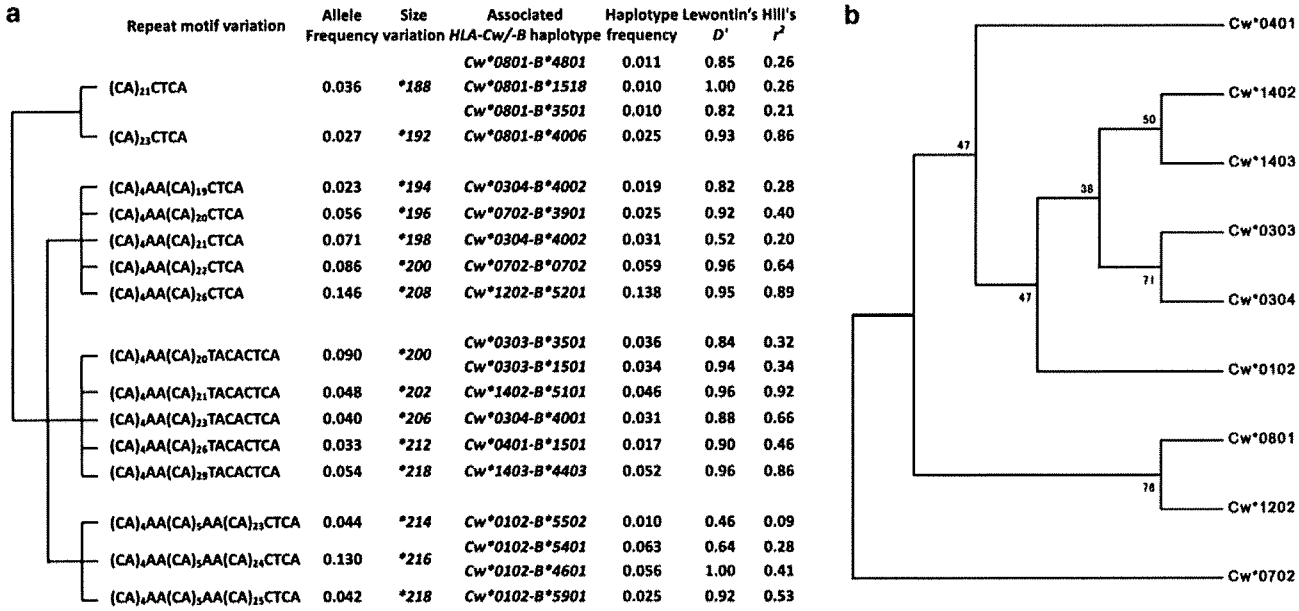

Figure 2 Phylogenetic relationship between *C1_2_5* motif and *HLA-Cw/-B* haplotype. (**a**) Phylogenetic tree predicted from the repeat motif structures at the *C1_2_5* locus. *HLA-Cw/-B* haplotypes associated with *C1_2_5* alleles were indicated with two LD coefficients, *D'* and r^2. Representative *HLA-B* alleles were shown. (**b**) Phylogenetic analysis of exon 4 sequences of *HLA-Cw*. Phylogenetic tree was constructed by the UPGMA method. The numbers for interior branches refer to the bootstrap values in percentage with 1000 replications.

Table 3 Overall LD among *HLA-B*, *HLA-Cw* and *C1_2_5* loci

LD pair[a]	*Hedrick's multiallelic D'*	*Cramer's V*
HLA-B locus and C1_2_5 locus	0.85	0.70
HLA-B locus and C1_2_5 motif	0.88	0.60
C1_2_5 locus and HLA-Cw locus	0.87	0.64
C1_2_5 motif and HLA-Cw locus	0.91	0.73
HLA-Cw locus and HLA -B locus	0.88	0.82
HLA-Cw/-B haplotype and C1_2_5 locus	0.94	0.85
HLA-Cw/-B haplotype and C1_2_5 motif	0.95	0.74

Overall LDs for each pair were statistically significant ($P<0.05$).
[a]C1_2_5 locus and C1_2_5 motif indicate allele (fragment size) and repeat motif structure, respectively.

co-evolve. For example, the change of *C1_2_5* found in the *Cw*0304-B*4002* haplotypes was attributable to the differences in the number of repetitive units, which can be explained by a strand-slippage mechanism. On the other hand, the difference of repeat motifs in *C1_2_5*198* and *C1_2_5*206* associated with the identical allele, *Cw*0304*, was characterized by distinct *HLA-B* alleles, *B*4002* and *B*4001*, respectively. It was unlikely that these two *Cw*0304*-linked haplotypes were shaped by a simple recombination event between *HLA-Cw* and *-B* loci, as the motif structures of *C1_2_5* were different between them. Instead, *Cw*0304* might originally exist in two different haplotype lineages.

Comparison of EHH profile showed that the length of LD varied depending on the HLA haplotypes. One possible explanation for the variation includes the diversity of pairing between the alleles of *HLA-B* and *-Cw*. Indeed, the *HLA* allele with a short-range LD profile showed larger diversity due to the repeated recombination events over time, thereby providing the LD decay between the landmark allele and the linked markers. On the other hand, haplotypes with a long-range LD profile might be of recent origin. In general, human genetic geography showed high continuity, and it is well known that the MHC haplotypes in neighboring populations were introduced to Japan through multiple routes.[28] Therefore, the MHC haplotype structures in the Japanese population might be shaped by multiple immigrations.

Each repeat motif observed in the *C1_2_5* locus was in tight LD with a particular *HLA-Cw* allele and in part with an *HLA-B* allele, which consisted of *HLA-Cw/-B* haplotypes. The mutation rate at a microsatellite is known to depend on the intrinsic features, including repeat number, length and motif size.[29] For example, microsatellites with greater number of repeats showed higher mutation rates due to the increased probability of slippage.[30] In contrast, interruption of perfect repeat array had a great impact on the stability of microsatellite alleles.[31] Indeed, interrupted motif within repeat tracts that were correlated with *HLA-DR/-DQ* haplotypes was described for *DQCAR*.[32]

In conclusion, we revealed that unique mutational dynamics at *C1_2_5* locus could serve as a useful resource for tracing haplotype lineage in the Japanese population. Analysis of *C1_2_5* structures along with *HLA-Cw/-B* haplotypes in other ethnic groups will show the lineages of haplotypes. Statistical methodology for predicting the *HLA* allele and its haplotype carried on the chromosome have been established using informative SNPs inside and/or outside the *HLA* genes.[33,34] However, the use of bi-allelic SNPs as a marker requires more efforts to obtain the information than the use of multi-allelic microsatellite markers, because many *HLA* alleles show a mosaic structure shaped by multiple polymorphic backgrounds. Microsatellite markers will shed light on the haplotype lineage in a different perspective from the SNP-based tagging approach.

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Impact of novel TRIM5α variants, Gly110Arg and G176del, on the anti-HIV-1 activity and the susceptibility to HIV-1 infection

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Objective: TRIM5α is one of the factors contributing to intracellular defense mechanisms against HIV-1 infection. We investigated the association of TRIM5α sequence variations with the susceptibility to HIV-1 infection in Japanese and Indian.

Design: Sequence variations in TRIM5α were investigated in HIV-1-infected patients and ethnic-matched controls. Functional alterations caused by rare variants were analyzed.

Methods: We sequenced TRIM5α-exon 2 in both Japanese (94 HIV-1-infected patients and 487 controls) and Indian (101 HIV-1-infected patients and 99 controls). Frequency of variants and haplotypes were compared between the HIV-1-infected patients and controls. Functional analyses were performed for two rare variants, Gly110Arg and G176del.

Results: The frequency of 43Tyr-allele in the Indian HIV-1-infected patients was significantly lower than that in the ethnic-matched controls (odds ratio = 0.52, 95% confidence interval = 0.31–0.89, $P = 0.015$). A similar tendency was observed in Japanese sample, although it was not statistically significant (odds ratio = 0.67, 95% confidence interval = 0.43–1.05, $P = 0.095$). On the other hand, haplotype analyses revealed that the haplotype carrying the 43Tyr-allele was significantly associated with the reduced susceptibility to HIV-1 infection in both ethnic groups. Functional analysis revealed that Gly110Arg variant weakened the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α, whereas the truncated G176del-TRIM5 enhanced the antiviral activity of coexpressed TRIM5α. Epidemiological data were consistent in that Gly110Arg and G176del were associated with the susceptibility to and protection from HIV-1 infection, respectively.

Conclusion: Both common and rare variants of TRIM5α are associated with the susceptibility to HIV-1 infection.

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Keywords: association, HIV-1, polymorphism, susceptibility, TRIM5

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Introduction

Although the cellular and humoral immune systems are known to play crucial roles in the defense against retroviral infection, mammals have also evolved defense mechanisms within cells. Several lines of evidence indicated that restriction factors within host cells inhibit viral replication more effectively than the immune system, and TRIM5α is one of the factors involved in the intracellular defense against retroviruses [1,2]. It was reported that TRIM5α from rhesus monkeys restricted HIV-1 production at a postentry, preintegration stage in the viral life cycle through rapid degradation of HIV-1 Gag polyproteins [3,4], whereas human TRIM5α restricted HIV-1 only weakly and potently restricted N-tropic murine leukemia virus [5,6].

TRIM5 is a member of the tripartite-motif containing superfamily and includes a Really Interesting New Gene (RING) domain, B-box 2 domain and coiled-coil domain [7]. Alternative splicing of TRIM5 gene generates several isoforms of TRIM5 proteins. One isoform, TRIM5α, contains the carboxy-terminal B30.2 (SPRY) domain that is essential for anti-HIV-1 activity, and sequence differences in the SPRY domain contribute to the differences in the anti-HIV-1 activity among primate species [4,8–13].

It is well known that the infection by HIV-1 and progression to AIDS are variable among human individuals, which are considered to be controlled by diversity in the human genome [14,15]. As TRIM5α has crucial roles in the restriction of viral replication within the host cells, it is a good candidate gene controlling the susceptibility to or protection from HIV-1 infection and/or progression to AIDS. Actually, several studies have demonstrated that common TRIM5α functional polymorphisms, His43Tyr and Arg136Gln, were associated with the susceptibility to HIV-1 infection. However, the significance of association has not been established [16–20].

In this study, we investigated two ethnic populations, Japanese and Indian, for the polymorphism in TRIM5α-exon 2 and its association with the HIV-1 infection. We found that a TRIM5α haplotype carrying the 43Tyr-allele was associated with the reduced susceptibility to HIV-1 infection in both ethnic groups. In addition, we identified two rare variants, G176del and Gly110Arg, which affected the anti-HIV-1 activity and showed suggestive associations with the HIV-1 infection.

Material and methods

Participants

Protocol of the present study was approved by the Ethics Review Board of the Medical Research Institute, Tokyo Medical and Dental University and that of All India Institute of Medical Science. At the set-up of the cohort in 1995, all the HIV-1-infected Japanese hemophiliac patients had been infected for longer than 10 years and they were asymptomatic without any antiviral measures. Blood samples were collected from 94 well characterized patients who were selected from the cohort after obtaining written informed consent [21,22]. Control DNA samples were prepared from Epstein–Barr virus-transformed human B cell lines established from randomly selected healthy donors with written informed consent ($n = 487$), which were purchased from the Japan Health Sciences Foundation. The DNA samples from HIV-1-infected individuals were prepared from the blood sample by using QuickGene DNA whole blood kit S (FUJIFILM, Tokyo, Japan). In addition, blood DNA samples were obtained with written informed consent from 101 HIV-1-infected Indian patients and 99 healthy Indian volunteers in the related hospitals of All India Institute of Medical Sciences, New Delhi. DNA samples from whites ($n = 96$) and African–Americans ($n = 96$) were obtained from the Coriell Institute for Medical Research (Camden, New Jersey, USA).

Identification and genotyping of nucleotide variations in TRIM5α-exon 2

Primer sets were designed to amplify the genomic segments covering the entire TRIM5α-exon 2 as follows: sense primer (5′-TTGGTCCCATTTTAACC TTCC-3′) and antisense primer (5′-AAGGCAGT TAA TGTCAAAGGC-3′). Genomic DNA was subjected to PCR amplification followed by sequencing on both strands using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Polymorphisms were identified using the Sequencher program (Gene Code Co., Ann Arbor, Michigan, USA).

Cloning and expression of TRIM5α

The generation of recombinant Sendai viruses (SeVs) expressing human TRIM5α, human/African green monkey (AGM) chimeric TRIM5α [18], cynomolgus monkey TRIM5α lacking SPRY domain [CM-TRIM5α-SPRY(−)-HA] [23] and AGM TRIM5α lacking coiled-coil domain [AGM-TRIM5α-CC(−)-HA] has been described previously [24]. All TRIM5α variants carried a hemagglutinin (HA)-tag (YPYDVP-DYAA) at the C-terminus. The Gly110Arg mutation was introduced into both human TRIM5α and human/AGM chimeric TRIM5α by PCR site-directed mutagenesis. To generate SeV expressing G176del carrying an HA-tag at the N-terminus (HA-G176del-TRIM5), the amplified PCR fragment from genomic DNA carrying G176del was cloned into a pSeV18+b(+) vector. Recombinant SeVs expressing human 110Arg-TRIM5α, human/AGM chimeric 110Arg-TRIM5α and HA-G176del-TRIM5 were recovered as described previously [12]. The second passages in embryonated

chicken eggs were used as the stock viruses in all experiments.

Western blot analysis

MT4 cells (1×10^6) infected with recombinant SeVs expressing HA-tagged TRIM5α proteins were lysed in lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). Western blot analyses with anti-HA high-affinity rat monoclonal antibody (Roche, Indianapolis, Indiana, USA) and anti-CypA affinity rabbit polyclonal antibody (Sigma, St Louis, Missouri, USA) were performed as described previously [19].

Viral infection

MT4 or CEM-SS cells (1×10^5) were infected with SeV expressing human TRIM5α, human 110Arg-TRIM5α, human/AGM chimeric TRIM5α, human/AGM chimeric 110Arg-TRIM5α, CM-TRIM5α-SPRY(−) or AGM-TRIM5α-CC(−) at a multiplicity of infection of 10 plaque-forming units (PFUs) per cell and incubated at 37°C for 9 h. To examine the effects of G176del-TRIM5 on the full length TRIM5α, 5 PFU per cell of each SeV expressing either CM-TRIM5α-SPRY(−), AGM-TRIM5α-CC(−) or HA-G176del-TRIM5 was simultaneously inoculated to MT4 cells with SeV expressing human/AGM chimeric TRIM5α. Cells were then superinfected with 30 ng of p24 of an X4 HIV-1 strain NL43 or 30 ng of p25 of HIV-2 GH123. The culture supernatants were collected periodically and the level of p24 or p25 was measured by RETROtek antigen ELISA kit (ZeptoMetrix, Buffalo, New York, USA).

Statistical analysis

All statistical analyses in this study were performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, California, USA). Correction for multiple testing was done by multiplying the P value by the number of tested markers to obtain the corrected P (P_c) value. Haplotype association analyses were performed by using SNPAlyze version 6.0 standard (DYNACOM Co., Ltd., Tokyo, Japan). Meta-analysis was performed using a Mantel–Haenszel method. P values less than 0.05 were considered to be statistically significant.

Results

Associations of TRIM5α-exon 2 polymorphism with susceptibility to HIV-1 infection

We identified 10 different nucleotide variations in the TRIM5α-exon 2 in this study and eight of them were reported in previous studies. Most of the sequence variations, except for His43Tyr, Val112Phe and Arg136Gln were observed with low frequencies (allele frequency less than 0.05) in the tested populations. Associations between the TRIM5α polymorphisms and the susceptibility to HIV-1 infection were summarized in Table 1. The frequency of 43Tyr-allele in Indian HIV-1-infected patients was significantly lower than that in the ethnic-matched controls [odds ratio (OR) (95% confidence interval (CI)) = 0.52 (0.31–0.89), $P = 0.015$ by χ^2 test]. A similar tendency was observed in Japanese, although it did not reach statistical significance [OR (95% CI) = 0.67 (0.43–1.05), $P = 0.095$ by χ^2 test]. A meta-analysis of data from two populations demonstrated the significant association corrected for multiple testing [OR (95% CI) = 0.61 (0.43–0.85), $P = 0.0004$, $P_c = 0.004$]. When we analyzed the data for HIV-1 loads after 7–8 years during the observation period, which were available for 75 Japanese HIV-1-infected patients, no significant correlation between the His43Tyr genotype and HIV-1 loads was observed (data not shown).

Two novel polymorphisms, Gly110Arg and G176del, were identified only in the Japanese samples. The Gly110Arg variant was more frequent in the HIV-1-infected patients than in the controls [OR (95% CI) = 13.12 (2.53–68.21), $P = 0.002$ by Fisher's exact test]. On the other hand, the G176del variant, a deletion of a G at the coding nucleotide position 176 from the initiation site of translation, which may result in a truncated TRIM5 protein product, was found only in the Japanese controls.

TRIM5α haplotype and susceptibility to HIV-1 infection

The associations between the susceptibility to HIV-1 infection and TRIM5α haplotypes composed of five sequence variations with relatively high frequency, His43Tyr, Gly110Arg, Val112Phe, Thr128Thr and Arg136Gln, were investigated in Japanese and Indian populations (Table 2). In both populations, the frequency of a common haplotype 43Tyr-110Gly-112Val-128Thr-136Arg was significantly low in the HIV-1-infected patients. This result was consistent with the association of 43Tyr with the reduced susceptibility to HIV-1 infection, because this haplotype was in tight linkage disequilibrium with the 43Tyr-allele.

Anti-HIV-1 activity of TRIM5α was attenuated by Gly110Arg substitution

To investigate the functional significance of Gly110Arg on the anti-HIV activity of TRIM5α, we constructed a SeV containing a C-terminal HA-tagged human 110Arg-TRIM5α (Fig. 1a). As shown in Fig. 1b, expression level of variant 110Arg-TRIM5α was comparable to that of wild-type human TRIM5α. A human/AGM chimeric TRIM5α, which possessed the SPRY domain of AGM TRIM5α, was also generated to enhance the weak anti-HIV-1 activity of human TRIM5α (Fig. 1a). As shown in Fig. 1b, the expression level of human/AGM chimeric

Table 1. Allele frequencies of TRIM5α-exon 2 sequence variations and associations of them with HIV-1/AIDS susceptibility.

	Japanese			Indian			White	African–American
Sequence variations[a]	Control ($n=487$)	HIV-1-infected patients ($n=94$)	Odds ratio (95% confidence interval)	Control ($n=99$)	HIV-1-infected patients ($n=101$)	Odds ratio (95% confidence interval)	Control ($n=96$)	Control ($n=96$)
Gly31Ser	0.000	0.000	ND	0.000	0.000	ND	0.000	0.032
His43Tyr	0.184	0.133	0.67 (0.42 – 1.05)	0.227	0.134****	0.52 (0.31 – 0.89)	0.115	0.068
Cys58Tyr	0.000	0.000	ND	0.000	0.000	ND	0.000	0.011
G176del	0.005	0.000	ND	0.000	0.000	ND	0.000	0.000
Asp109Asp	0.000	0.000	ND	0.000	0.000	ND	0.000	0.005
Gly110Arg	0.002	0.021***	13.14 (2.53 – 68.21)	0.000	0.000	ND	0.000	0.000
Gly110Glu	0.000	0.000	ND	0.000	0.000	ND	0.005	0.005
Val112Phe	0.052	0.043	0.80 (0.37 – 1.70)	0.192	0.198	1.04 (0.63 – 1.71)	0.052	0.021
Thr128Thr	0.000	0.011**	ND	0.000	0.000	ND	0.000	0.000
Arg136Gln	0.105	0.144	1.48 (0.94 – 2.32)	0.177	0.173	0.98 (0.58 – 1.64)	0.349	0.177

ND, not defined.
[a]The numbers of sequence variations, except for G176del, are referenced by the amino acid coding position of TRIM5α. G176del is a deletion of a G at the coding nucleotide position 176 from the initiation site of translation.
**$P<0.05$ in Fisher's exact test, when compared with control.
***$P<0.01$ in Fisher's exact test, when compared with control.
****$P<0.05$ in χ^2 test with Yates correction, when compared with control.

Table 2. Haplotype frequencies of four common haplotypes for TRIM5α-exon 2 and association of them with HIV-1/AIDS susceptibility.

	Japanese				Indian			
Haplotype (His43Tyr-Gly110Arg-Val112Phe-Thr128Thr-Arg136Gln)	Control ($n=487$)	HIV-1-infected patients ($n=94$)	*P* value in permutation test	Odds ratio (95% confidence interval)	Control ($n=99$)	HIV-1-infected patients ($n=101$)	*P* value in permutation test	Odds ratio (95% confidence interval)
43His-110Gly-112Val-128Thr-136Arg	0.659	0.721	0.129	1.35 (0.96 – 1.91)	0.404	0.495	0.076	1.45 (0.97 – 2.15)
43Tyr-110Gly-112Val-128Thr-136Arg	0.184	0.089	0.001*	0.44 (0.26 – 0.75)	0.227	0.134	0.008*	0.52 (0.31 – 0.89)
43His-110Gly-112Val-128Thr-136Gln	0.103	0.090	0.645	0.87 (0.51 – 1.49)	0.177	0.173	0.909	0.98 (0.58 – 1.64)
43His-110Gly-112Phe-128Thr-136Arg	0.051	0.031	0.251	0.61 (0.26 – 1.44)	0.192	0.198	0.802	1.04 (0.64 – 1.71)

*$P<0.01$ in permutation test.

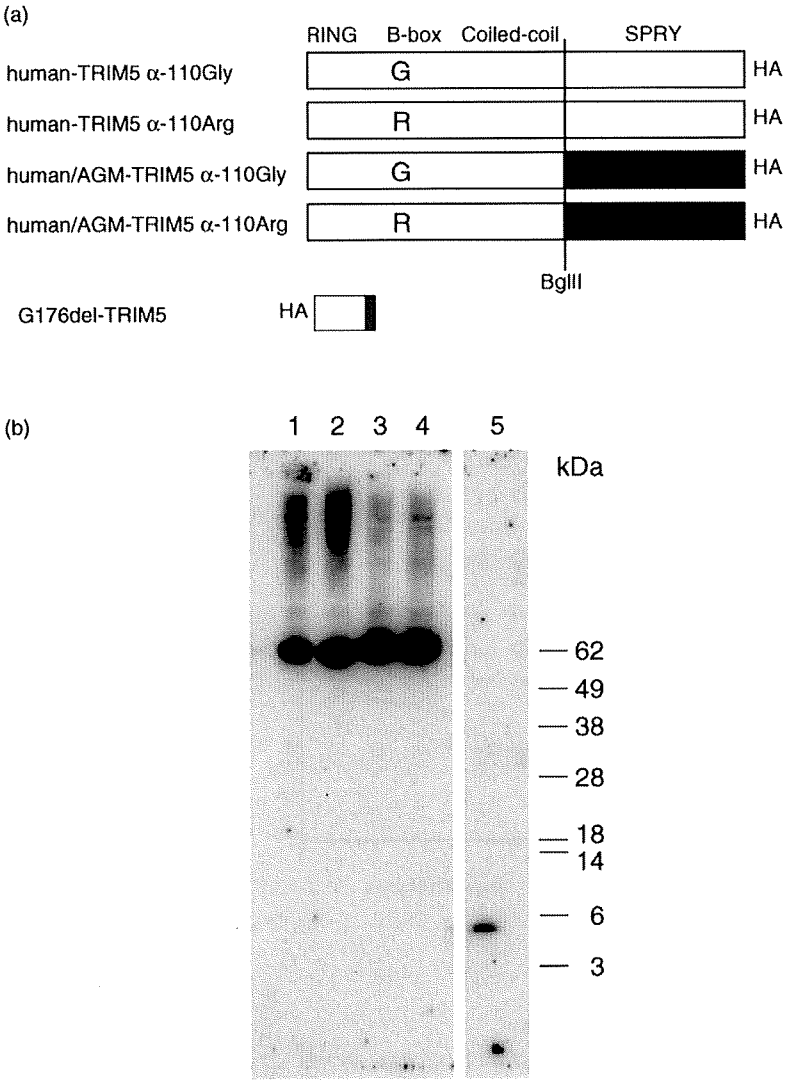

Fig. 1. Expression of TRIM5α constructs used in this study. (a) Schematic representation of TRIM5α fused with a hemaglutinin (HA)-tag. Domain structures of TRIM5α are shown at the top. White and black bars denote human and African green monkey (AGM) sequences, respectively. Gray bar denotes the G176del-specific 16 amino acid residues generated by the frameshift. A BglII site was used to exchange carboxy-terminal B30.2 (SPRY) domains between human and AGM TRIM5α. 'G' or 'R' denotes the amino acid residue at the 110th position. WT denotes wild type. (b) Western blot analysis of TRIM5 protein expressed by recombinant Sendai virus (SeV). MT4 cells were infected with a SeV containing a HA-tagged variant (110Arg) human TRIM5α (lane 1), wild-type human TRIM5α (lane 2), human/AGM chimeric 110Arg-TRIM5α (lane 3), human/AGM chimeric wild-type-TRIM5α (lane 4) and G176del-TRIM5 (lane 5). Sixteen hours after the infection, cells were lysed and subjected to SDS-PAGE. HA-tagged proteins were detected by anti-HA antibody.

110Arg-TRIM5α was similar to that of human/AGM chimeric wild-type-TRIM5α.

These TRIM5α constructs were tested for their ability to restrict the X4-tropic HIV-1 strain NL43 and HIV-2 strain GH123. MT4 cells infected with recombinant SeV expressing each of the TRIM5α constructs were super-infected with HIV-1 NL43 or HIV-2 GH123. We used SeV expressing cynomolgus monkey TRIM5α lacking the SPRY domain CM-TRIM5α-SPRY(−) as a negative control for functional TRIM5α, as overexpression of TRIM5α lacking the SPRY domain exerted a dominant negative effect on the endogenous human TRIM5α [24]. We also used SeV expressing AGM-TRIM5α lacking the coiled-coil domain AGM-TRIM5α-CC(−) as a non-interfering control [24]. As shown in Fig. 2a, both wild-type (110Gly) and variant (110Arg) human/AGM chimeric TRIM5α strongly restricted HIV-1 NL43. On the other hand, both wild-type and variant human TRIM5α showed only weak anti-HIV-1 activity. There was, however, a small increase of HIV-1 in cells expressing the human/AGM chimeric 110Arg-TRIM5α than the cells with the human/AGM chimeric TRIM5α. In the case of HIV-2, virus grew to higher titers in cells

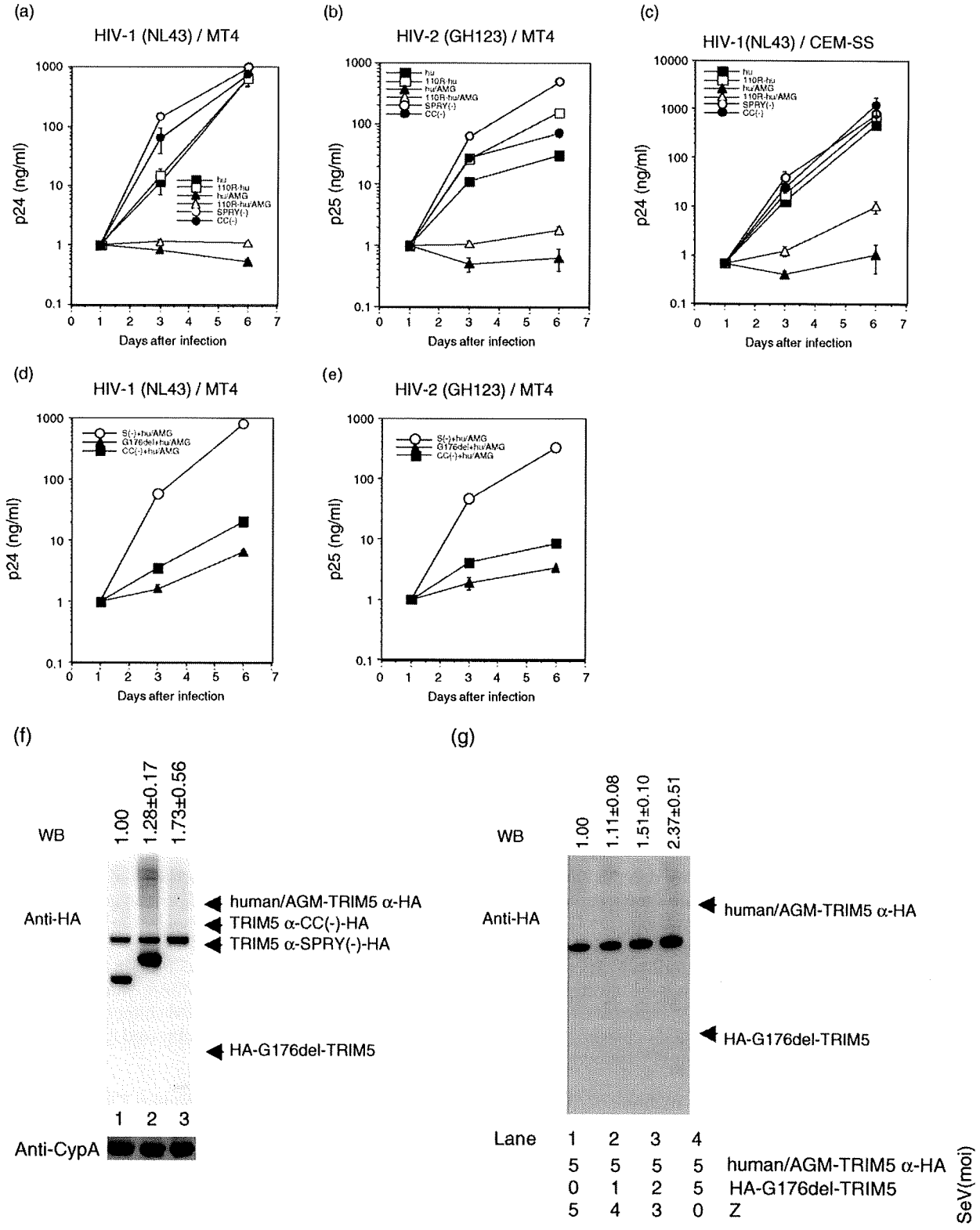

Fig. 2. Effect of TRIM5α variants on the anti-HIV-1 and anti-HIV-2 activities. Human MT4 (a, b) or CEM-SS (c) cells were infected with recombinant Sendai virus (SeV) carrying human wild-type TRIM5α (■; hu), human 110Arg-TRIM5α (□; 110R-hu), human/African green monkey (AGM) chimeric TRIM5α (▲; hu/AGM), human/AGM chimeric 110-ArgTRIM5α (△; 110R-hu/AGM), CM-TRIM5α-SPRY(−) (○; SPRY(−)) or AGM-TRIM5α-CC(−) (●; CC(−)). Nine hours after infection, cells were inoculated with HIV-1 NL43 (a and c) or HIV-2 GH123 (b). Culture surpernatants were periodically assayed for levels of p24 (a and c) or p25 (b). MT4 cells were simultaneously infected with two recombinant SeVs at 5 plaque-forming unit (PFU) per cell for

expressing dominant negative TRIM5α-SPRY(−) than in cells with noninterfering TRIM5α-CC(−), demonstrating the anti-HIV-2 activity of endogenous human TRIM5α (Fig. 2b). Both wild-type and variant human TRIM5α exhibited weak but apparent anti-HIV-2 activity, and HIV-2 grew to higher titers in cells expressing the human 110Arg-TRIM5α than in cells with the human wild-type-TRIM5α (Fig. 2b). In human/AGM chimeric version, wild-type TRIM5α completely restricted HIV-2 (Fig. 2b). In contrast, HIV-2 grew to slightly higher titers in cells expressing the human/AGM chimeric 110Arg-TRIM5α than in cells expressing the wild-type human/AGM chimeric TRIM5α (Fig. 2b). These results indicated that the Gly110Arg variant weakened the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α in MT4 cells.

We recently found that the expression of TRIM5α protein introduced by SeV varied depending on cell types, that is, it was much lower in CEM-SS than in MT4 cells [25]. To evaluate the anti-HIV-1 activity of variant TRIM5α at more physiological levels of expression, we performed experiments using CEM-SS (Fig. 2c). Neither wild-type nor variant human TRIM5α exhibited anti-HIV-1 activity, probably due to the low level expression of TRIM5α in CEM-SS cells. However, HIV-1 grew to approximately 10 times higher levels in cells expressing the human/AGM chimeric 110Arg-TRIM5α than in cells with the wild-type chimeric TRIM5α, suggesting that the anti-HIV-1 activity of TRIM5α in CEM-SS cells was also reduced by the Gly110Arg substitution. Therefore, we concluded that the Gly110Arg polymorphism affected both the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α.

Truncated G176del-TRIM5 enhanced antiviral activity of coexpressed TRIM5α

To express the G176del-TRIM5, we added an HA-tag at its N-terminus, because the expression of G176del-TRIM5 protein tagged with HA at the C-terminus could not be detected. Although the expression of HA-fused protein was clearly visualized by anti-HA antibody, its expression was much lower than the full-length TRIM5α (Fig. 1b). In cells infected with SeV expressing the G176del-TRIM5, HIV-2 grew to the same titers as those in cells infected with SeV expressing a nonfunctional mutant TRIM5α-CC(−), indicating that the G176del-TRIM5 lost the anti-HIV-2 activity (data not shown). We then investigated whether the G176del-TRIM5 showed any effects on the anti-HIV activity of coexpressed full-length TRIM5α, because all individuals carrying the G176del variant were in the heterozygous state. As shown in Fig. 2d and 2e, both HIV-1 and HIV-2 were restricted in cells simultaneously expressing the human/AGM chimeric TRIM5α and TRIM5α-CC(−). As expected, both HIV-1 and HIV-2 grew to high titers in cells expressing the human/AGM chimeric TRIM5α and the dominant negative mutant TRIM5α-SPRY(−) [24]. In contrast, both HIV-1 and HIV-2 were severely restricted in cells expressing the human/AGM chimeric TRIM5α and G176del-TRIM5 as compared within cells expressing the human/AGM chimeric TRIM5α and TRIM5α-CC(−). These results suggested that the G176del-TRIM5 enhanced the antiviral activity induced by the full-length TRIM5α.

Next, we investigated whether the truncated G176del-TRIM5 could affect the expression of TRIM5α. Expressions of the human/AGM chimeric TRIM5α in cells expressing either TRIM5α-SPRY(−), TRIM5α-CC(−) or G176del-TRIM5 are shown in Fig. 2f. Amount of human/AGM chimeric TRIM5α in cells coexpressing the G176del-TRIM5 was 1.7 times higher than that in cells coexpressing the TRIM5α-SPRY(−). When we infected a constant amount of SeV expressing the human/AGM TRIM5α in combination with the increasing amounts of SeV expressing the G176del TRIM5 variant, we found that the expression level of human/AGM TRIM5α was increased by the G176del TRIM5 (Fig. 2g).

Discussion

It is widely accepted that within host cells, there are restriction factors that oppose retroviral replication more effectively than the conventional arms of the immune

Fig. 2. *(continued)*
each SeV. CM-TRIM5α-SPRY(−) and human/AGM chimeric TRIM5α (○; S(−) + hu/AGM), AGM-TRIM5α-CC(−) and human/AGM chimeric TRIM5α (■; CC(−) + hu/AGM), or hemaglutinin (HA)-G176del-TRIM5 and human/AGM chimeric TRIM5α (▲; G176del + hu/AGM) were simultaneously inoculated. Nine hours after the infection, cells were superinfected with HIV-1 NL43 (d) or HIV-2 GH123 (e) and culture surpernatants were periodically assayed for levels of p24 (d) or p25 (e). The means with standard deviations of triplicate samples are shown. (f) Western blottings for TRIM5 protein and cyclophilin A from MT4 cells infected with SeV expressing the HA-tagged human/AGM chimeric TRIM5α (human/AGM-TRIM5α-HA) coexpressed with the AGM-TRIM5α-CC(−)-HA (lane 1; TRIM5α-CC(−)-HA), coexpressed with the CM-TRIM5α-SPRY(−)-HA (lane 2; TRIM5α-SPRY(−)-HA), or with the HA-G176del-TRIM5 (lane 3: HA-G176del-TRIM5). The relative amounts of human/AGM chimeric TRIM5α are shown on the top with the standard deviation of six independent samples. (g) MT4 cells were infected with SeV expressing the HA-tagged human/AGM chimeric TRIM5α coinfected with SeV expressing the HA-G176del-TRIM5 or an empty vector parental Z strain. The multiplicity of infection in each SeV is shown on the bottom. The relative amounts of human/AGM chimeric TRIM5α are shown on the top with standard deviation of triplicate samples.

system [1,2]. Because TRIM5α has crucial roles in the intracellular defense mechanisms against HIV-1 [2–4], sequence variations in TRIM5α might be associated with the susceptibility to HIV-1 infection and/or progression to AIDS. In this study, we demonstrated the association of 43Tyr-allele with the reduced susceptibility to HIV-1 infection in two ethnically distinct populations. In addition, we identified two novel rare variants, Gly110Arg and G176del, both of which had an impact on the anti-HIV-1 activity and susceptibility to HIV-1 infection.

The association of His43Tyr with the HIV-1 infection or AIDS progression has been tested in several studies, but the results were not consistent [16–20]. We found that the 43Tyr-allele was less frequent in the HIV-1-infected patients than in the ethnic-matched controls in both Japanese and Indian populations. The study sizes were not very large, but two independent ethnic populations did exhibit the same trends for the association with His43Tyr. We previously analyzed HIV-1-infected long-term nonprogressors and standard progressors in France and Japan for the TRIM5α polymorphisms and failed to find any differences in the frequency of 43Tyr-allele between these two HIV-infected groups both in France and Japan [19]. However, the allele frequency of 43Tyr in the Japanese HIV-1-infected patients we analyzed in the previous study was 0.143, which was similar to that in the present study (0.133, Table 1). Interestingly, several studies have reported that the anti-HIV-1 activity of TRIM5α with 43Tyr was lower than that with 43His [16,18]. In our previous study, we also showed that the anti-HIV-1 activity of TRIM5α with 43Tyr was lower than that with 43His, although the difference in anti-HIV-1 activity between the 43His-TRIM5α and 43Tyr-TRIM5α was very small [19]. In spite of the lower anti-HIV-1 activity of the 43Tyr-TRIM5α, several epidemiological studies have shown that the 43Tyr-allele was associated with the reduced susceptibility to HIV-1 infection [16,18], as demonstrated in this study. The reasons for the discrepancy between the epidemiological and functional effects of His43Tyr remain unclear at the moment. On the other hand, van Manen *et al.* [20] recently reported that homozygous status for 43Tyr was associated with the accelerated disease progression in white populations, which was consistent with the effect of His43Tyr variation on the anti-HIV-1 activity. Further epidemiological studies will be required to clarify the impact of His43Tyr on the susceptibility to HIV-1 infection and AIDS progression.

We also showed that the impact of His43Tyr on the susceptibility to HIV-1 infection was slightly different between Japanese and Indian. The frequency of 43Tyr-allele in the Indian HIV-1-infected patients was significantly lower than that in the Indian controls, but the significant difference was not found in Japanese. Different distribution of HIV-1 subtypes might be one of the reasons for the different contribution of 43Tyr-allele in the susceptibility, because all of the Indian patients examined in this study were infected with HIV-1 subtype C, whereas only subtype B was observed in our Japanese patients, as was found in the previous reports [26,27]. Kaumanns *et al.* [28] have reported that the antiretroviral activities of TRIM5α differed among the HIV-1 subtypes, although the differences in the in-vitro antiretroviral effect of TRIM5α between the subtypes C and B were not evident.

In this study, one focus was the functional impact of two rare TRIM5α variants found in our epidemiological studies. First, our findings indicated that the 110Arg variant weakened the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α in human T-cell lines. This variant was observed more frequently in the Japanese HIV-1-infected patients than in the controls. This variation substitutes the smallest amino acid glycine with a positively charged amino acid arginine at the 110 amino acid position of TRIM5α and is located next to the amino acid residue 109Gly, which is suspected to be a zinc-coordinating residue in the B-box 2 domain [29]. This drastic change in amino acid character might change the structure of TRIM5α, in which an intact B-box 2 domain was essential for the antiretroviral activity of TRIM5α and disruption of the TRIM5α B-box domain by specific amino acid substitution resulted in loss of retroviral restriction [8,30–32]. The 3D structure of the amino acid residues 11–133 of TRIM5α was modeled by SWISS-MODEL, an Automated Comparative Protein Modeling Server (http://swissmodel.expasy.org/SWISS-MODEL.html) [33]. As shown in Fig. 3, residue 110 constituted one of the β sheets in the N-terminal half of TRIM5α. Interestingly, the location of residue 110 was close to residue 43 in the modeled 3D structure of TRIM5α (Fig. 3b and 3c). As described previously, His43Tyr was reported to affect antiretroviral activity. These data suggested that residue 110 might be one of the key amino acid residues in the TRIM5α structure like residue 43.

Second, we found that the truncated G176del-TRIM5 enhanced the antiviral activity of coexpressed full-length TRIM5α. Coinfection of SeVs expressing the G176del-TRIM5 and human/AGM-TRIM5α was accompanied by the increased protein level of full-length human/AGM-TRIM5α. The amount of human/AGM chimeric TRIM5α in cells coinfected with SeVs expressing the G176del-TRIM5 was 1.7 times higher than in cells coinfected with SeVs expressing the TRIM5α-SPRY(−). These data suggested that the truncated TRIM5α was degraded rapidly, resulting in a delay of the degradation process of full-length TRIM5α and leading to the augmentation of protein levels. Recently, we observed that coexpression of a splice variant of TRIM5, TRIM5γ, increased the amount of TRIM5α.

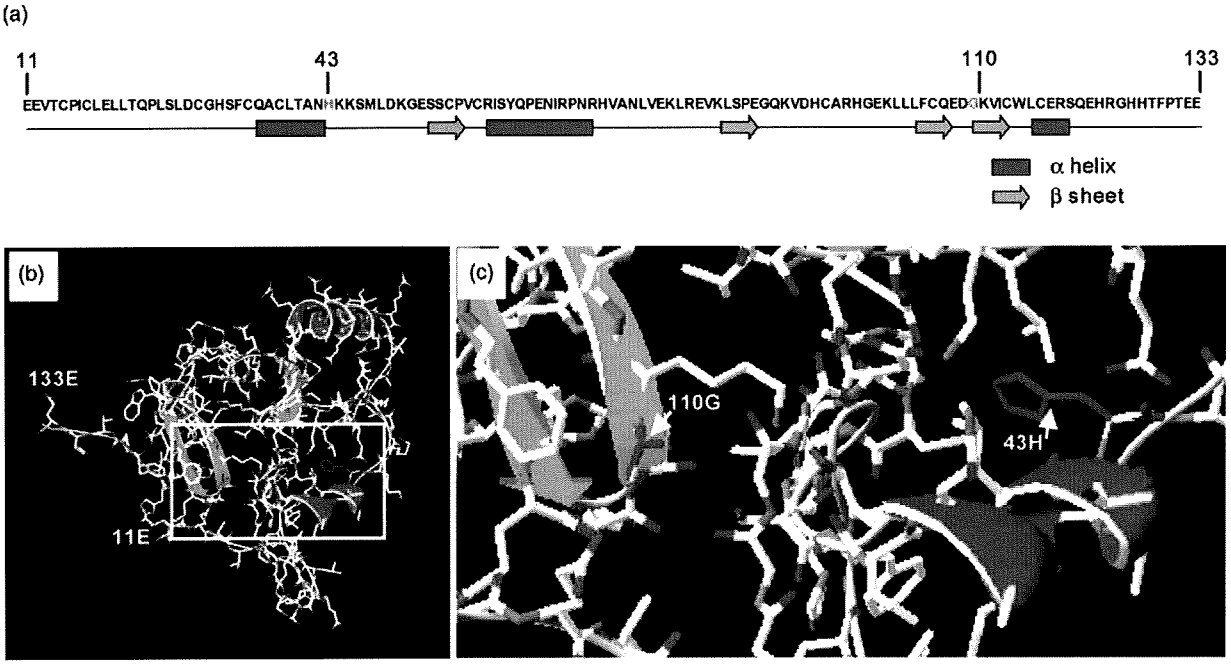

Fig. 3. Structural model of variant TRIM5α. (a) The primary structure is illustrated by the amino acid sequence of residues 11–133. One-letter amino acid code is used. The secondary structure is diagrammatically represented below the sequence showing the regions of the polypeptide chain, which are folded into the α helices (red boxes), the β sheets (green arrows) and random coils (black lines). (b) The 3D structure of amino acids 11–133 was modeled by SWISS-MODEL. (c) A magnified view, which is enclosed with a white square in (b), is shown. The arrows indicate the 43His and 110Gly residues.

TRIM5γ itself was unstable and its expression was lower than TRIM5α (Maegawa, unpublished data).

In this study, we identified three individuals harboring the G176del variant in the heterozygous state only in the Japanese controls, not in the HIV-1-infected patients. It appeared that the homozygous state for the G176del-allele would increase the susceptibility to HIV-1 infection, because it should result in null TRIM5α activity. It follows from the enhanced anti-HIV-1 activity of full-length TRIM5α by the truncated G176del-TRIM5 that the heterozygous state for the 176del-allele might mask the reduction in TRIM5α gene number and thus might not have a serious effect on the susceptibility to HIV-1 infection.

We demonstrated the association of common variant 43Tyr with the reduced susceptibility to HIV-1 infection in Japanese and Indian. We also identified two rare variants, 110Arg and G176del, which decreased and increased, respectively, the anti-HIV-1 activity in human cells expressing TRIM5α. We suggested that the sequence variations of TRIM5α were tightly linked to the susceptibility to or protection against the HIV-1 infection. However, further epidemiological studies using larger population samples will be required to clarify the impact of these rare variants on the HIV-1/AIDS susceptibility. In an effort to understand the genetic factors controlling the HIV-1 infection and AIDS progression, considerable attention should be paid to rare variants in addition to common variants in the candidate genes.

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The 'immunologic advantage' of HIV-exposed seronegative individuals

I would like to commend Dr Masaaki Miyazawa and his colleagues on the excellent comprehensive review article on HIV-seronegative individuals who have been exposed to HIV (ESN) [1]. However, I would like to point out some omissions in their article.

The authors assert 'HIV-specific $CD4^+$ T-cell responses were initially described in heavily infected men enrolled from the MACS cohort' and cite two publications from 1991 and 1992. They neglect to report in the article that these men were identified from a study done by Imagawa and colleagues in the MACS 2 years earlier that described a group of 31 heavily exposed HIV-seronegative men from whom HIV was isolated and who were PCR-positive for HIV [2].

The authors offer the hypothesis that infection resulting in at least one complete cycle of viral replication allows the effective presentation of viral peptides with a binary complex for a human leukocyte antigen (HLA) class I molecule and cite a 2002 publication. However, we proposed that HIV was cleared in ESN men in 1991 [3]. Further, in 1994, we proposed that more efficient MHC antigen presentation to $CD8^+$ cells may prime these cells to clear HIV [4].

The authors assert 'ESN individuals are characterized by a generalized immune activation. This observation was made when $CD4^+/CD25^+$, $CD38^+/CD45RO$ and both HLADR expressing $CD4^+$ and $CD8^+$ activated T lymphocytes were analyzed in the peripheral blood of ESN individuals.' Three papers are cited, all published since 2000. The observation of an increase in $CD8^+$ cells and of $CD8^+/CD25+$ cells among ESN was, however, originally made in 1994 [4] and 1996 [5].

The authors further state that 'A plausible explanation could be that immune activation is a favorable factor in inducing an immune resistance to primary HIV infection in ESN individuals'. We strongly agree, and published that hypothesis in 1996 [5].

The authors cite several references to genetic factors that may mediate resistance. We would like to bring their attention to our earlier 1996 study that identified a significantly higher proportion of TAP 1.4 alleles and a combination of TAP 1.4 and 2.3 alleles in ESN [5].

In conclusion, we are pleased to see increasing interest in individuals who appear to be resistant to HIV infection. It is our opinion that this group may hold the key to identifying and inducing a protective immune response in susceptible individuals and are especially important, given the recent vaccine failures.

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The 'immunologic advantages' of HIV-exposed seronegative individuals: authors' reply

We appreciate the constructive comments made by Professor Detels [1] on our recent review [2] and would like to address them here.

It is true that the high-risk exposed homosexual individuals examined for HIV-1 peptide-specific T-cell responses and reported in the 1992 paper [3] were from the Multicenter AIDS Cohort Study (MACS) cohort; this is acknowledged on p. 163 of our review [2]. The MACS samples were made available through the efforts of the late Janis Giorgi and were analyzed by one of us (M.C.) in Dr Giorgi's laboratory at UCLA. The resulting paper [3] was the foundation of all the follow-up work. The fact that these individuals had previously been examined by Imagawa *et al.* [4] was clearly stated in the 1992 paper [3]; however, in the paper published in 1989, Imagawa and colleagues, including Dr Giorgi, verified the possible presence of infectious HIV-1 and provirus in the context of prolonged latency before seroconversion, and the possibility of observing HIV-1-specific T-cell responses in these individuals was not addressed. As the section of the review Dr Detels is referring to is devoted to cell-mediated immune responses, we started our descriptions with the references that described T-cell responses in exposed seronegative (ESN) individuals.

Dr Detels states that Imagawa and Detels [5] proposed in their 1991 letter the possible clearance of HIV in ESN men. We have the utmost respect for their pioneering work, but they actually concluded that the original results [4] and the follow-up studies [5] were more consistent with the hypothesis of 'incomplete infection', and not 'clearance'. Although 'incomplete infection' might have meant an initial establishment of HIV infection and later clearance of the virus, it should be noted that no clear concept of reduced susceptibility to HIV acquisition due to cell-mediated immunity was proposed in this letter.

The 1994 paper mentioned by Dr Detels [6] indicated a possible resistance to HIV-1 infection in high-risk homosexual men who remained seronegative despite having had more than 45 anal intercourse partners. However, what the authors observed in the resistant group were levels of neutrophils and $CD8^+$ T cells higher than those seen in the susceptible group, and immune activation defined by relevant markers was not directly examined at this stage. In the 1996 paper [7], the authors observed a higher percentage of $CD25^+$ cells among $CD8^+$ T cells and proposed that $CD8^+$ T cells might have cleared HIV-1. Although this hypothesis is intriguing, what we described in the section on immune activation in our review [2] was more generalized immune activation, and not the possible eradication of HIV-1^+ cells by antigen-specific T cells.

As to the *TAP1* and *TAP2* polymorphisms reported in the same paper [7], it was not our intention to cover all reported genetic factors that are associated with susceptibility to HIV infection in our review [2], and the *TAP* polymorphism was included in the effects of the major histocompatibility complex. As the *TAP* loci are located between the *DQB1* and *DPB1*, it is possible that other loci linked to the described *TAP* alleles might actually have influenced the host immune responses. As the authors from the same group discussed in their later paper [8], whether the epidemiological observations on the *TAP1* and *TAP2* polymorphism reflect functional differences in peptide selectivity remains unknown.

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Retrovirology

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Mouse APOBEC3 affects the production of virus-neutralizing antibodies by restricting early retroviral replication, not by altering the B-cell repertoire

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Recent genetic analyses have indicated that polymorphisms in the mouse *APOBEC3* locus constitute the *Rfv3* gene that influences the production of virus-neutralizing antibodies in mice infected with Friend leukemia retrovirus [1,2]. Mice of the resistant genotype preferentially express the exon 5-lacking transcript in higher levels, while susceptible mice express the full-length transcript in lower levels [2,3]. Mouse APOBEC3 expressed in the resistant strains restricts the replication of mouse retroviruses *in vitro* through a mechanism independent of its deaminase activity [2]. However, the mechanisms through which mouse APOBEC3 affects the production of virus-neutralizing antibodies remain unclear. To address this question, we analyzed retroviral replication and the production of virus-neutralizing antibodies in mice of different *APOBEC3* genotypes and those lacking its expression.

Strain A mice with the susceptible *APOBEC3* genotype nevertheless produced high levels of virus-neutralizing antibodies when they possessed the H-2^b haplotype, and class-switching to IgG was observed in the presence of virus-specific T helper cells. Further, APOBEC3-deficient mice produced virus-neutralizing antibodies when their T helper cells had been primed with the viral antigen. Friend virus-induced derangements in the hematopoiesis and resultant splenomegaly are not directly responsible for the delayed antibody responses, because higher levels of viremia and lower antibody responses were observed upon infection with nonpathogenic Friend murine leukemia helper virus in the absence of APOBEC3. In mice of the resistance-associated *APOBEC3* genotype lower levels of viremia were observed even before the detection of virus-neutalizing antibodies in the blood, indicating that the polymorphisms in the *APOBEC3* locus affect the production of neutralizing antibodies as a result of restricted retroviral replication.

Conclusion

B cells from the mice of susceptible *APOBEC3* genotypes produce neutralizing antibodies in the presence of virus-specific T helper cells. Polymorphisms in the *APOBEC3* affect the levels of viremia prior to the production of neutralizing antibodies, indicating that APOBEC3 may indirectly influence B cell functions by restricting viral replication.

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SHORT COMMUNICATION

Development of a new disinfectant with very strong anti-influenza viral activity: a preliminary report

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Abstract
Objectives We evaluated the effectiveness and safety of a disinfectant newly developed by our laboratories for use against influenza viruses.
Methods The effectiveness of our new disinfectant against avian, swine and human influenza viruses was tested in ovo. The acute toxicity of this disinfectant to two different cultured cell lines was investigated.
Results This new disinfectant showed very strong anti-influenza viral activity in the in ovo tests. All of the influenza viruses tested were inactivated very quickly. Following exposure to the disinfectant, the infectivity of all viral strains tested had been eliminated within $\leq$10 min. The infectant showed a weak acute toxicity in vitro.
Conclusion This new disinfectant is expected to be useful for preventing viral infection during a new influenza pandemic.

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Keywords Avian influenza virus · Human influenza virus · New disinfectant · Strong activity · Swine influenza virus

Introduction

Outbreaks of highly pathogenic avian influenza and other emerging and re-emerging diseases have caused serious economical and social disturbances worldwide [1–4]. Although the pandemic H1N1 subtype influenza virus has rapidly spread throughout the world since the end of April 2009, the production of a new influenza vaccine is still insufficient. However, the preparation of large amounts of medicine effective against influenza was also difficult prior to the occurrence of this latest pandemic. Therefore, there is a need to develop possible control methods, such as an easily obtained, effective disinfectant to prevent the virus from spreading. Our laboratories have succeeded in developing a new disinfectant which consists mainly of an iron ion. Tests have demonstrated that this disinfectant in very efficient in rapidly inactivating bacteria and influenza viruses.

Materials and methods

Experiment 1

The new disinfectant was prepared as follows. First, solution A was made by dissolving 0.96 g $FeCl_3 6H_2O$ in 200 ml distilled water. Next, solution B was prepared by dissolving 1 g L-cysteine, 0.1 g ascorbic acid, 0.05 g potassium sorbate and 0.1 g sodium lauryl sulfate in 800 ml distilled water. Solutions A (200 ml) and B (800 ml) were then mixed and 3 *N* HCl was added to this mixture to adjust it to pH 3. This new disinfectant is a colorless and transparent liquid.

Table 1 Time taken to completely inactivate influenza A viruses following contact with the new disinfectant

Virus strain[a]	Time to complete disinfection (minutes)	
	1:9[b]	1:99[b]
H1N1 $10^{8.25}EID_{50}$/0.2 ml	>10	10
10-times diluted	10	10
H3N2 $10^{8.75}EID_{50}$/0.2 ml	10	10
10-times diluted	10	10
H4N6 $10^{8.50}EID_{50}$/0.2 ml	>10	10
10-times diluted	10	10
H5N3 $10^{8.25}EID_{50}$/0.2 ml	>10	2
10-times diluted	2	2
H6N2 $10^{7.75}EID_{50}$/0.2 ml	>10	10
10-times diluted	10	10
H7N7 $10^{8.25}EID_{50}$/0.2 ml	10	10
10-times diluted	10	10

EID_{50}, Median egg infectious doses (virus titre)

[a] H1N1, A/swine/Iowa/15/30; H3N2, A/Aichi/2/68; H4N6, A/duck/Czech/56; H5N3, A/whistling swan/Shimane/499/83; H6N2, A/duck/Massachusetts/3740/65; H7N7, A/whistling swan/Shimane/42/80

[b] Ratio of virus to the new disinfectant

This disinfectant was tested on six strains of influenza virus, namely, A/swine/Iowa/15/30 (H1N1), A/Aichi/2/68 (H3N2), A/duck/Czech/56 (H4N6), A/whistling swan/Shimane/499/83 (H5N3), A/turkey/Massachusetts/3740/65 (H6N2) and A/whistling swan/Shimane/42/80 (H7N7) [5, 6]. Prior to this investigation, these viruses were grown in the allantoic cavity of 10-day-old embryonated SPF hen's eggs for 2 days at 37°C. The allantoic fluid (virus fluid), which has a very high titre of hemagglutination (HA) activity, was collected and stored at −80°C. All virus strains were titrated by inoculating 10-day-old embryonated SPF hen's eggs via the allantoic cavity. Virus titres were expressed as median egg infectious doses (EID_{50}) [7]. The titres of all virus strains tested in these experiments were greater than $\log 10^{7.7}$ EID_{50}/0.2 ml (Table 1).

Two strengths of virus fluid were tested: undiluted and diluted tenfold in phosphate buffered saline (pH 7.2). 10- and 100-ml samples of both the diluted and undiluted virus fluids were poured into small tubes, made up to 1 ml with the new disinfectant solution, shaken carefully and left at room temperature. After incubations of 2, 10 and 60 min, respectively, the presence of surviving virus was determined by inoculating the virus fluid into the allantoic cavity of 10-day-old SPF hen's eggs.

Experiment 2

The acute toxicity of this disinfectant to cultured cell lines was investigated. CV-1 monkey kidney cells and Jurkat human lymphoma cells were seeded at densities of 5×10^5 cells/well and 1.0×10^5 cells/well, respectively, in 24-well tissue culture plates and incubated for 24 h in tissue culture media. Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) was used to culture the CV-1 cells and RPMI-1640 supplemented with 10% heat-inactivated FBS was used for Jurkat. The new disinfectant and chrolhexidine gluconate as a positive control were added to duplicate wells at the final concentrations indicated in Table 2. The percentages of viable cells were determined by the trypan blue dye exclusion method 48 h later. Negative control wells were those without the test reagents. The percentages of growth inhibition were calculated by using the following formula:

Percentage growth inhibition = [(percentage dead cells in a test well) − (percentage dead cells in a control well)]/[100 − (percentage dead cells in a control well)].

Table 2 Cell toxicity of the different disinfectants tested

Test reagent	Final concentration (% of the working solution)[a]	Percentage inhibition of cell growth[b]	
		CV-1	Jurkat
New disinfectant	10.0	60.2 ± 5.9	48.4 ± 4.2
	1.0	28.4 ± 3.8	22.9 ± 10.5
	0.1	18.2 ± 3.3	4.6 ± 3.0
	0.01	13.1 ± 9.1	0
	0.001	5.3 ± 2.3	1.6 ± 1.5
	0.0001	0	0
Chrolhexidine gluconate (positive control)	100	100	100
	10	99.3 ± 0.9	100
	1.0	82.0 ± 1.2	87.0 ± 14.6
	0.1	63.2 ± 5.4	31.0 ± 18.6
	0.01	19.9 ± 7.5	15.6 ± 6.6
	0.001	0.6 ± 0.8	8.6 ± 7.6

[a] The new disinfectant is meant to be used undiluted, and thus the working concentration is 100%. At the lowest test dilution, the new disinfectant was added at 10% (v/v) strength to the culture media. The recommended working dilution for chrolhexidine gluconate for disinfection of the skin is 0.1–0.5%; therefore, for the highest test concentration, a Hibitane concentrate containing 5% (w/v) chrolhexidine gluconate was added at 1/50 (v/v) to the culture media

[b] Data are presented as the mean ± standard error of the mean (SEM), calculated from the results of three repeated experiments

Results and discussion

The results of experiment 1 (Table 1) show that all human, a swine and avian influenza A viruses belonging to the H1N1, H3N2, H4N6, H5N3, H6N6 and H7N7 subtypes lost at least 10^6 EID_{50} of their infectivity following contact with the new disinfectant for 10 min at room temperature,

thereby demonstrating that this disinfectant has a very strong anti-influenza virus activity. We did not use the H5N1 virus in this investigation for the following reason: it is difficult to get a sufficient high titre of H5N1 virus since the virulence of this highly pathogenic avian influenza virus is so severe that infected chick embryos died less than 16 h post inoculation and, therefore, the virus titre in allantoic fluid was generally low. We did succeed in generating a high pathogenicity with this H5N3 virus from an avirulent one by passaging it in chicks [5].

In experiment 2, as shown in Table 2, the cytotoxicity of the new disinfectant at the working concentration (100%) is weaker than that of chrolhexidine gluconate (0.1–0.5%).

Based on our results, the new disinfectant has a quick and strong anti-influenza viral activity, and its toxicity is rather weak. All human, swine and avian influenza viruses tested at a titre $>10^{7.7}$ completely lost their infectivity following contact with this new disinfectant for at least 10 min at room temperature. The acute toxicity of the new disinfectant is much weaker than that of chrolhexidine gluconate. We therefore suggest that this new disinfectant is both a safe and a promising disinfectant and that it can be used in any area where outbreaks of emergent infectious diseases, such as influenza, including that caused by the H1N1 subtype influenza virus, are occurring.

We are currently elucidating the underlying mechanisms of the anti-viral activity of this new disinfectant. We expect to find that the usual anti-bacterial activity of metallic ions is involved and also that the activity of this new disinfectant is stimulated by the existence of another unknown factor.

Our new disinfectant maintains the efficacy stated above for at least 3 years at room temperature (data not shown). It is reasonable to expect that this disinfectant will prove useful in preventing infection from the pandemic H1N1 subtype influenza virus and from other kinds of pathogens.

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ORIGINAL PAPER

Administration of Ag85B showed therapeutic effects to Th2-type cytokine-mediated acute phase atopic dermatitis by inducing regulatory T cells

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Abstract Increase in the number of patients with atopic dermatitis (AD) has been recently reported. T helper (Th) cells that infiltrate AD skin lesions are Th2-type dominant; reduced exposure to environmental Th1-cytokine-inducing microbes is believed to contribute to the increased number of AD patients. Regulatory type immune responses have been also associated with the occurrence of AD. It has been reported that antigen 85B (Ag85B) purified from mycobacteria is a potent inducer of Th1-type immune response in mice as well as in humans. In this study, we have examined the effect of plasmid DNA encoding Ag85B derived from *Mycobacterium kansasii* on AD skin lesions induced by oxazolone (OX) application. Th2-cytokine mediated mouse AD model with immediate type response followed by a late phase reaction was developed by repeated applications of low-dose OX to sensitized mice. Mice were immunized with plasmid DNA encoding cDNA of Ag85B before OX sensitization or during repeated elicitation phase. Both therapies were associated with significant suppression of immediate type response, clinical appearance, dermal cell infiltration, reduced IL-4 production, and augmented IFN-γ mRNA expression compared to placebo-treated mice. Additionally, increased number of Foxp3$^+$ regulatory T cells were observed in the skin sections in Ag85B treated mice. The results of this study suggest that Ag85B DNA vaccine is a potential therapy for Th2 type dermatitis.

Keywords Atopic dermatitis · Antigen 85B · Regulatory T cell

Abbreviations

AD	Atopic dermatitis
Th	T helper
BCG	*Bacillus Calmette-Güerin*
Treg	Regulatory T cell
Ag85B	Antigen 85B
OX	Oxazolone

Introduction

It is known that acute phase skin lesion in atopic dermatitis (AD) is associated with enhanced secretion of T helper (Th) 2-type cytokines [8]. Increased incidence of atopic disorders has been reported in industrialized countries; according to the hygiene hypothesis, the increase in the incidence of patients may be explained by a better lifestyle and less exposure to environmental microbes [5, 7, 28]. Environmental microbes such as mycobacteria or certain virus may promote Th1-type immune response and thus reducing atopy-associated Th2-type reaction. For instance, the study

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carried out in Japanese *Bacillus Calmette-Güerin* (BCG)-vaccinated school children showed that responders to tuberculin had a lower prevalence of atopic disease compared to tuberculin non-responders [28]. BCG-treated mice showed suppression of experimental allergic responses [12]. More recently, it has been shown that microbial stimulation can induce regulatory T (Treg) cells with the ability to suppress both Th1-type and Th2-type inflammation [35]. In the experimental model of pulmonary inflammation, *Mycobacterium vaccae* reduces allergic pulmonary inflammation significantly by increasing the number of Treg cells that secretes IL-10 and TGF-β[37]. These observations indicate that shift from Th2 to Th1 type immune response by mycobacteria may be used for the prevention and treatment of atopic disorders.

The specific antigens eliciting Th1-type immune responses in mycobacteria have not been elucidated so far; a recent study suggested that one of the specific proteins for Th1 development is antigen 85B (Ag85B) [31]. Ag85B is a 30-kDa major protein secreted from all *Mycobacterium* species and that belongs to the Ag85 family[4]. The Ag85B can induce a strong Th1-type immune response in mice as well as in humans [31], and DNA vaccines encoding Ag85B have been reported to protect animals from tuberculosis infection by inducing Th1 response [34, 36]. We have previously reported enhancement of anti-tumor specific CTL response using Ag85B-transfected tumor cells, and by inducing Th1-type immune responses as a vaccine adjuvant [22, 30].

The purpose of the present study was to evaluate the therapeutic efficacy of Ag85B derived from *M. kansasii* in acute phase dermatitis. Repeated applications of hapten such as oxazolone (OX) on BALB/c mice causes delayed type hypersensitivity in the beginning that changes to an immediate-type response in the late phases with elevated IgE production, and deviation of Th cell responses. The skin lesions that appear in late phases are compatible with the clinical findings as well as cytokine profile observed in AD [19, 21]. In all Ag85B-treated AD mice, the immediate type reaction is effectively suppressed and IL-4 is significantly reduced. The results of this study provide evidence for the potential usefulness of Ag85B as a novel approach for the treatment of Th2 type-mediated dermatitis such as AD.

Materials and methods

Animals

Six-week-old BALB/c male mice were purchased from Japan SLC Co. (Shizuoka, Japan) and used at the age of 7 weeks. Animal care was done according to ethical guidelines, and approved by the Institutional Board Committee for Animal Care and Use of Mie University.

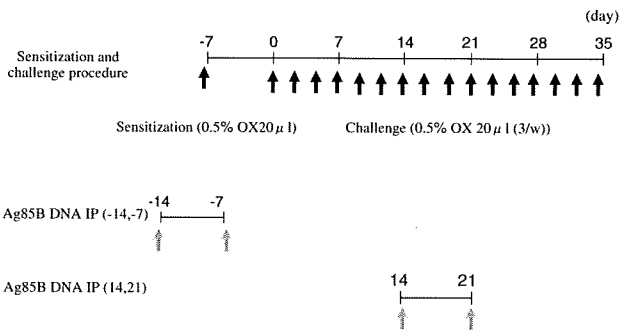

Fig. 1 Model of chronic contact hypersensitivity, and treatment with Ag85B DNA

Sensitization and challenge of animals

Oxazolone was purchased from Sigma (St Louis, MO, USA), and dissolved in acetone/olive oil (1:1). As shown in Fig. 1, mice were initially sensitized by pasting 20 μl of 0.5% OX solution to their left ear 7 days prior to the first challenge (day -7) and then 20 μl of 0.5% OX solution was repeatedly applied on the left ear three times per week from day 0. The ear swelling response was expressed as the difference between before and 30 min after application. The Ag85B expression vector pcDNA-Ag85B of *M. kansasii* open reading frame lacking a signal sequence has been constructed into KpnI–ApaI sites of pcDNA3.1 as described previously [22]. Plasmid DNAs were purified using the Plasmid Mega Kit (Qiagen, Chatsworth, CA, USA). The empty plasmid pcDNA3.1 was used as a control. Plasmid DNAs were diluted with sterilized physiological saline. Hundred micrograms per mouse of plasmid DNA was injected intraperitoneally on day -14, -7 to evaluate prophylactic effects, or on day 14 and 21 for the assessment of therapeutic effects.

Histological analysis

Skin specimens obtained 30 min after the final challenge were fixed in 10% buffered neutral formaldehyde and embedded in paraffin. Sections prepared of 7 μm thickness were stained with hematoxylin and eosin (H&E), or truidine blue.

Immunohistochemistry

The left ear was sacrificed on day 35, and was embedded in Tissue-Tek OCT compound (Miles, Elkhart, USA), frozen in liquid nitrogen, and cut with a cryostat into 7 μm-thick sections. The tissue preparations were then incubated with