

Identification of novel HLA-A*0201-restricted CD8⁺ T-cell epitopes on hepatitis delta virus

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Hepatitis delta virus (HDV) superinfection causes a poor prognosis in hepatitis B virus-infected patients and effective therapy is lacking. Cytotoxic T-lymphocyte (CTL) responses play an important role in the pathogenesis of chronic viral hepatitis; however, the CD8⁺ T-cell epitopes of HDV have never been defined. Potential HLA-A*0201-restricted HDV peptides were selected from the SYFPEITHI database and screened by T2 cell-stabilization assay. HLA-A*0201 transgenic mice on a C57BL/6 background were injected intramuscularly with an HDV DNA vaccine. Splenocytes were stained directly *ex vivo* with HLA-A*0201-peptide tetramers after immunization. Epitope-specific CTL responses were confirmed by cytotoxic assays. HLA-A2, chronically infected HDV patients were also enrolled, to assess the existence of HDV-specific CD8⁺ T cells, based on findings in animals. Following HDV DNA vaccination, nearly 0.9% of the total splenic CD8⁺ T cells were specific for peptides HDV 26–34 and HDV 43–51 in HLA-A*0201 transgenic mice, which was significantly higher than the number found in non-transgenic mice or in transgenic mice that had been immunized with control plasmid. HDV 26–34- and 43–51-specific CTL lines were able to produce CTL responses to each peptide. Interestingly, HDV 26–34- and HDV 43–51-specific CD8⁺ T cells were also detectable in two chronically infected HDV patients in the absence of active HDV replication. In conclusion, HDV 26–34 and 43–51 are novel HLA-A*0201-restricted CTL epitopes on genotype I HDV. HDV 26–34- and 43–51-specific CTLs have been detected in chronic hepatitis delta patients without active disease. Evoking CTL responses to HDV may be an alternative approach to controlling HDV viraemia in patients with chronic hepatitis delta.

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INTRODUCTION

Hepatitis delta virus (HDV), a defective, 1.7 kbp, single-stranded RNA virus, needs the envelope of hepatitis B virus (HBV) for assembly and transmission (Rizzetto *et al.*, 1980; Wu *et al.*, 1991). Superinfection of HDV in HBV carriers can lead to fulminant hepatitis (Wu *et al.*, 1994). Most patients progress to chronic hepatitis, becoming a group at high risk of cirrhosis and hepatocellular carcinoma (Govindarajan *et al.*, 1986; Wu *et al.*, 1995a). The effect of interferon or lamivudine in the treatment of chronic hepatitis delta (CHD) is transient and a satisfactory treatment regimen is unavailable (Farci *et al.*, 1994; Lau *et al.*, 1999). It is necessary to develop an alternative approach to save these patients from unfavourable outcomes.

CD8⁺ cytotoxic T lymphocytes (CTLs) play a key role in immunity against intracellular pathogens. In HBV and hepatitis C virus (HCV) infection, multi-specific CD8⁺ T-cell responses to HBV and HCV are related strongly to virus clearance (Chisari & Ferrari, 1995; Maini *et al.*, 2000; Thimme *et al.*, 2001); however, the pathogenic mechanism of chronic HDV infection is undetermined. HDV transgenic mice that express hepatitis delta antigen (HDAg) in the liver do not develop liver damage, providing evidence that HDV is not a cytopathic virus (Guillhot *et al.*, 1994). The facts that a high HDV load was detected in HIV-infected CHD patients whose circulating T-cell numbers were suppressed and that activity of HDV-induced liver disease is related to CD4⁺ T-cell response to HDV suggest that

immune mechanisms play a significant role in chronic HDV infection (Roingard *et al.*, 1992; Nisini *et al.*, 1997). Strategies to induce an HDV-specific CTL response are promising ways to control chronic HDV infection.

HDV has two forms of viral proteins: large and small HDAGs (Casey & Gerin, 1995). These two antigens are identical in sequence, except that the large HDAG (L-HDAG) contains an additional 19 aa at the C-terminus compared to the small HDAG (S-HDAG). The immunogenic domains of HDAG, recognized by anti-HDV antibodies derived from chronic HDV-infected patients, include aa 2–7, 63–74, 86–91, 94–100, 159–172, 174–195 and 197–207 (Wang *et al.*, 1990). Epitopes of HDAG that are recognized by CD4⁺ T-cells of HDV-infected patients are aa 26–41, 50–65, 66–81 and 106–121 (Nisini *et al.*, 1997). The CD8⁺ T-cell epitopes on HDAG have never been defined; however, the S-HDAG C-terminus, aa 77–195, has been suggested to contain possible epitopes (Karayiannis *et al.*, 1993). Identification of the CTL epitopes on HDV is crucial work in vaccine design and study of HDV pathogenesis.

A prophylactic or therapeutic HDV vaccine has potential use for HBV carriers who are at risk of HDV superinfection and for CHD patients. Our previous study demonstrated that HDV DNA vaccines can produce a T helper (Th) 1 immune response and that cellular immunity can be generated by DNA vaccines that encode L- or S-HDAG (Huang *et al.*, 2000, 2003). CTL response against HDAG can also be induced by DNA vaccines in mice (Mauch *et al.*, 2001). HLA-A*0201 transgenic mice provide a useful model for characterizing CTL epitopes of HLA-restricted viral antigen and evaluating CTL responses. In this study, we used an HDV DNA vaccine in HLA-A*0201 transgenic mice and identified novel CTL epitopes that are restricted by the HLA-A*0201 molecule on HDAG. HDV-specific CTLs were also detected in two chronically infected HDV patients without active disease.

METHODS

Plasmids. The L-HDAG gene was amplified by PCR with pairs of primers ($\delta x25$: 5'-GGCTCTAGAGTAAGAGTACTGAGG-3' and $\delta EcoRV$: 5'-ATGATATCCCACCCGAAGAG-3') from plasmid TWD2667 (GenBank accession no. AF104263) that contained the HDV coding region (genotype I) in the PCR II vector, and was cloned into the *XbaI*-*EcoRV* sites in plasmid pcDNA3.1(-) (Invitrogen) to produce plasmid p2667L. Expression of HDAG by the plasmid was reported previously (Huang *et al.*, 2000, 2003). Plasmid pcDNA3.1(-) was used as a negative control. Plasmid DNA was purified from transformed *Escherichia coli* DH5 α (Gibco-BRL) by using a Qiagen Giga plasmid purification kit.

Screening for HLA-A*0201-restricted peptides. Twelve potentially HLA-A*0201-restricted peptides from sequence TWD2667 (GenBank accession no. AF104263) were selected by using the SYFPEITHI database (Table 1) (<http://www.syfpeithi.de/>; Rammensee *et al.*, 1999). One HBV core peptide, aa 18–27 (FLPSDFPFI, a common sequence for HBV genotypes B and C), which is known to be capable of binding to HLA-A*0201, was used as a positive control. All peptides were synthesized commercially by Sigma-Genosys.

Table 1. Sequences of peptides used in this study

Peptide	Sequence	SYFPEITHI score
Known HLA-A*0201-restrictive peptide:		
HBV core 18–27	FLPSDFPFI	22
High-score HLA-A*0201-restrictive peptides:		
HDV 26–34	KLEDLERDL	22
HDV 29–37	DLERDLRKI	22
HDV 36–44	KIKKKIKKL	24
HDV 43–51	KLEDENPWL	22
HDV 107–115	ALENKRKQL	21
HDV 114–122	QLAAGGKHL	23
HDV 121–130	HLSKEEEEEEL	22
HDV 143–152	RTAGPSVGGV	23
Medium-score HLA-A*0201-restrictive peptides:		
HDV 165–174	GGFVNPMLSV	15
HDV 173–182	SVPESPFRT	12
HDV 177–186	SPFSRTGEGEGL	14
HDV 180–188	SRTGEGLDV	14

T2 cell-stabilization assay. T2 cells (ATCC, Manassas, VA, USA) produce the HLA-A*0201 gene, but are unable to present endogenous antigens. A T2 cell-stabilization assay was performed as described previously with some modification (Matsumura *et al.*, 2002). T2 cells were seeded in 96-well U-bottom plates at a density of 3×10^5 ml⁻¹ and incubated for 18 h with each peptide (80 μ g ml⁻¹) at 26 °C. After incubation, brefeldin A was added to a final concentration of 10 μ g ml⁻¹, followed by incubation at 37 °C for 3 h. The cells were washed and stained with fluorescein isothiocyanate-labelled mouse anti-human HLA-A2 antibody (clone: BB7.2; Pharmingen). Samples were run on a FACScan flow cytometer and the data were analysed by using CellQuest software (Becton Dickinson Immunocytometry System). Binding activity of each peptide was calculated by a fluorescence ratio (mean fluorescence of T2 cells loaded with peptide:mean fluorescence of T2 cells without peptide). According to the results of the T2 assay, HLA-A*0201-peptide tetramers were synthesized commercially by Proimmune (Altman *et al.*, 1996). These tetramers had been evaluated in three healthy, HBV-negative volunteers. Background signals were <0.03 % of total CD8⁺ T cells for all tetramers.

Target cells. A human B lymphoblastoid cell line (B-LCL) that carries the HLA-A2 molecule (wu-LCL) and T2 cells were used as target cells. Another B-LCL (fmc-LCL) that carries the HLA-A11 molecule served as a control. HLA phenotypes were classified by using Micro SSP HLA DNA typing trays (One Lambda).

Animal model and immunization. C57BL/6-TgN (HLA-A2.1)1Enge transgenic mice (Jackson Laboratory, ME, USA) were used in this study. These homozygous mice had previously been used to predict HCV CTL epitopes (Shirai *et al.*, 1995). Control C57BL/6 mice were obtained from the National Laboratory for Animal Breeding and Research Center, Taipei, Taiwan. Mice were housed at the SPF room, Laboratory Animal Facility, Taipei Veterans General Hospital. Animals received humane care and the protocol was approved by the Animal Committee of Taipei Veterans General Hospital. Groups of four mice were immunized with DNA at 8–10 weeks of age. Mice were anaesthetized and given intramuscular injections into the bilateral quadriceps with a total dose of 100 μ g plasmid DNA dissolved in 100 μ l sterilized normal saline. All mice received an injection of cardiotoxin (Sigma) 1 week before DNA immunization. Each mouse was given booster doses at 3 and 6 weeks after the first immunization. Mice were immunized as

follows: group 1 (HLA-A2.1 transgenic mice) with p2667L; group 2 (HLA-A2.1 transgenic mice) with pcDNA3.1(-); and group 3 (C57BL/6 mice) with p2667L. Mice were sacrificed 2 weeks after the last immunization. Splenocytes from immunized mice were stained directly *ex vivo* with HLA-A*0201-peptide tetramers and rat anti-mouse CD8 antibody (PharMingen). Samples were run on a FACScan flow cytometer and the data were analysed by using CellQuest software (Becton Dickinson). Animal experiments were repeated twice to verify their validation.

Induction of peptide-specific CTL lines. Spleen cells from immunized mice were suspended in RPMI medium that contained 10% fetal bovine serum. For stimulation *in vitro*, red blood cell-depleted spleen cells ($5 \times 10^6 \text{ ml}^{-1}$) were mixed with each of the HDV peptides ($0.1 \mu\text{g ml}^{-1}$) and incubated at 37°C for 2 days. Splenocytes were transferred to anti-mouse CD3 antibody-coated dishes in the presence of recombinant mouse interleukin 2 (IL2, 10 U ml^{-1}) and anti-mouse CD28 antibody, then incubated for another 10 days. Splenocytes were restimulated with each of the peptides and mouse IL2 for 10 days. CTL lines were confirmed by staining with HLA-A*0201-peptide tetramers.

Cytotoxic assays. CTL lines were evaluated for their cytotoxic response in the presence of specific peptides ($1 \mu\text{g ml}^{-1}$) and target cells ($5 \times 10^4 \text{ ml}^{-1}$, $100 \mu\text{l}$). A 4 h ^{51}Cr -release assay was performed in 96-well V-bottom plates by using ^{51}Cr -labelled target cells. The percentage of specific lysis was calculated by the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release). Experimental release represented mean counts min^{-1} released by target cells in the presence of effector cells. Total release represented the radioactivity released after lysis of target cells with 1% Triton X-100. Spontaneous release represented the radioactivity counted in medium derived from target cells alone.

Chronic HDV-infected patients and peripheral blood mononuclear cell (PBMC) preparation. Nine chronically infected HDV patients were screened for their HLA phenotypes. Among them, four patients who expressed HLA-A2 phenotype were found and these were recruited in this study. The study was approved by the Institutional Review Board of Taipei Veterans General Hospital. All four patients tested positive for serum HBsAg and anti-HDV antibodies by using radioimmunoassay kits (Abbott Laboratories) and tested negative for anti-HCV antibodies. Two patients had abnormal serum alanine transaminase (ALT) levels and were chronically positive for HDV RNA. The other two patients, who had been positive for HDV RNA in the past, but whose serum ALT levels had become normal and whose HDV RNA was undetectable since 1996, were defined as being in disease remission. PBMCs were purified from venous blood by using Ficoll-Histopaque gradient centrifugation techniques. PBMCs ($2 \times 10^6 \text{ ml}^{-1}$) were stimulated with human anti-CD3 antibody in the presence of recombinant human IL2 (50 U ml^{-1}) and incubated in RPMI 1640 medium (Gibco) for 7 days. The expanded PBMCs were used for HLA-A*0201-peptide tetramer and mouse anti-human CD8 antibody (Serotec) staining. Stimulated PBMCs from patient 1 were further restimulated with cognate peptide (HBV core 18-27, HDV 26-34 or HDV 43-51) at $0.1 \mu\text{g ml}^{-1}$ for 7 days to derive short-term lines. The PBMCs from three non-HLA-A2 chronically HDV-infected patients (two HLA-A11 and one HLA-A24) and two healthy individuals who were positive for hepatitis B surface antibodies (anti-HBs Ab) after successful HBV vaccinations served as controls. Control PBMCs were stimulated with human anti-CD3 antibody and IL2 and stained under the same conditions as described above.

HBV DNA and HDV RNA detection. HBV DNA was quantified by using a Cobas Amplicor HBV monitor (Roche). The detection limit of this assay was $200 \text{ copies ml}^{-1}$. Serum HDV RNA was

detected by RT-PCR and sequenced as described previously (Wu *et al.*, 1994). HBV and HDV genotypes were classified by PCR-RFLP as reported previously (Mizokami *et al.*, 1999; Wu *et al.*, 1995b).

ELISPOT assay for gamma interferon (IFN- γ). A human IFN- γ ELISPOT assay kit (R&D Systems) was used to determine whether the stimulated PBMCs were functional, by following the manufacturer's protocol. To summarize, stimulated PBMCs were washed and restimulated with the cognate peptide (HBV core 18-27, HDV 26-34 or HDV 43-51) at $0.1 \mu\text{g ml}^{-1}$. All of the PBMCs (2×10^4 in $100 \mu\text{l}$ medium) were pipetted into the wells and incubated at 37°C overnight. Biotinylated polyclonal antibody that was specific for human IFN- γ was added, followed by alkaline phosphatase conjugated to streptavidin. 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride was used as substrate. Images of spots were captured by using a dissection microscope, then counted using ImageMaster TotalLab v1.10 software (Amersham Biosciences) (Huang *et al.*, 2003). The number of specific spot-forming cells (SFCs) was determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells with medium only.

Statistical analysis. The Mann-Whitney rank sum test was used to compare the results between the groups. A *P* value of <0.05 was considered to be significant.

RESULTS

T2 cell-stabilization assay

As shown in Fig. 1, the fluorescence ratios for HBV core 18-27 and HDV 43-51 increased twofold compared to baseline, suggesting that these two peptides had a strong affinity with the HLA-A*0201 molecule. The fluorescence

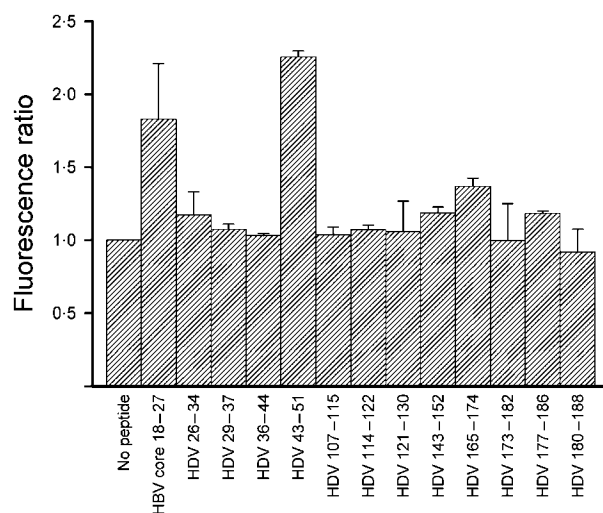


Fig. 1. T2 cell-stabilization assay. HLA-A*0201-binding activity of peptides was assessed by the T2 cell-stabilization assay. HBV core 18-27, a previously defined HLA-A*0201-restricted CTL epitope, was used as the control. Fluorescence ratio was calculated by (mean fluorescence of T2 cells loaded with peptide)/(mean fluorescence of T2 cells without peptide). Values show the mean \pm SD for three independent experiments.

ratios of peptides HDV 26–34, 143–152, 165–174 and 177–186 were also 10% higher than baseline.

Direct HLA-A*0201–peptide tetramer complex staining *ex vivo*

One HBV peptide core, 18–27, and six HDV peptides, 26–34, 43–51, 107–115, 114–122, 143–152 and 165–174, were selected to construct HLA-A*0201–peptide tetramer complexes (namely Tc 18–27, Td 26–34, Td 43–51, Td 107–115, Td 114–122, Td 143–152 and Td 165–174, respectively), following the results of the T2 cell-stabilization assay and SYFPEITHI scores. Peptides HDV 107–115 and 114–125 were chosen as they are within a known HDV major histocompatibility complex (MHC) class II-restricted T-cell epitope (Accapezzato *et al.*, 1998). HDV 177–186 was not able to form an HLA-A*0201–peptide tetramer, due to instability.

Two weeks after the last immunization, pooled spleen cells from groups of mice were stained directly *ex vivo* with HLA-A*0201–peptide tetrameric complexes. As shown in Fig. 2, five of the seven tetramers gave background levels, but two showed a significantly enhanced response in HLA-A2.1 transgenic mice after HDV DNA vaccination. Tetramer-positive CD8⁺ T cells accounted for 0.77 ± 0.37 and 0.94 ± 0.09 % of total CD8⁺ T cells for Td 26–34 and Td 43–51, respectively, in HLA-A2.1 transgenic mice immunized with p2667L. These numbers were significantly higher than those detected in the control groups ($P=0.029$ by Mann–Whitney rank sum test). Nearly 0.9% of CD8⁺ T cells in HLA-A*0201 transgenic mice were specific for HDV peptides 26–34 and 43–51 after DNA immunization.

Peptide-stimulated CTL lines trigger HDV-specific cytotoxicity

Spleen cells from immunized mice were stimulated *in vitro* to generate CTL lines as described in Methods. The HDV 26–34- and HDV 43–51-specific CTL lines were used as effector cells. T2 cells and wu-LCL, which express HLA-A2

molecules, served as target cells. Another cell line, fmc-LCL, which expresses HLA-A11 and an HLA-A*0201-restricted peptide, HBV core 18–27, were used as controls. As shown in Fig. 3, the CTL lines specifically lysed HLA-A2-expressing target cells in the presence of HDV 26–34 (Fig. 3a) or HDV 43–51 (Fig. 3b). There was no lytic effect for peptides HDV 26–34, HDV 43–51 or HBc 18–27 in the fmc-LCL cell line. These findings confirmed that both CTL lines could target HDV peptides 26–34 and 43–51 specifically.

HLA-A2 HDV chronically infected patients

Four HLA-A2 chronically infected hepatitis delta patients were enrolled to determine whether HDV epitope-specific CTLs exist in humans (Table 2). All patients were positive for anti-HDV. Two patients (patients 1 and 2) were defined as being in remission, with loss of HDV RNA and persisting normal serum ALT levels after several years of chronic hepatitis. The stored sera of these two patients from 1991 and 1992 were available for detection of HDV RNA and sequencing. The other two patients (patients 3 and 4) had active liver disease and were positive for HDV RNA, with persisting abnormal ALT levels (greater than two times the upper limit of normal). HDV sequences that were isolated from patients 1–3 belonged to genotype I and the sequence isolated from patient 4 belonged to genotype IIa (Fig. 4). PBMCs were stimulated with human anti-CD3 antibody and human IL2 for 7 days. The stimulated cells were stained with HLA-A*0201–peptide tetramer complexes. As shown in Fig. 5a, significant frequencies of Td 26–34- and Td 43–51-positive CD8⁺ T cells were detected in patients 1 and 2, whose disease was in remission. In addition, Tc 18–27-positive CD8⁺ T cells were also detected in patient 1. This finding confirmed that the epitope-specific CTLs identified above could be detected in HDV-infected patients. Tc 18–27-, Td 26–34- or Td 43–51-positive CD8⁺ T cells ranged from 0.01 to 0.02% of the total CD8⁺ T cells in patients 3 and 4 (Fig. 5b). Td 107–115-, 114–122-, 143–152- and 165–174-positive cells were all <0.03% of total CD8⁺ T cells among the four patients. Staining of stimulated

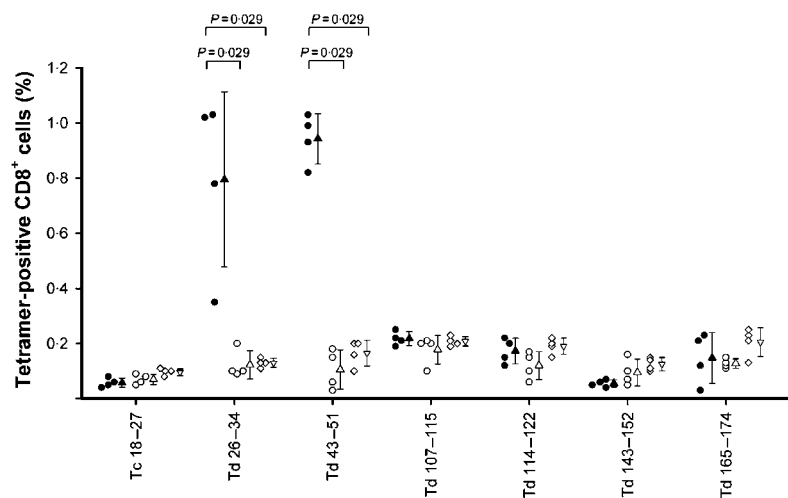


Fig. 2. Direct tetramer staining *ex vivo*. HLA-A*0201–peptide tetramer complexes were used to stain splenocytes from HLA-A2.1 transgenic mice that had been immunized with p2667L (●) or pcDNA3.1(-) (○), or C57BL/6 mice that had been immunized with p2667L (◇). Tc 18–27 was used as a control. Values are frequencies (%) of tetramer-positive cells in total CD8⁺ T cells. Frequencies of Td 26–34- and Td 43–51-positive CD8⁺ T cells were significantly higher in HLA-A2.1 transgenic mice that had been immunized with p2667L.

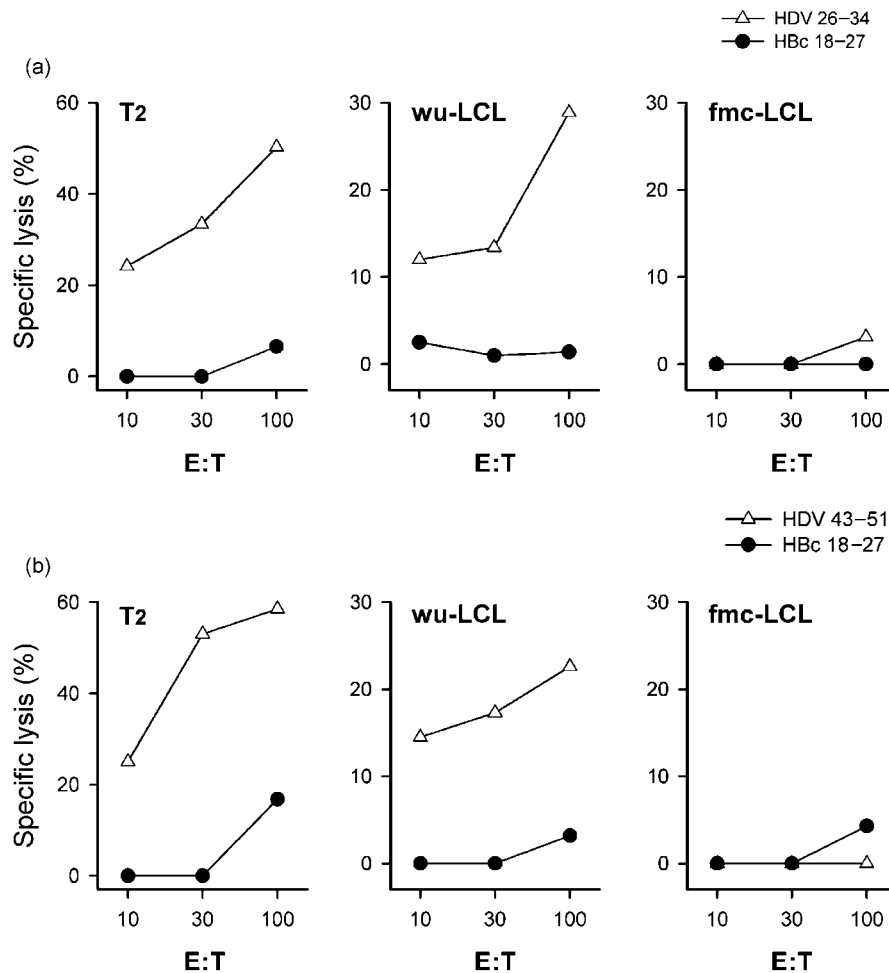


Fig. 3. ^{51}Cr -release assays. Cytotoxic assay by (a) HDV 26–34- or (b) HDV 43–51-specific CTL lines (Δ). HBV core 18–27 (\bullet), a known HLA-A*0201-restricted CTL epitope, was used as control. ^{51}Cr -labelled T2 cells, wu-LCL cells and fmc-LCL cells were pulsed with HDV peptides or HBV core 18–27 and incubated with CTL lines at each effector : target (E : T) ratio in a standard ^{51}Cr -release assay.

PBMCs from three non-HLA-A2 chronically infected HDV patients and one healthy control are shown in Fig. 5(c, d). Frequencies of tetramer-positive cells were all below the background levels found in the controls. Data for another HBV-vaccinated healthy control are not shown, as the results were similar to those in Fig. 5(d). We restimulated PBMCs from patient 1 with cognate peptide (HBV core 18–28, HDV 26–34 or HDV 43–51) for 7 days. As shown in

Fig. 5(e), the frequencies of peptide-specific CD8^+ T cells increased after peptide stimulation.

ELISPOT assay

To determine whether the stimulated PBMCs were functional, an ELISPOT assay for $\text{IFN-}\gamma$ was performed. Stimulated PBMCs from patient 1 were restimulated with

Table 2. Demographic data of HLA-A2 chronically infected HDV patients

Patient	Age (years)	Sex	Serum ALT (U l^{-1})	HDV RNA (by RT-PCR)	HBV DNA (copies ml^{-1})	HBV/HDV genotype
1	61	M	30	Negative	<200	B/I
2	59	M	25	Negative	<200	B/I
3	27	F	207	Positive	5.36×10^6	C/I
4	67	M	161	Positive	<200	C/IIa

	11	21	31	41	51
Patient 1	DGREGILEQW	VNGRKKLEDL	<u>ERDLR</u> IKKKK	IKKLEDENPW	LGNIKGILGK
Patient 2	---E---	-----	-----V---	-----	-----
Patient 3	-----	-----	-----	-----	-----
	12	22	32	42	52
Patient 4	GT--E---KW	ITT--- <u>A-E-</u>	<u>-K-</u> ---AR-T	V- <u>R--E-</u> ---	---L--IR-

Fig. 4. Partial HDV sequences of four HLA-A2 chronic HDV infection patients. HDV sequences of patients 1 and 2 were isolated from sera stored in 1991 and 1992, respectively. HDV sequences isolated from patients 1 to 3 belong to genotype I, and the sequence isolated from patient 4 belongs to genotype IIa HDV. HDV CTL epitopes identified in this study are underlined.

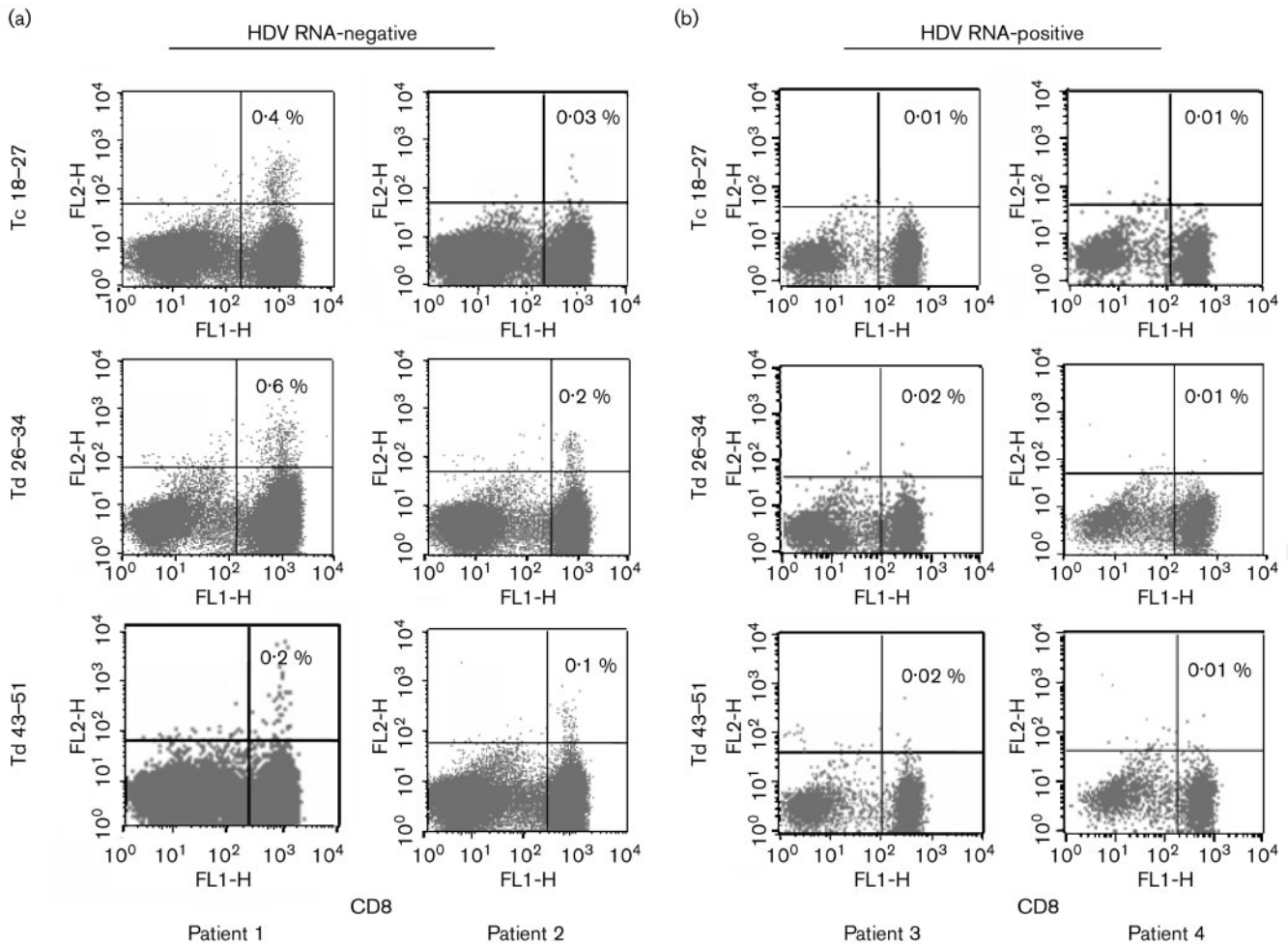
HBV core 18–27, HDV 26–34 or HDV 43–51. The number of peptide-specific SFCs was calculated by subtracting the number of spots in the absence of antigen from those in the presence of antigen. As shown in Fig. 6, the mean numbers of IFN- γ -positive SFCs against HBV core 18–27, HDV 26–34 and HDV 43–51 from triplicate studies were 87 ± 3 , 82 ± 4 and 50 ± 5 per 2×10^4 PBMCs. These findings were concordant with the results of tetramer staining, shown in Fig. 5(e).

DISCUSSION

Virus-specific CTLs play a major role in the clearance of intracellular pathogens. DNA vaccines can induce a broad

range of immune responses and currently show promise for prevention and treatment of persistent viral infections (Martins *et al.*, 1995). HDV DNA vaccines can generate CTL responses in mice (Huang *et al.*, 2000, 2003; Mauch *et al.*, 2001), but further antiviral approaches are needed before application in humans. Identification of the CTL epitopes on HDV is important for the development of T-cell vaccines.

The large form (L-HDAg) plasmid construct, instead of the small form, was adopted in this study as L-HDAg encompasses the whole 214 aa length of HDAg. HDAg is a nuclear protein that is usually detected in the nuclei of infected hepatocytes in chronically infected hepatitis delta



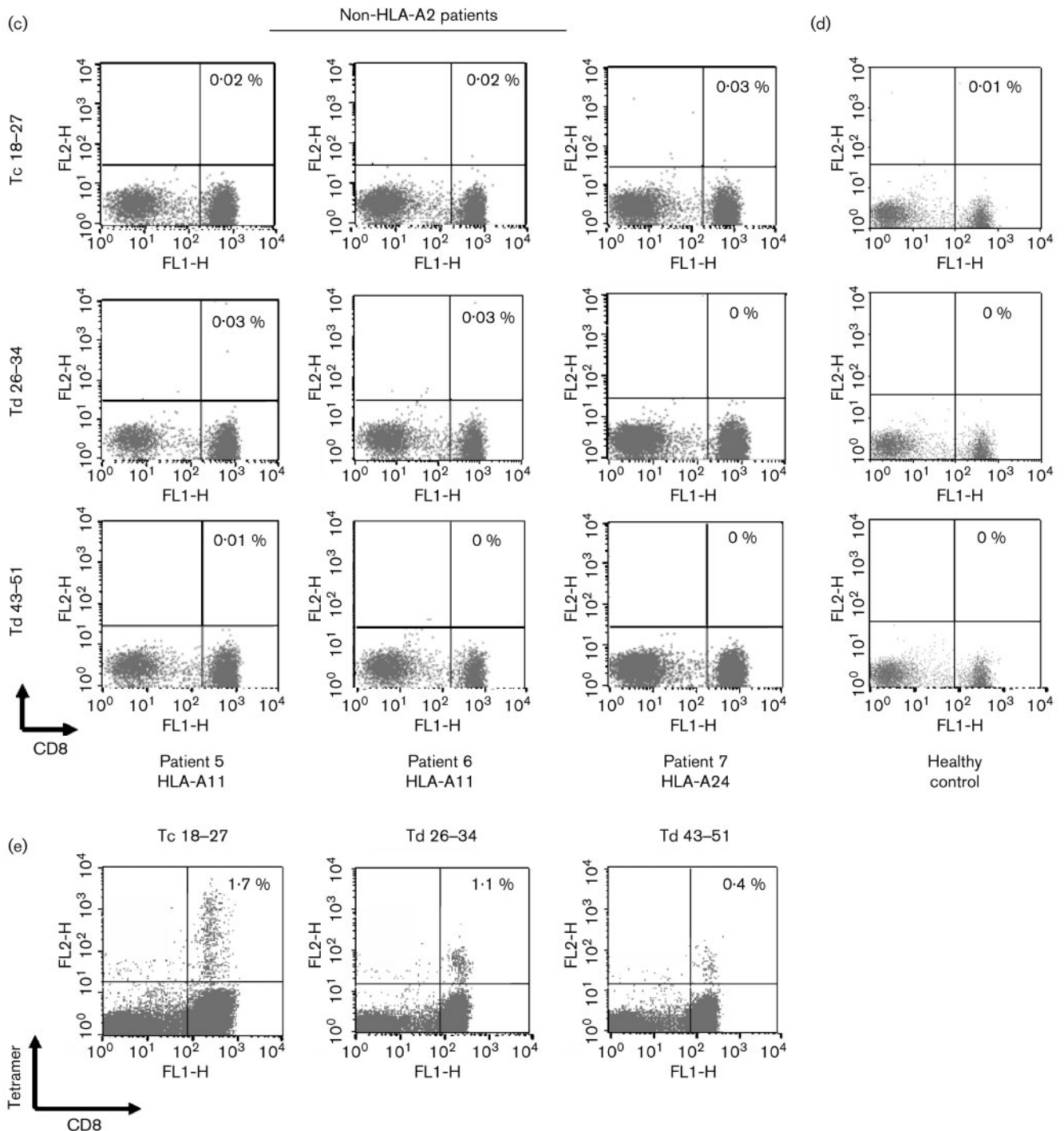


Fig. 5. Frequencies of tetramer-positive CD8⁺ T cells in PBMCs of HLA-A2 and non-HLA-A2 chronically infected HDV patients and a healthy control. (a) Patients 1 and 2 were negative for HDV RNA. (b) Patients 3 and 4 were positive for HDV RNA. (c) Patients 5–7 were HLA-A11 or HLA-A24. (d) The healthy control was positive for anti-HBs after HBV vaccination. (e) Frequencies of tetramer-positive CD8⁺ T cells in PBMCs from patient 1 after stimulation *in vitro* with cognate peptides for 7 days. In total, 50 000 cells were analysed and the frequency of tetramer-positive cells was calculated. Values represent the percentage of tetramer-positive CD8⁺ T cells in total CD8⁺ T cells.

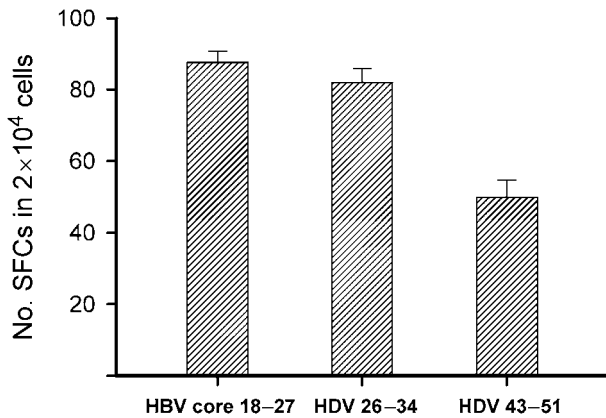


Fig. 6. IFN- γ ELISPOT assay for quantification of cytotoxic responses in patient 1. Stimulated PBMCs were restimulated with peptides HBV core 18-27, HDV 26-34 or HDV 43-51. The number of peptide-specific SFCs in 2×10^4 cells was calculated by subtracting the number of spots in the absence of antigen from that in the presence of antigen.

patients (Chang *et al.*, 1988). Our findings suggest that HDAg can be processed in the cytoplasm and the resultant peptides presented on the surface of infected cells for recognition by CD8⁺ T cells.

It is recognized that the HLA-A*0201-restricted peptide-binding motif has unique properties, with leucine, isoleucine or methionine at position 2 and valine or a residue with an aliphatic hydrocarbon side chain at the C-terminus (Falk *et al.*, 1991; Hunt *et al.*, 1992). We selected the potential HLA-A*0201-restricted peptides in HDAg for analysis by using the SYFPEITHI database (Rammensee *et al.*, 1999). It is worth noting that not every peptide with a high SYFPEITHI score had binding affinity for T2 cells. In the T2 cell-stabilization assay, HDV 26-34 had a mean fluorescence ratio of 1.17, but is an HLA-A*0201-restricted peptide. Both the SYFPEITHI database and T2 cell-stabilization assay can serve as screening tools, but a further confirmation study is needed to identify HLA-A*0201-restricted peptides. In addition, we should point out that our approach for initial screening may have overlooked some epitopes, as the SYFPEITHI database and the T2 cell-stabilization assay are not perfect screening methods.

Recently, HHD mice (which are deficient for the β 2-microglobulin gene and mouse MHC class I H-2D^b molecules) have been reported to facilitate identification and characterization of HLA class I-restricted virus T-cell epitopes (Pascolo *et al.*, 1997). In this study, even though C57BL/6-TgN(HLA-A2.1)1Enge transgenic mice were not devoid of H-2 class I molecules, we identified two novel HLA-A*0201-restricted epitopes in HDAg. According to the results in non-transgenic C57BL/6 mice and the control plasmid group, the two epitopes that were identified in HLA-A2.1 transgenic mice were confirmed as being

HLA-A2.1-specific. Previous studies suggested that HDAg CTL epitopes are located within the S-HDAg C-terminus (Karayiannis *et al.*, 1993), but the eight peptides chosen within the region were not the candidate HLA-A*0201-restricted CTL epitopes. We cannot exclude the possibility that other CTL epitopes, in addition to the peptides studied, exist in HDAg; further studies in HHD mice are needed for clarification.

HLA-A*0201-peptide tetramers have been used to detect the frequency of epitope-specific CD8⁺ T cells in this study. About 0.9% of total CD8⁺ T cells are specific for epitopes HDV 26-34 and 43-51 following DNA immunization. This finding corroborates our recent report on BALB/c mice that showed that HDV-specific, IFN- γ -secreting CD8⁺ splenocytes numbered approximately 0.9% of the total after DNA-based immunization (Huang *et al.*, 2003). After stimulation *in vitro*, both CTL lines were able to trigger specific CTL responses to peptides HDV 26-34 or HDV 43-51.

The two HLA-A*0201-restricted HDV epitopes that are targeted by the HDV DNA vaccine were identified in mice. We wondered whether these epitope-specific CTLs existed in HLA-A2 HDV-infected patients. Interestingly, of the four patients studied, two had HDV-specific CTLs detected by HLA-A*0201 tetramers. For patient 1, the frequencies of peptide-specific CD8⁺ T cells increased significantly after peptide stimulation. ELISPOT assays also confirmed that those cells were functional and cytotoxic to HBV and HDV. It is convincing that both epitopes are potentially HLA-A*0201-restricted human HDV epitopes. So far, the pathogenesis of chronic HDV infection is unclear. Dual HBV and HDV infection adds complications to the disease. In chronic HBV infection, an effective HBV-specific CD8⁺ T-cell response can inhibit virus replication (Maini *et al.*, 2000). Evidence shows that the frequencies of HBV core 18-27-specific CD8⁺ T cells are lower in most chronically infected hepatitis B patients with higher viral load than in patients controlling the virus (Maini *et al.*, 2000). Coincidentally, we detected HDV 26-34- and 43-51-specific CTLs in two chronically infected HDV patients without HDV viraemia. From the clinical data, patients 1 and 2 both had persistently normal serum ALT levels and undetectable HDV RNA levels by RT-PCR for at least 3 years. HDV sequences isolated from the stored sera of the two patients from 10 years previously were identical to those of Td 26-34 and Td 43-51. In addition, not only HDV-, but also HBV-specific CTLs were detectable in patient 1. In contrast, no significant amounts of HDV-specific CTLs were detected in patient 3, who had HBV and HDV viraemia and high serum ALT levels. In patient 4, the HBV load was lower than the detection limit of the assay kit. This HBV DNA may be undetectable, due to the suppression effect of HDV (Wu *et al.*, 1995a). The divergence in nucleotide sequences among different HDV genotypes ranges from 23 to 34% (Casey *et al.*, 1993; Wu *et al.*, 1995c). Sequence variation between genotype I and IIa HDV may have caused the

HDV-specific CD8⁺ T cells in patient 4 to be undetectable by the tetramers used, which were tailor-made for genotype I HDV. However, the data from patient 4 served as a background control for this study. Genotype I is the most prevalent genotype of HDV in the world and a previous report suggested that genotype I HDV had a worse prognosis than genotype II (Wu *et al.*, 1995b). There is an urgent need to identify CTL epitopes on genotype I HDV for vaccine design and antiviral therapy; consequently, we focused on genotype I HDV in this study. According to current knowledge on HBV and HCV, it is plausible that an effective HDV-specific CTL response might be related to HDV clearance. Coexistence of HBV- and HDV-specific CTLs is needed to control both viruses in patients with chronic hepatitis delta. If so, strategies to induce CTL responses against HDV epitopes 26–34 and 43–51 have the potential to treat chronic hepatitis delta. The data from this study also show that the presence of HDV-specific CTLs was coincidentally detectable in two chronically HDV-infected patients without HDV activity. Further study using a larger number of patients is needed to support this idea. However, this kind of study is not easy, due to the rapid decline in the patient population and the fact that only a minority of patients belong to genotype I HDV in Taiwan (Wu *et al.*, 1995b, 1998; Huo *et al.*, 1997).

In conclusion, HDV 26–34 and HDV 43–51 are novel HLA-A*0201-restricted CTL epitopes in genotype I HDV. HDV 26–34- and HDV 43–51-specific CTLs happen to be detected in chronically infected hepatitis delta patients without active disease. Evoking CTL responses to HDV by using an HDV DNA vaccine may be an alternative approach to controlling HDV viraemia in patients with chronic hepatitis delta.

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