that of the haplotypes containing *CCR2 64I*, *CCR5 –1835T*, or *CCR5 P1*. Phylogenetic analysis of all the haplotypes defined in Fig. 2 showed that there were two major groups (H1-H5 and H6-H13) (Fig. 3), one of which contained all the haplotypes with *CCR2 64I*, *CCR5 –1835T*, or *CCR5 P1*. These data indicate that all the HIV-1 disease-modifying *CCR5* haplotypes were in fact very similar to each other regardless of the direction of their effects on HIV-1 diseases.

Effects on promoter activity by sequence polymorphisms in the regulatory sequences of the CCR5 gene

To compare *CCR5* promoter activity among the three major haplotype groups, I, II, and III, we constructed a series of firefly luciferase reporter fusions containing various lengths of the 5′-non-coding region of *CCR5*, and analysed their promoter activity in monocytic U937 cells. Two distinct promoters for the *CCR5* gene, upstream (Pu) and downstream (Pd), have been identified (Mummidi *et al.*, 1997). Constructs labelled with Pu (Pu-I, Pu-IIa, Pu-IIb, and Pu-III) contained both Pu and Pd, while those labelled with Pd (Pd-I and Pd-III) contained only Pd (Fig. 4). The IIa promoter corresponded to *H6*, *H7*, and *H8*, while its IIb counterpart corresponded to *H9*.

Our results showed that promoters of haplotype groups II and III expressed higher luciferase activity than those of haplotype group I in both constructs (Fig. 4). These results are especially relevant in view of the fact that studies of HIV-1-infected individuals homozygous for a haplotype analogous to haplotype group III (*the CCR5 P1 lacking CCR2 64I and CCR5Δ32*) found that they progressed to AIDS more rapidly than those with other *CCR5* promoter genotypes (Martin *et al.*, 1998; McDermott *et al.*, 1998). One of these studies also reported that a *CCR5* promoter bearing *–2459 A* (*59029 A* in numbering system C), specific for haplotype groups II and III, expressed higher promoter activity than one bearing *–2459G* (McDermott *et al.*, 1998). In addition, Mummidi *et al.* (2000) demonstrated

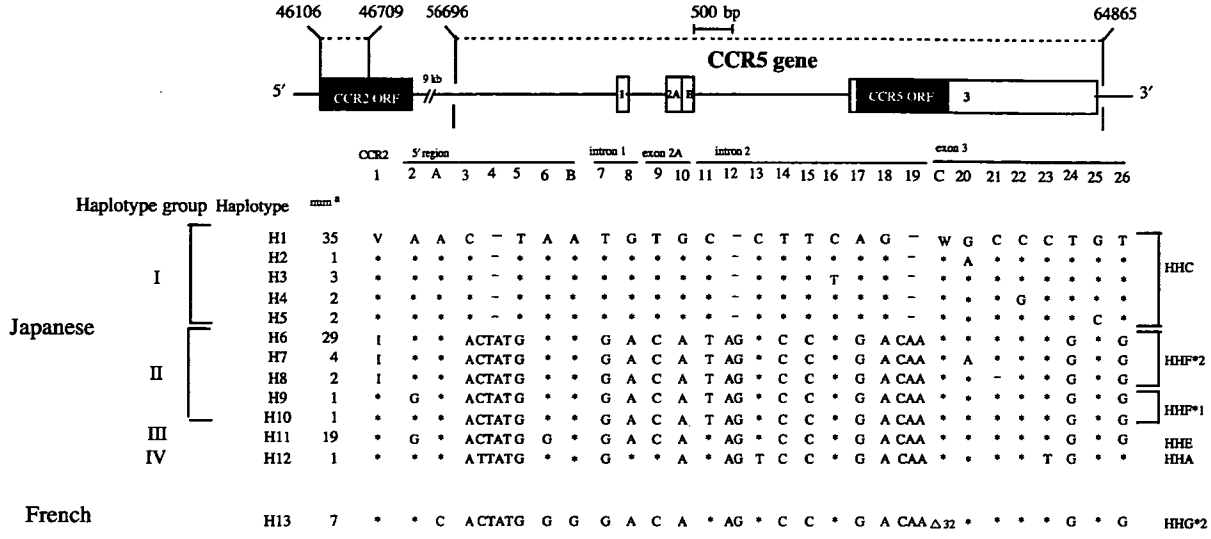

Figure 2. Map of *CCR2, CCR5* and nucleotide polymorphisms. White and black boxes indicate non-coding exons and open reading frames (ORF), respectively. Dotted lines signify the sequenced regions. PCR and sequence screening of eight segments of the *CCR2* and *CCR5* gene identified 29 nucleotide positions with genetic polymorphisms. Twenty-six polymorphic sites observed in Japanese are numbered consecutively across the top. A, B, and C denote polymorphisms specifically observed in the French samples. Combining the 29 polymorphic positions indicated 13 *CCR2-CCR5* haplotype alleles designated H1 through H13, with H1 being exactly the same as the GenBank U95626 sequence. Asterisks indicate nucleotides identical to those of H1; dashes represent deletion sites. W shows *CCR5* coding sequences without 32 bp deletion, while Δ32 indicates 32 bp deletion of the *CCR5* coding region. V and I in position 1 indicate *CCR2 64V* and *CCR2 64I*, respectively. [a]Number in this column represents the actual numbers of haplotypes identified in the 100 sequenced chromosomes. The human haplogroups according to Mummidi *et al.* (2000) are shown on the right.

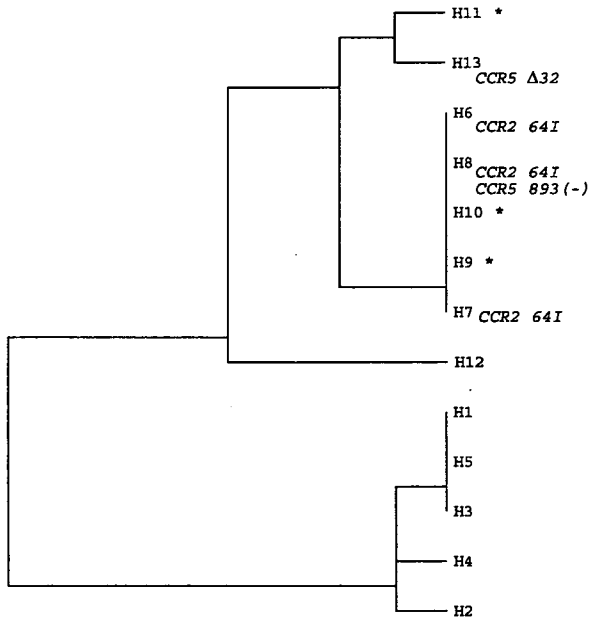

Figure 3. A phylogenetic tree of 13 *CCR2-CCR5* haplotypes defined in Fig. 2. The tree was produced with the UPGMA (unweighted pair-group method with arithmetic averages) method. Haplotypes with *CCR5 Δ32, CCR2 64I, CCR5 893(-)* are shown. Asterisks denote haplotypes with *CCR5 P1 lacking CCR5 Δ32 and CCR2 64I.*

that the *HHC* (haplotype group I) promoter construct demonstrated lower promoter activity than that of *HHF* (haplotype group II) and of *HHE* (haplotype group III). Our study further identified *–2135C* and *–2086A*, which are linked to *–2459A*, as mutations responsible for elevated promoter activity, since Pd-III was shown to express higher luciferase activity than Pd-I (Fig. 4).

To examine the effects of *CCR5 –1835T* on expression level, we generated two series of constructs with part of the intron containing position –1835 and the splicing acceptor site located immediately upstream of *CCR5* exon 4 (LSA and PdSA in Fig. 4). Again, promoters from haplotype groups II and III expressed higher luciferase activity than the promoter from haplotype group I in both the LSA and the PdSA constructs, and since there were no other differences between haplotype groups II and III, this suggests that *CCR5 –1835T* has no effect on *CCR5* expression.

Novel method using PCR-restriction fragment length polymorphism for detecting HIV-1 disease-accelerating haplotype

As described above, in Japanese subjects, *–2852G* was associated with 90% of the disease-accelerating haplotype, this is, *CCR5 P1 lacking CCR5Δ32* and *CCR2 64I*, since no *CCR5Δ32* was identified in these individuals. The remaining 10% were accounted for by individuals with *CCR5 –1835T* without *CCR2 64I*. To confirm the strong linkage disequilibrium between *–2852G* and the disease-accelerating allele observed in 50 Japanese, we established a PCR-RFLP method to detect *–2852G* (see Materials and Methods) and used it to genotype 80 additional (38 HIV-1-infected and 42 non-HIV-1-infected) Japanese. *CCR5 C-2135T, G-2086A*, and *C-1835T* were genotyped by direct-sequencing of PCR product R5-4

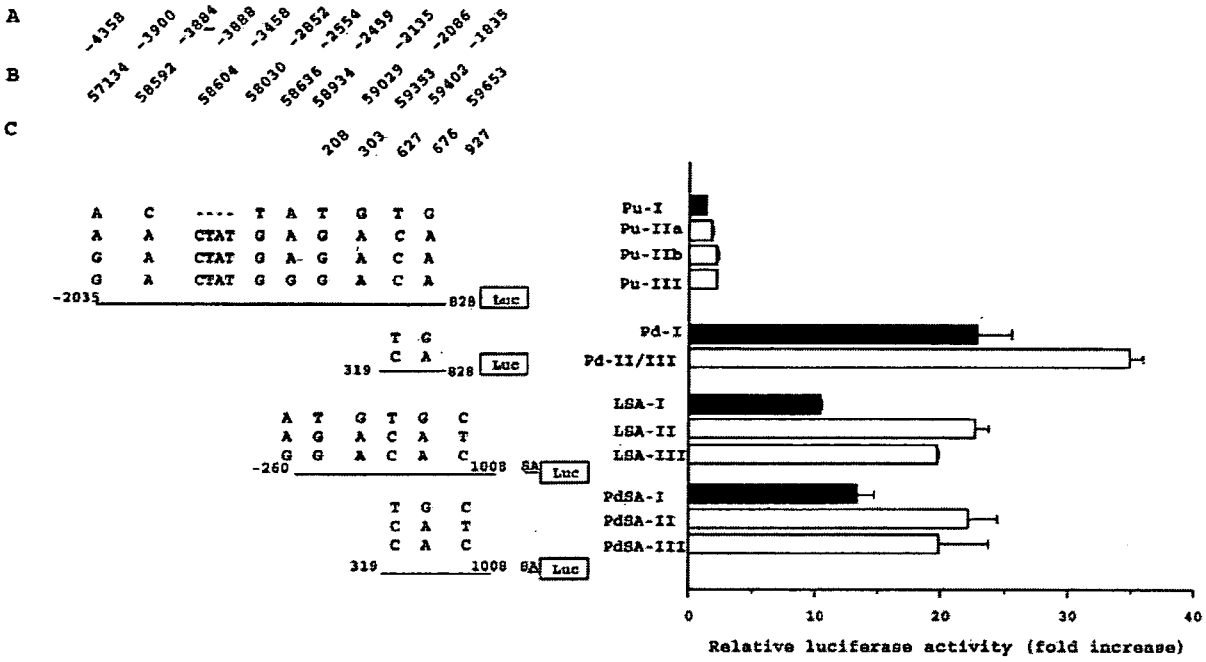

Figure 4. Effect of sequence polymorphism in CCR5 regulatory region on promoter activity. The promoter regions inserted into the pGL3-basic vector are shown by solid lines with the first and last nucleotides enumerated according to numbering system C. SA indicates 0.25 kb of the 5′-non-coding region containing a splice acceptor immediately upstream of the CCR5 open reading frame. Nucleotides at the polymorphic positions are marked. Boxes represent firefly luciferase open reading frames. The relative luciferase activity of each construct is represented by a solid bar (haplotype group I), a grey bar (haplotype group II), and an open bar (haplotype group III). Data shown are representative of five independent experiments with similar results. Error bars indicate fluctuations between measurements of relative luciferase activity in two independent clones of a construct.

Table 4. Linkage disequilibrium between *CCR5 –1835T* and *CCR2 64I* in Japanese and Thai subjects

		CCR2 V64I		
Subjects	*CCR5 C-1835T*	VV	VI	II
Japanese	CC	58	0	0
	CT	2	54	0
	TT	0	1	15
Thais	CC	123	0	0
	CT	0	61	0
	TT	0	0	2

(Table 2), and *CCR2 V64I* was genotyped by PCR-RLFP according to the method described by Smith *et al.* (1997). Frequencies of *CCR2 64I* and *CCR5 –1835T* of 130 Japanese subjects were 33% and 34%, respectively, and were higher than those observed in Caucasians and African-Americans (Table 3). As shown in Table 4 and Table 5, we confirmed the strong linkage disequilibrium between *CCR2 64I* and *CCR5 –1835T* (D′ = 1). We also found the strong linkage disequilibrium between *CCR5 –2852G* and the disease-accelerating haplotype (D′ = 1) since *–2852G* was found in 55 out of 58 (95%) of the disease-accelerating haplotype in 130 Japanese subjects.

In Thai subjects, we previously observed that *CCR2 64I* alleles were 100% associated with *CCR5 –1835T* (unpublished results). We therefore examined whether *–2852G* was associated with the disease-accelerating haplotype in 186 Thai subjects (97 non-HIV-1-infected and 89 HIV-1-infected individuals). Frequencies of *CCR2 64I*, *CCR5 –2852G*, *–2135T*, *–2086 A*, and *–1835T* of 186 Thai subjects were shown in Table 3. As expected, *CCR2 64I* alleles were associated with *CCR5 –1835T* without any exceptions (Table 4). Furthermore, *CCR5 –2852G* was also 100% associated with the disease-accelerating haplotype, *CCR5 P1* lacking *CCR2 64I* and *CCR5Δ32* (Table 5). These results indicated that only one allele (*–2852G*) could be used as a representative of the disease-accelerating haplotype, *CCR5 P1* lacking *CCR2 64I* and *CCR5Δ32* in the Thai population.

Discussion

Polymorphisms in human *CCR5* genes were initially studied for their effects on susceptibility to HIV-1 infection and rate of disease progression to AIDS in HIV-1-infected individuals. Subsequently, these polymorphisms were evaluated for their roles in other human phenotypes such as those accounting for differences in renal transplantation outcomes (Fischereder *et al.*, 2001), myocardial infarction (Gonzalez *et al.*, 2001; Valdes *et al.*, 2002), and autoimmune diseases (Garred *et al.*, 1998; Gomez-Reino *et al.*, 1999; Spagnolo *et al.*, 2005). Our systematic survey of natural sequence variations in an 8.1-kb region of the

Table 5. Linkage disequilibrium between *CCR5 -2852G* and *CCR5* promoter haplotypes in Japanese and Thai subjects

		CCR2 V64I, CCR5 T-2135C, CCR5 G-2086 A, CCR5 C-1835T genotyping					
Subjects	*CCR5 A-2852G*	*V-P1/V-P1*	*V-P1/I-P1*	*V-P1*/Others	*I-P1/I-P1*	*I-P1*/Others	Others/Others
Japanese	AA	0	1	2	15	37	29
	AG	0	17	20	0	0	0
	GG	9	0	0	0	0	0
Thais	AA	0	0	0	2	46	78
	AG	0	15	39	0	0	0
	GG	6	0	0	0	0	0

V-P1: haplotype group III, *CCR5 P1* haplotype (*-2135C, -2086 A,* and *-1835C*) lacking *CCR2 64I.*
I-P1: haplotype group II, *CCR5 P1* haplotype (*-2135C, -2086 A,* and *-1835C*) with *CCR2 64I.*
Others: haplotype group I (*-2135T, -2086G,* and *-1835C*) and haplotype group IV (*-2135T, -2086 A,* and *-1835C*).

entire *CCR5* gene as well as *CCR2V64I* in 50 Japanese individuals revealed several Asian-specific characteristics of the *CCR2-CCR5* haplotype structure that were not seen in those of Caucasians and African-Americans. In particular, we were able to show that the G allele at position -2852 from the *CCR5* open reading frame may represent the *CCR5* promoter haplotype with a higher promoter activity (*CCR5 P1*) without *CCR2 64I* in a *CCR5Δ32*-negative Thai population. For other ethnic groups, the method for *CCR5* promoter haplotype determination is complicated since single-nucleotide polymorphisms (SNPs) at several different positions need to be determined (Martin *et al.*, 1998; McDermott *et al.*, 1998; Mummidi *et al.*, 2000). It would therefore be useful to be able to analyse only one SNP especially in resource-constrained setting.

Our results for *CCR5* promoter activity also confirmed the previously reported observation that polymorphisms of *CCR5* affect its promoter activity (McDermott *et al.*, 1998; Mummidi *et al.*, 2000). Other studies have shown that the SNP at position -2459 affects the binding of certain factor(s) to the *CCR5* promoter region (Bream *et al.*, 1999; Mummidi *et al.*, 2000). However, our study demonstrated that constructs lacking position -2459 still showed differences in promoter activity and that *-2135C* and *-2086 A* were responsible for elevated promoter activity. Further studies will be needed, however, to identify and clarify the exact molecular mechanisms underlying the effects of those SNPs on *CCR5* promoter activity.

On the other hand, we could not detect any effects on luciferase expression level by *CCR5 -1835T*, which is in strong linkage disequilibrium with disease-delaying *CCR2 64I*. Recently, we reported that *CCR2 64I* substitution increases the stability of CCR2A, which is a splicing variant of CCR2B (Nakayama *et al.*, 2004). CCR2A was shown to bind CCR5 while most of CCR2A molecules are retained in cytoplasm. Therefore, cell surface expression levels of CCR5 co-expressed with CCR2A (64I) were lower than those of CCR5 co-expressed with CCR2A (64 V) (Nakayama *et al.*, 2004). Our results reported here also supported the notion that *CCR2 64I* but not *CCR5 -1835T* is directly responsible for the effects of those SNPs on HIV-1 disease progression (Mummidi *et al.*, 1998).

Phylogenetic analysis of human *CCR5* haplotypes clearly demonstrated that all the protective alleles, *CCR2 64I* (*CCR5 -1835T*), *CCR5Δ32* and *CCR5 893(-)*, belong to haplotype groups with higher promoter activity. These results suggest that a certain selective pressure existed that favoured low levels of CCR5 expression during human evolution. CCR5 has been shown to be involved in several inflammatory diseases (Garred *et al.*, 1998; Gomez-Reino *et al.*, 1999), and *CCR5Δ32* to be associated with a reduction in the risks for patients with multiple sclerosis (Barcellos *et al.*, 2000; Kantor *et al.*, 2003) and asthma (Hall *et al.*, 1999). It is therefore possible that impaired function or low levels of CCR5 expression may be beneficial for the survival of individuals with autoimmune or inflammatory diseases.

It is noteworthy that four of the five *CCR5 G668A* (*R223Q*) alleles were found in the *H7* haplotype, which also contains *CCR2 64I* (Fig. 2). Previously, *CCR5 668 A* was reported to be associated with slower disease progression in HIV-1-infected individuals (Zheng *et al.*, 2002). However, since *CCR5 668 A* is associated with another disease-delaying allele, *CCR2 64I*, an epidemiological study is needed to compare individuals with *CCR5 668 A* with those who carry *CCR2 64I* without *CCR5 668 A* in order to evaluate the effects of *CCR5 668 A* on HIV-1 disease progression.

Our study also suggested that not only the frequency but also the degree of linkage disequilibrium of SNPs varies between Japanese and Thai subjects, both Asian. Among the Japanese, 58 of 260 chromosomes were found to carry *CCR5* -1835T, and 55 of these 58 chromosomes also carried *CCR2 64I*. No haplotype carrying *CCR2 64I - CCR5 -1835C* was found among the 260 Japanese chromosomes. Studies of *CCR5* and *CCR2* polymorphisms of Caucasian individuals revealed that both *CCR2 64I - CCR5 -1835C* and *CCR2 64 V - CCR5 -1835T* did exist in Caucasians (Mummidi *et al.*, 1998). On the other hand, *CCR2 64I* was completely associated with *CCR5 -1835T* in 372 Thai chromosomes. It is likely that the *CCR2 64I* mutation only occurred on a *CCR5 -1835T* bearing chromosome and that the *CCR2 64I - CCR5 -1835C* and *CCR2 64 V - CCR5 -1835T* haplotypes were generated as the results of a recombination event between

the *CCR2 64I-CCR5 –1835T* and the *CCR2 64 V – CCR5 –1835C* haplotypes. In Thais, however, that kind of recombination never occurred or only at a very low frequency. At the very least, our results presented here point to the need for detailed linkage studies of genetic polymorphisms, such as the HapMap project, in all ethnic groups, since degree of linkage disequilibrium can vary even among Asian groups.

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Letter to the Editor

Host Genetic Analysis of HIV Type 1 Subtype CRF01_AE (E)-Infected Thai Patients with Different Rates of Disease Progression

EDITOR: HIV-1 infection is generally characterized by a long-term, chronic disease course, gradually progressing to AIDS.[1,2] However, there are a few, but strikingly different, variant scenarios. A small fraction of HIV-1-infected individuals remains normal, both clinically and immunologically, for 10 or more years after seroconversion. Conversely, another fraction is featured by extremely rapid disease progression taking place even within 1 year. Determining host factors involved in these different disease courses would be extremely helpful for better understanding and control of HIV infection.

It is known that polymorphisms in human genes involved in viral cell entry and modulation of immune responses play a substantial role in disease progression of European-American and African-Americans infected with HIV-1 subtype B.[3] However, there are few such data from Asian populations. Furthermore, diverse strains of HIV-1 circulate in different geographical areas and the effects of host genetic polymorphisms may differ due to antigenic or other differences between these HIV-1 subtypes. In the present study, we conducted a "proof of principle" study in previously defined and well-characterized HIV-1-infected Thai patients with different rates of disease progression. We show here that host genetic polymorphisms tend to be associated with rates of disease progression in HIV-1-subtype CRF01_AE (E)-infected Thai patients.

EDTA blood was obtained with written informed consent from 20 HIV-1-infected (all with documented CRF01_AE) Thai patients. Immunological and virological features of these patients were extensively studied and reported previously.[4] All 20 patients had similar CD4 cell counts (558–613 cell/μl) at the start of observation. Nine patients were considered as "progressors" (PRs), since they showed a decline of more then 50 CD4 cells per year and lost more than 50% of them during 3–6 year periods of observation. All RPs were symptomatic or had CD4 cell counts less than 200/μl at the end of the follow-up period. The remaining 11 patients were considered as "slow progressors" (SPs), since they showed virtually no CD4 cell decline during observation periods of similar length. We previously reported that SPs retained the immune competence of antibody responses over time.[4]

We genotyped these 20 patients for the following seven polymorphisms: CCR2 64I,[5] SDF-1 3′A,[6] IL4 -589T,[7] RANTES -28G,[8] RANTES -403A,[8] CCR5 m303,[9] and CCR5 P1.[10] Herein, we did not observe any RANTES -28G or CCR5 m303, probably because of a relatively low frequency of those alleles in Thais.[11,12] Table 1 summarizes the other genotype and allele frequencies observed in these 20 patients. The mutant allele frequency of genetic polymorphism was analyzed by Hardy–Weinberg equilibrium and the distribution of each genotype frequency in the PRs and SPs was compared using Fischer's exact test.

CCR2 64I, SDF-1 3'A, and IL-4 -589T were considered to be protective alleles against HIV-1 disease progression.[7,10,11,13] Consistently, we observed a trend toward higher frequencies of those protective alleles in SPs than in PRs. However, none of the differences reached statistical significance, maybe due to the small sample size. CCR5 P1 and RANTES -403A were previously shown to be associated with a more rapid course of HIV-1 disease progression, and the frequencies of those alleles tended to be slightly lower in SPs than in PRs.[5,8,10] In this study, however, the differences did not reach statistical significance (Table 1).

Among genetic polymorphisms studied here, we observed the lowest p value (Table 1, $p = 0.14$) for SDF-1 3′A. SDF1-3′A was elevated in the SRs compared to PRs (0.5 vs. 0.222). Interestingly, the previous study demonstrated that the frequency of this allele in healthy Thais was 0.375,[14] being higher than that of RPs and lower than that of SPs. An international meta-analysis of large numbers of HIV-1-infected European- and African-Americans,[13] however, did not confirm our observation of the potential protective effect of SDF-1 3′A against HIV-1 disease progression in Thai subjects. In contrast, our recent study in infants and another study in adults[15] have shown an apparent protective effect of SDF-1 3′A against HIV-1 transmission in Thais. It is possible that the SDF-1 3′A polymorphism exerts a stronger effect on HIV-1 (CRF01_AE) infection in Thais than in European- and African-Americans with subtype B infections.

The mean number of protective alleles for PRs was lower than that of SPs (mutant allele frequency 2.1 vs. 3.1), and the mean number of accelerating alleles for PRs was higher than that of SPs (mutant allele frequency 1.0 vs. 0.7). Interestingly, 73% (8 out of 11) of SRs compared to 22% (2 out of

TABLE 1. CCR2, SDF1, IL-4, CCR5 PROMOTOR, AND RANTES PROMOTOR GENOTYPE DISTRIBUTION IN HIV-1-INFECTED INDIVIDUALS WITH DIFFERENT RATES OF DISEASE PROGRESSION

Genotype	*Progressors (RPs)* (n = 9)	*Slow progressors (SPs)* (n = 11)	p *value*[a]
CCR2 +/+	7	6	
CCR2 +/64I	0	4	
CCR2 64I/64I	2	1	
Frequency of CCR2-64I	0.222	0.273	1
SDF1 +/+	6	3	
SDF1 +/3'A	2	5	
SDF1 3'A/3'A	1	3	
Frequency of SDF1-3'A	0.222	0.5	0.14
IL-4 +/+	2	1	
IL-4 +/−589T	3	3	
IL-4 −589T/−589T	4	7	
Frequency of IL-4 −589T	0.611	0.773	0.45
CCR5 +/+	5	8	
CCR5 +/P1	4	3	
CCR5 P1/P1	0	0	
Frequency of CCR5-P1	0.222	0.136	0.68
RANTES +/+	5	7	
RANTES +/−403A	3	3	
RANTES −403A/−403A	1	1	
Frequency of RANTES −403A	0.278	0.227	1

[a]Fisher's exact test; "+" denotes alleles other than the indicated allele.

9) of PRs carried more than three protective alleles (data not shown, Fisher's exact test, $p = 0.07$). These results suggest that some of the alleles protective against HIV-1 disease progression in European- and African-Americans also modulate HIV-1 disease progression in HIV-1 subtype CRF01_AE (E)-infected Thais, although we tested a small number of patients. In addition, there may be some differences in the repertoire of host genetic polymorphisms that play a role in disease progression in Asian subjects as compared to European or African-American subjects. The other limitation of the present study is our working definition of RPs and SPs without knowing the exact time of infection. Nevertheless, our data are intriguing and suggest that further analysis with at least 500 CRF01_AE-infected Thai subjects, ideally followed from a known time of infection, is indicated to confirm or refute these findings, and to define the usefulness of human genetic polymorphisms in predicting the clinical course of HIV-1 infection in Thais.

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Impact of glycosylation on antigenicity of simian immunodeficiency virus SIV239: induction of rapid V1/V2-specific non-neutralizing antibody and delayed neutralizing antibody following infection with an attenuated deglycosylated mutant

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Infection of rhesus macaques with a deglycosylation mutant, Δ5G, derived from SIV239, a pathogenic clone of simian immunodeficiency virus (SIV), led to robust acute-phase viral replication followed by a chronic phase with undetectable viral load. This study examined whether humoral responses in Δ5G-infected animals played any role in the control of infection. Neutralizing antibodies (nAbs) were elicited more efficiently in Δ5G-infected animals than in SIV239-infected animals. However, functional nAb measured by 90% neutralization was prominent in only two of the five Δ5G-infected animals, and only at 8 weeks post-infection (p.i.), when viral loads were already below 10^4 copies ml^{-1}. These results suggest a minimal role for nAbs in the control of the primary infection. In contrast, whilst Ab responses to epitopes localized to the variable loops V1/V2 were detected in all Δ5G-infected animals at 3 weeks p.i., this response was associated with a concomitant reduction in Ab responses to epitopes in gp41 compared with those in SIV239-infected animals. These results suggest that the altered surface glycosylation and/or conformation of viral spikes induce a humoral response against SIV that is distinct from the response induced by SIV239. More interestingly, whereas V1/V2-specific Abs were induced in all animals, these Abs were associated with vigorous Δ5G-specific virion capture ability in only two Δ5G-infected animals that exhibited a functional nAb response. Thus, whereas the deglycosylation mutant infection elicited early virion capture and subsequent nAbs, the responses differed among animals, suggesting the existence of host factors that may influence the functional humoral responses against human immunodeficiency virus/SIV.

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INTRODUCTION

The precise role of antibody (Ab) responses in the containment of human immunodeficiency virus (HIV) remains a subject of intense study and debate. Besides the classical direct virus neutralization properties, antibodies are also capable of blocking infection via other pathways such as antibody-dependent complement-mediated inactivation of virus (Aasa-Chapman *et al.*, 2005) and antibody-dependent cellular lysis (Ahmad & Menezes, 1996; Forthal *et al.*, 2001). Acquiring an understanding of these various mechanisms for their exploitation in the

development of candidate vaccines has been a major challenge.

The envelope protein (Env) of HIV/simian immunodeficiency virus (SIV) comprises an exterior protein (gp120) and a transmembrane (TM) protein (gp41), and trimers of the gp120/gp41 complexes form viral spikes that promote binding to receptors and co-receptors on the cell membrane for entry into the target cells (Wyatt & Sodroski, 1998). The major viral receptors of HIV/SIV include CD4 and a variety of co-receptors such as CCR5 or CXCR4. One desirable target epitope for neutralizing antibody (nAb) that shows relative conservation across clades is the binding site for the co-receptor (Burton *et al.*, 2004; Zolla-Pazner, 2004); however, this site is conformationally cryptic within the viral spike up until immediately after binding of the viral spike to CD4, providing an effective shielding mechanism to the virus. Another distinct feature of HIV/SIV Env is the extensive glycosylation that also effectively prevents access to antibodies directed at the epitopes (Chen *et al.*, 2005; Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). The gp120 protein possesses 18–33 Asn–X–Ser/Thr sequences, signals for the attachment of *N*-linked carbohydrate side chains (Leonard *et al.*, 1990; Ohgimoto *et al.*, 1998; Regier & Desrosiers, 1990; Zhang *et al.*, 2004). As the carbohydrate moiety is generally weakly immunogenic and is recognized to a large extent as self by the host immune system, the massive glycans on the surface of viral spikes constitute an immunologically silent facade (Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). As a result, mature viral spikes are protected from nAb and other host immune responses by a massive carbohydrate 'glycan shield' (Chen *et al.*, 2005; Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). In fact, a prominent role of carbohydrates of HIV/SIV in evasion from immune surveillance has been reported previously as follows. Variants of SIV that have evolved to acquire additional glycans in the variable regions of Env have increased neutralization resistance compared with the parental virus (Chackerian *et al.*, 1997; Cheng-Mayer *et al.*, 1999). Similarly, the appearance of neutralization escape mutants has been associated with altered glycosylation in HIV-1 evolved during the course of infection (Wei *et al.*, 2003). Conversely, infection with SIV239 mutants with deglycosylated Env (lacking *N*-linked glycosylation sites) in the variable loops V1/V2 of gp120 elicited markedly increased titres of nAb (Reitter *et al.*, 1998). We have reported that a deglycosylation mutant, Δ5G, lacking *N*-linked glycosylation sites at aa 79, 146, 171, 460 and 479 in gp120 of SIV239 displayed an attenuated phenotype when used to infect rhesus macaques (Mori *et al.*, 2001; Ohgimoto *et al.*, 1998). In addition, animals infected with Δ5G exhibited almost sterile protection against rechallenge with SIV239 (Mori *et al.*, 2001).

Thus, we suggest that studies aimed at identifying the mechanisms underlying the early and potent immune control of deglycosylated SIV may provide knowledge for the formulation of effective HIV/SIV vaccines. Studies performed herein were therefore directed at attempts to define more precisely the early humoral responses (both virus-specific nAb and non-nAb) generated after infection with Δ5G in rhesus macaques and to compare these responses with those observed in macaques inoculated with wild-type SIV239, with the rationale that results from such studies may help to identify their potential contribution towards viral control of primary infection.

METHODS

Viruses. The molecular pathogenic clone of SIV239 (Regier & Desrosiers, 1990) and its derived deglycosylated mutant, Δ5G, were used in this study. Δ5G was derived by mutagenesis of an SIV239 infectious DNA clone so that the asparagine residues for *N*-glycosylation at aa 79, 146, 171, 460 and 479 in gp120 were converted to glutamine residues (Fig. 1a) (Ohgimoto *et al.*, 1998). Viral stocks of SIV239 and Δ5G were prepared as reported previously (Mori *et al.*, 2001).

Peptides. A series of 72 consecutive 25 mer peptides overlapping by 13 aa were synthesized based on the entire SIV239 Env sequence (Env-1–72). These peptides were synthesized by the Microchemical Facility, Emory University School of Medicine, Atlanta, USA. Another set of 15 mer peptides overlapping by 11 aa around the V1/V2 region in gp120 (V1V2-1–12) was synthesized by Sigma-Aldrich Japan based on the Δ5G sequence (see Fig. 5b). All peptides were dissolved in DMSO diluted in PBS.

Animal infection. Juvenile rhesus macaques originating from Myanmar (Burma) (Mm12, Mm13, Mm20, Mm23 and Mm26) or from Laos (Mm07, Mm22 and Mm25) were used following the results of screening for SIV, simian T-cell lymphotropic virus, B virus and type D retrovirus infection, which were all negative prior to inception of the study. All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare as outlined by the National Institute of Infectious Diseases and National Institute of Biomedical Innovation. Animals were infected intravenously with Δ5G or SIV239 as described previously (Mori *et al.*, 2001).

Plasma viral load measurements. SIV infection was monitored by measuring the plasma viral RNA load using a highly sensitive quantitative real-time RT-PCR. Viral RNA was isolated from plasma samples from infected animals using a commercial viral RNA isolation kit (Roche Diagnostics). SIV *gag* RNA was amplified and quantified using a method originally developed by Hofmann-Lehmann *et al.* (2000) using a TaqMan EZ RT-PCR kit (Applied Biosystems). The detection sensitivity of plasma viral RNA by this method was 100 viral RNA copies per ml plasma (given as copies ml^{-1}).

Neutralization assay. SIV neutralization was tested according to a protocol using CEMx174/SIVLTR-SEAP cells, originally described by Means *et al.* (1997). To measure low levels of nAb, IgG was purified from plasma as described below and concentrated virus stocks were used.

Anti-gp120 Ab ELISA and anti-Env peptide ELISA. Recombinant SIV239 gp120 and Δ5G gp120 were expressed utilizing a Sendai virus vector as described previously (Mori *et al.*, 2005; Yu *et al.*, 1997). Culture supernatant containing approximately 2 μg secreted SIV gp120 ml^{-1} was diluted with an equal amount of PBS, dispensed into each well of an ELISA plate and allowed to incubate at 4 °C overnight.

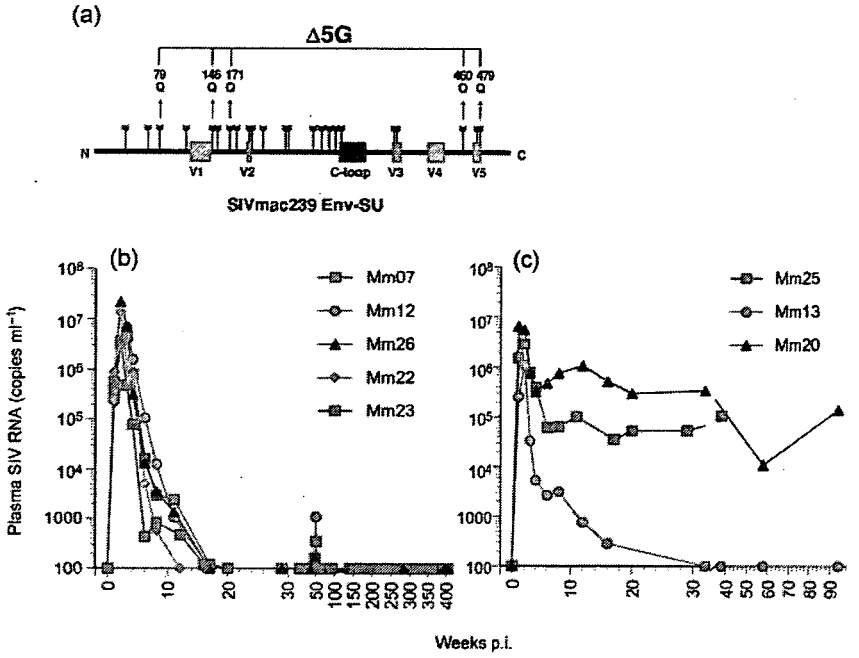

Fig. 1. Plasma SIV RNA loads in animals infected with Δ5G or SIV239. (a) *N*-Glycosylation sites in SIV239 gp120 and deglycosylation sites in Δ5G gp120. The locations of 23 *N*-glycosylation sites in SIV239 gp120, variable regions (V1–V5) and cysteine loops (C-loop) are shown. Δ5G was deglycosylated by N→Q substitutions at aa 79, 146, 171, 460 and 479 in Env (Ohgimoto *et al.*, 1998). (b, c) Plasma viral load in Δ5G-infected (b) and SIV239-infected (c) animals was measured in plasma samples using sensitive real-time RT-PCR to indicate when viral loads declined below 100 copies ml^{-1}. Three Δ5G-infected animals (Mm07, Mm12 and Mm26) were challenged with SIV239 at 48 weeks p.i.; thus, slightly increased viral loads were detected in those animals during weeks 49–51 p.i. (Mori *et al.*, 2001).

For the peptide ELISA, each peptide was diluted to 0.5 μM with 50 mM carbonate buffer (pH 9.5) and captured on Nunc Immobilizer amino plates (Nalge Nunc) at 4 °C overnight. A 1 : 100 dilution (150 μl) of the plasma sample to be tested was dispensed into antigen-immobilized plates and incubated at 37 °C for 1 h. Ab responses were detected using peroxidase-conjugated goat anti-monkey IgG and *o*-phenylenediamine. Absorbance was measured at 490 nm.

Removal of linear V1/V2 epitope-specific Abs from IgG fractions. A mixture of the peptides (V1V2-9, -10 and -11; see Fig. 5b) was conjugated to a HiTrap NHS-activated HP column (GE Healthcare). IgGs from plasma samples were fractionated using a mAb trap kit (GE Healthcare) and applied to the peptide-conjugated column. The flow-through fractions devoid of anti-V1V2-9, -10 and -11 peptide-specific Abs were collected. The concentration of IgG was determined using a protein assay kit (Bio-Rad).

Virion capture assay. The virion capture assay was modified using a method reported by Nyambi *et al.* (1998). ELISA plates were coated with the IgG samples described above at a concentration of 20 μg ml^{-1} in 50 mM carbonate buffer (pH 9.5) and incubated at 4 °C for 48 h. The plates were washed three times with PBS and blocked with 3 % BSA in PBS at 37 °C for 1 h. The plates were then washed three times with serum-free RPMI 1640. Δ5G or SIV239 virion solutions with a $p27^{gag}$ concentration of 15, 7.5 and 3.75 ng in 10 % fetal bovine serum/RPMI 1640 were added to each well of the IgG-coated plate and incubated at 37 °C for 3 h. The wells were washed five times with serum-free RPMI 1640 to remove unbound virus. The virus bound to IgG was lysed using MagNA Pure LC Lysis/Binding Buffer (Roche Diagnostics). The viral lysates were subjected to viral RNA purification using a MagNA Pure Compact nucleic acid purification kit (Roche Diagnostics). The copy number of the isolated SIV RNA was determined by real-time RT-PCR for SIV239 as described above.

Statistical analysis. Correlation analysis was done using Spearman's non-parametric rank test and the Mann–Whitney test using GraphPad Prism 4.0 software. Correlations were considered to be statistically significant for values of $P<0.05$.

RESULTS

Plasma viral loads of a quintuple deglycosylated SIV239 mutant in rhesus macaques

Eight rhesus macaques were infected intravenously with Δ5G ($n=5$) or SIV239 ($n=3$) (Mori *et al.*, 2001). Plasma viral RNA loads were assayed for up to 400 weeks p.i. and the data obtained in the Δ5G-infected (Fig. 1b) or SIV239-infected (Fig. 1c) animals were plotted. Both Δ5G and SIV239 replicated with similar kinetics during the early phase of primary infection for up to 4 weeks p.i. However, subsequent to this acute infection phase, virus replication was markedly different in the two groups of monkeys: SIV239-infected animals exhibited viral load set points around 10^5 copies ml^{-1} in two of three animals, with one animal (Mm13) having an undetectable viral load (<100 copies ml^{-1}) by 30 weeks p.i. (Fig. 1c). In contrast, the Δ5G-infected animals showed uniformly controlled viraemia reaching undetectable levels by 12–16 weeks p.i. and maintained this control for more than 6 years p.i. (Fig. 1b).

nAb response in Δ5G-infected animals

Although failure to detect a nAb response is characteristic of SIV239-infected rhesus macaques (Johnson *et al.*, 2003; Means *et al.*, 1997), the rapid control of viraemia in Δ5G-infected animals prompted us to determine whether nAb played a role in this control of viraemia. We hypothesized that the deglycosylation might lead to the elicitation of a markedly more vigorous nAb response than infection with SIV239. To maximize the detection sensitivity of weak nAb responses at early time points p.i., an assay that measures neutralization titres based on 50 % inhibition of virus replication (IC_{50}) in $CD4^+$ T-cell lines was initially used.

Consistent with the reported results in SIV239-infected animals, no appreciable nAb titre was detected in two animals (Mm13 and Mm25), despite the fact that viral load in Mm13 was distinctively decreased by 30 weeks p.i. However, we observed a rare animal (Mm20) that elicited a robust nAb response against SIV239 and a relatively delayed nAb response against Δ5G, despite the maintenance of a high viral load (Fig. 2a). These results indicated the lack of correlation of nAb response with viral load in SIV239-infected animals. In contrast, nAb was detected in two Δ5G-infected animals (Mm07 and Mm22) starting at 8 weeks p.i. and in two additional animals (Mm12 and Mm23) at 12 weeks p.i. (Fig. 2b, left panel). These titres peaked at either 12 or 18 weeks p.i., and the peak was followed by a decrease in titre that varied among animals. Mm12 and Mm23, which exhibited nAb induction at 12 weeks p.i., had essentially low titres, whilst Mm07 and Mm22, which exhibited nAb induction at an earlier time point, maintained vigorous nAb titres of $>1:100$. Of note, plasma from Mm26 did not contain detectable levels of nAb at any time p.i. In contrast, nAb against SIV239 was not induced in any of the Δ5G-infected animals (Fig. 2b, right panel). As low-level nAb may play a role in control of virus replication, purified IgG from the plasma samples was used to measure neutralizing activity. However, the results from the purified IgG corresponding to the plasma at a 1:3 dilution did not change the kinetics of nAb response in Δ5G-infected animals (data not shown).

In experiments where the passive administration of monoclonal HIV nAb successfully prevented the infection of macaques with simian–human immunodeficiency virus, the results unequivocally indicated that high titres of nAb were needed to achieve such protection (Nishimura *et al.*, 2002). In consideration of these results, data were recalculated based on a cut-off value of 90% inhibition of virus replication (IC_{90}) in $CD4^+$ T-cell lines. As a result, nAb responses were detected in only two of the animals, Mm07 and Mm22, but with titres of 1:100 and 1:500, respectively (Fig. 2b, middle panel). Next, we examined the correlation between viral load and nAb titre at 8 and 12 weeks p.i. and found that the correlation was not statistically significant (Fig. 2c).

Anti-gp120 Ab response in Δ5G-infected animals

Next, we measured binding Ab responses against gp120. When the plasma samples were assayed for levels of Ab that bound to SIV239 gp120 or Δ5G gp120, essentially identical values were obtained. Fig. 3 shows the data obtained using SIV239 gp120. Remarkably, anti-gp120 responses during the early period p.i. between the two groups of monkeys were distinct. Whereas anti-gp120-specific Ab responses

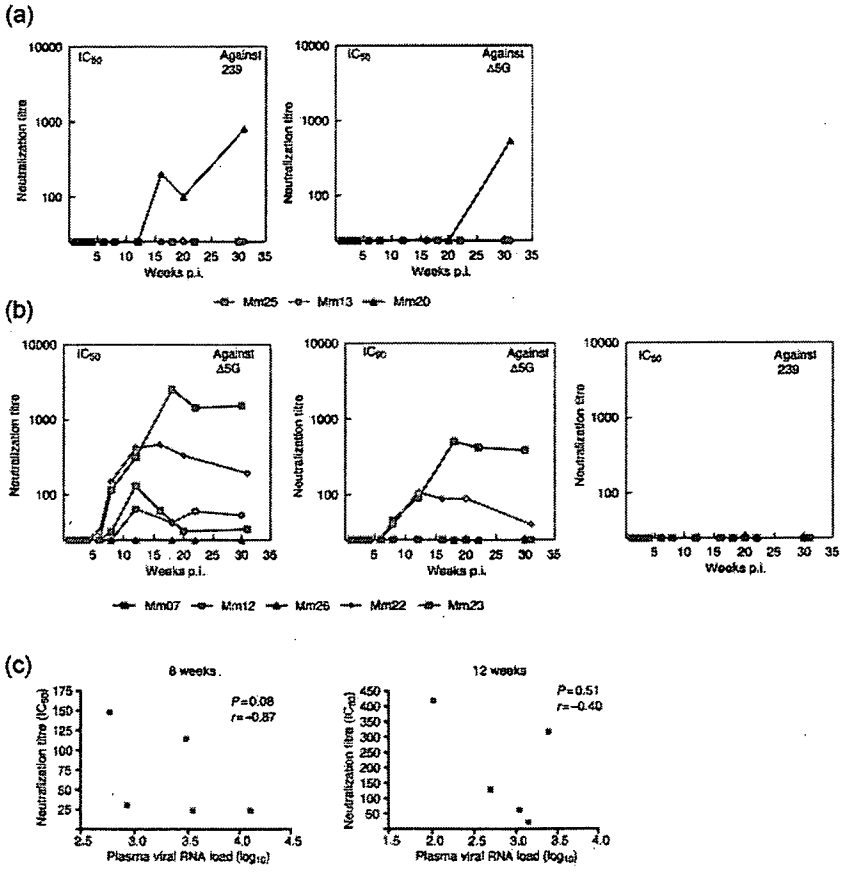

Fig. 2. nAb titres in SIV-infected animals. (a) nAb titres in SIV239-infected animals are indicated as the plasma dilution yielding 50% inhibition (IC_{50}) of SIV239 infection (left) or Δ5G infection (right) in CEMx174/SIVLTR-SEAP cells. (b) nAb titres in Δ5G-infected animals are indicated as the plasma dilution that yielded 50% inhibition (IC_{50}, left) and 90% inhibition (IC_{90}, middle) of Δ5G infection or 50% inhibition of SIV239 infection (right) in CEMx174/SIVLTR-SEAP cells. (c) Correlation between IC_{50} nAb titres and plasma viral RNA load at 8 and 12 weeks p.i. in Δ5G-infected animals.

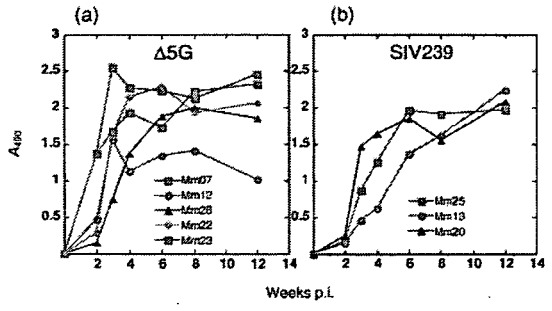

Fig. 3. Anti-gp120 Ab responses. Anti-gp120 Ab responses in Δ5G-infected (a) and SIV239-infected (b) animals were indicated as A_{490} using plasma diluted 1 : 100 in an ELISA.

peaked at 3–4 weeks p.i. in Δ5G-infected animals (Fig. 3a), those in SIV239-infected animals remained generally lower and required longer periods of time to reach their peak (Fig. 3b). Of note, whilst anti-gp120 Ab responses did not correlate well with nAb titres in the chronic phase in Δ5G-infected animals, the hierarchy detected in nAb titres (Mm07, Mm22, Mm23, Mm12 and Mm26, in descending order) was similar to that observed for gp120-binding antibodies at 2 weeks p.i. (Fig. 3a).

Ab responses to linear epitopes in gp120 and gp41 in Δ5G-infected animals differ from those detected in SIV239-infected animals

Next, we examined Ab-binding responses to linear epitopes in plasma samples from infected animals at 8 weeks p.i., as both nAb and anti-gp120-binding Ab were detected at this time point (Figs 2 and 3). We used 72 overlapping peptides encompassing the entire Env sequence of SIV239 for the detection of epitope-specific Ab in plasma samples from Δ5G-infected or SIV239-infected animals. As shown in Fig. 4 and Table 1, the plasma samples reacted with the peptides in six regions: two in gp120 and four in gp41. The regions in gp120 resided in the vicinity of V1/V2, designated region 1 (aa 109–193), and at the C terminus, designated region 2 (aa 493–529). Of note, only linear region 1 was directly affected by selected deglycosylation (aa 146 and 171). The regions in gp41 were located in the ectodomain for region 3 (aa 589–625) and region 4 (aa 660–685), and in the cytoplasmic domain for region 5 (aa 721–757) and region 6 (aa 841–879).

Although Ab responses to most of the peptides recognized in the plasma samples from Δ5G-infected animals were similar to those in SIV239-infected animals, a few peptides were recognized by Abs only in samples from Δ5G-infected animals, and Ab reactivity to some peptides was significantly different between the two groups (Fig. 4b, c and Table 1). Firstly, in region 1, whereas five peptides (Env-10, -12, -13, -14 and -15) were recognized by Abs from Δ5G-infected animals, only three peptides (Env-10, -12 and -13) reacted with Abs from SIV239-infected animals (Fig. 4b and c). Peptide Env-10 was detected by Abs from four Δ5G-infected animals, but from only one of the SIV239-infected animals. Similarly, peptides Env-12 and -13 were detected by Abs from five Δ5G-infected animals and two SIV239-infected animals. In contrast, peptides Env-14 and -15 were detected by Abs from Δ5G-infected animals but not SIV239-infected animals. The specificity of Δ5G infection in the reactivity of peptide Env-14 was statistically significant (P=0.0149) (Table 1). Secondly, the reactivity of Ab from Δ5G-infected animals with the peptides in regions 2, 3 and 4 was lower than that recorded with Ab from SIV239-infected animals (Fig. 4b and c). As shown in Table 1, the reduction in Ab reactivity from Δ5G-infected animals to peptide Env-51 (region 3) and peptide Env-56 (region 4) was significant (P=0.014 and 0.0053, respectively); however, the reduction in Ab response in region 2 was not significant. In addition, there were no significant differences in the Ab responses to the peptides in regions 5 and 6 between Δ5G-infected and SIV239-infected monkeys (Fig. 4b, c and Table 1).

A Δ5G-specific linear epitope resides in the region containing the third deglycosylation site (aa 171) between V1 and V2

As region 1 also contained the site of two mutations introduced to limit glycosylation in the Δ5G mutant, we focused additional studies on this region. To identify the Δ5G-specific epitope(s) in region 1, peptide ELISA was performed with 12 newly synthesized shorter peptides based on the Δ5G sequence spanning the V1/V2 region (Fig. 5). Ab reactivity to peptide Env-14 was mapped to peptides V1V2-9–11 (Fig. 5a). Thus, three linear epitopes (encompassed in peptides Env-10, V1V2-3 and V1V2-9–11) were identified within the V1/V2 region (Figs 4 and 5). Whilst two epitopes contained in peptides Env-10 and V1V2-3 were recognized by Ab from both SIV239- and Δ5G-infected animals, the epitope(s) corresponding to peptides V1V2-9–11 was specific to Δ5G infection (Fig. 5a). As the latter contained the third deglycosylation mutation (Figs 1 and 5b, aa 171), Δ5G specificity was probably secondary to the removal of *N*-glycan at this site in SIV239 gp120 (Fig. 5).

Δ5G-specific Ab responses to linear epitopes in Env elicited immediately following primary infection

In an effort to define the potential relevance of the linear epitope-specific Ab responses in the reduction of acute virus replication in Δ5G-infected animals, we examined the kinetics of Ab reactivity to 12 peptides: Env-10, V1V2-3 and V1V2-9, -10 and -11 for epitopes in region 1; Env-42 and -43 for epitopes in region 2; Env-50 and -51 for epitopes in region 3; Env-56 for epitopes in region 4; and Env-61 and -62 for epitopes in region 5 (Fig. 6). Whilst the induction kinetics of Ab to most peptides were variable in

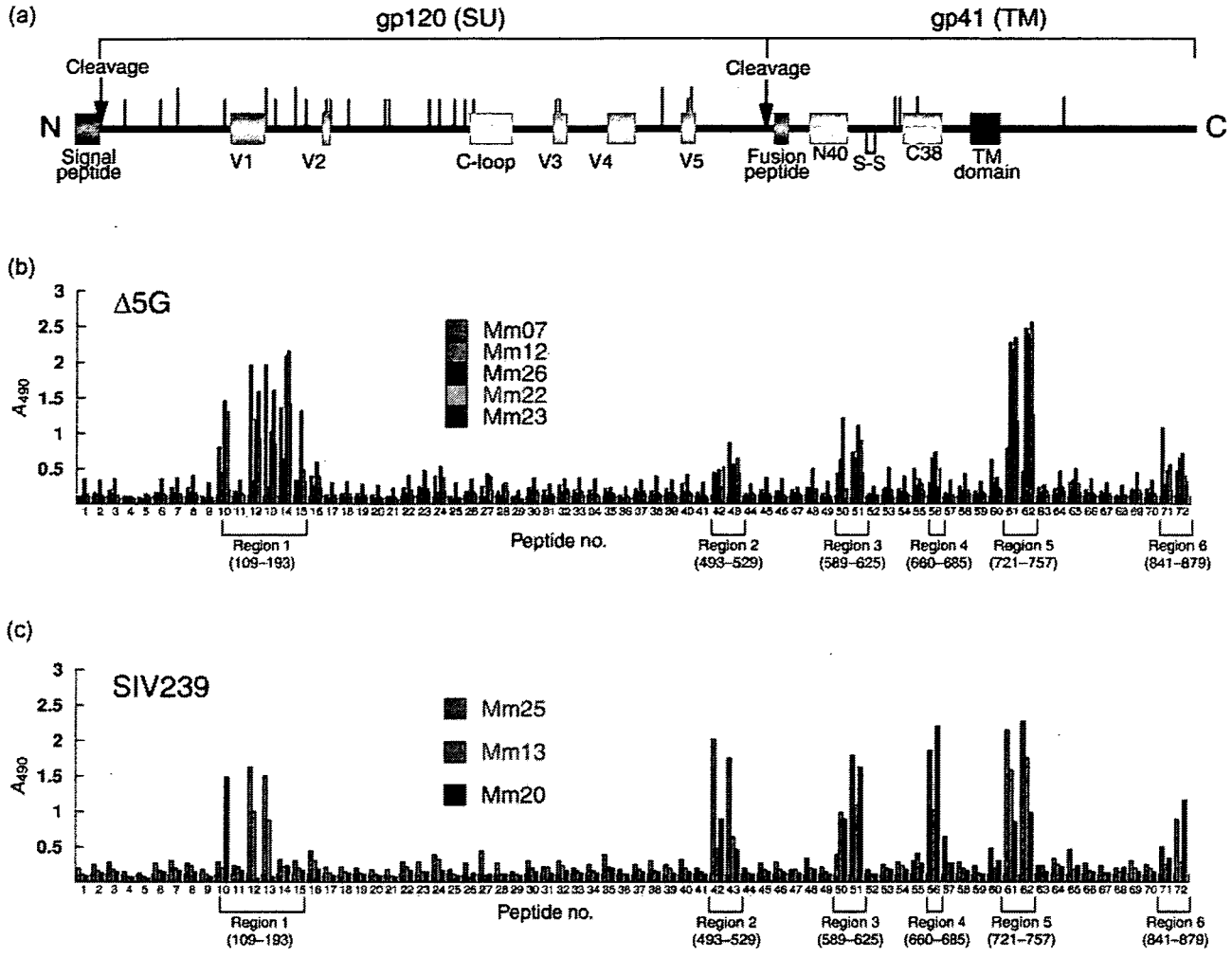

Fig. 4. Ab reactivity to synthetic overlapping peptides spanning the entire Env protein. (a) Diagram of SIV239 Env with the locations of the signal peptide (violet box), variable regions (pink boxes), cysteine loop (yellow box), fusion peptide (green box), N-terminal (N40) and C-terminal (C38) heptad repeats (light-blue boxes), membrane-spanning domain (blue box) and *N*-glycosylation sites (vertical bars) (Burns & Desrosiers, 1991; Choi *et al.*, 1994; Liu *et al.*, 2002). Red vertical bars indicate deglycosylation sites (aa 79, 146, 171, 460 and 479) in Δ5G. S-S indicates the indispensable disulfide bond for hairpin loop formation of the TM protein. (b, c) Plasma samples collected from animals infected with Δ5G (b) and SIV239 (c) at 8 weeks p.i. were used to examine Ab reactivity to 72 peptides (25 mers) overlapping by 13 residues each and spanning the entire Env protein. Reactivity was shown by A_{490}.

plasma from both groups of animals, Ab to V1V2-9, -10 and -11 was specific for Δ5G-infected animals, with rapid induction following primary infection. Ab responses to Env-61 and -62 were also induced rapidly in animals from the two groups; however, it has already been confirmed by SIV and HIV studies that a linear epitope covered by these peptides is the immunodominant epitope with no association with virus control (Eberle *et al.*, 1997; Kent *et al.*, 1992). In contrast to Ab responses to V1/V2 peptides, whilst Ab to peptides Env-51 and -56 in the gp41 ectodomain were detected in SIV239-infected animals, these reactions were low until at least 12 weeks p.i. in Δ5G-infected animals.

Properties of Ab against Δ5G-specific linear epitope

Although Ab reactivity to peptide V1V2-9, -10 and -11 was elicited specifically in Δ5G-infected animals, these Abs were non-nAbs, as these binding Abs were detected in all Δ5G-infected animals, including a nAb-undetectable monkey (Mm26), and before nAb was detected. In addition, we attempted to inhibit neutralization by the addition of excess concentrations of V1V2-9, -10 and -11 to the neutralization assay performed with plasma from Δ5G-infected animals collected at 8 and 12 weeks p.i. The reduction of nAb by the addition of an excess amount of

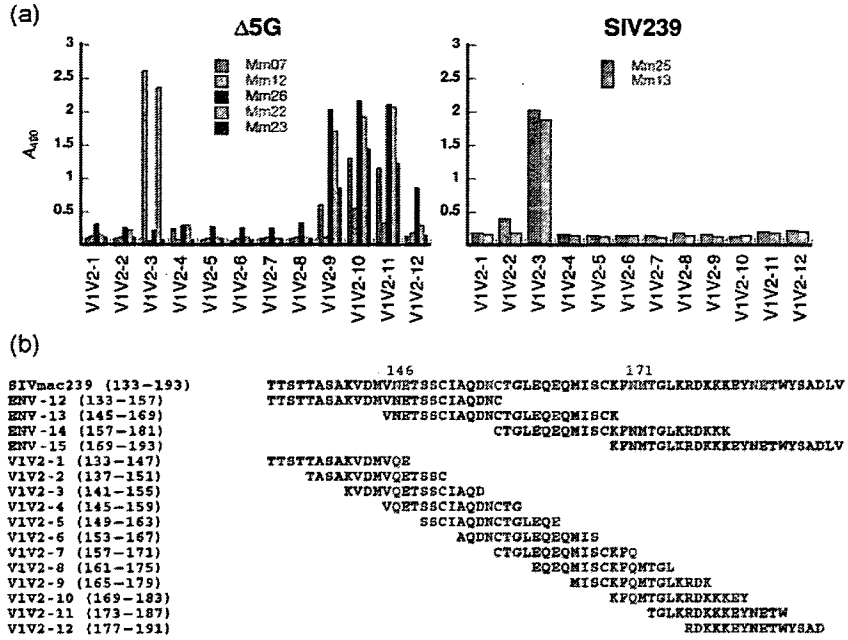

Fig. 5. Ab reactivity to linear epitopes in the V1/V2 region of gp120. (a) To define linear epitopes in the V1/V2 region, peptide ELISA was performed using 12 peptides (15 mers) overlapping by 11 residues each. (b) Sequences and positions of the 12 V1/V2 peptides used in (a) and the peptides Env-12 to -15.

peptide was not detected in any samples, confirming that the epitopes targeted by nAb and V1V2-specific Ab were distinct (data not shown).

Next, we tested plasma IgG samples from SIV-infected animals for the quantitative capture of whole virions. IgG fractions of plasma samples from SIV-infected animals collected at 3–4 weeks p.i. were compared for their capacity to capture Δ5G or SIV239 virions. IgG fractions from two Δ5G-infected animals (Mm07 and Mm22) exhibited remarkably higher virion capture activity than those from other animals (Fig. 7a); however, this capture activity was

Table 1. Epitope-specific Ab-binding regions in Env and influence of deglycosylation on Ab binding

Env subunit	Ab-binding region	Peptide no.	Amino acid range	Region	*P* value*
SU	Region 1	10	109–133	V1	0.6733
		12	133–157	V1	0.5678
		13	145–169	V1/V2	0.5563
		14	157–181	V1/V2	0.0149†
		15	169–193	V1/V2	0.2385
	Region 2	42	493–517	SU C terminus	0.0822
		43	505–529		0.3039
TM	Region 3	50	589–613	Ectodomain	0.4791
		51	601–625		0.0140†
	Region 4	56	660–685	Ectodomain	0.0053‡
	Region 5	61	721–746	Cytoplasmic domain	0.6818
		62	732–757		0.8188
	Region 6	71	841–865	Cytoplasmic domain	0.5237
		72	853–879		0.2451

*A *t*-test was performed by using data in Fig. 4 to determine differences in Ab reactivity between SIV239 infection and Δ5G infection.
†$P<0.05$; ‡$P<0.01$.