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A mutant HBs antigen (HBsAg)183–191 epitope elicits specific cytotoxic T lymphocytes in acute hepatitis B patients

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Summary

HBs antigen (HBsAg)183–191 (FLLTRILTI, R187 peptide) is a dominant human leucocyte antigen-A2 (HLA-A2)-restricted epitope associated with hepatitis B virus (HBV) infection in Caucasian populations. However, its prevalence is poorly understood in China, where there is a high incidence of HBV infection. In this report, we sequenced the region of HBsAg derived from 103 Chinese patients. Approximately 16·5% of the patients bore a mutant HBsAg183–191 epitope in which the original arginine (R187) was substituted with a lysine (K187 mutant peptide). Importantly, K187 still bound to HLA-A2 with high affinity, and elicited specific cytotoxic T lymphocyte (CTL) responses in HLA-A2/K^b transgenic mice. K187-specific CTLs were also generated successfully in acute hepatitis B (AHB) patients, indicating that this mutant epitope is processed and presented effectively. Our findings show that R187-specific CTLs can cross-react with the K187 peptide. These findings reveal that K187 still has the property of an HLA-A2 restricted epitope, and elicits a protective anti-HBV CTL response in humans.

Keywords: cytotoxic T lymphocyte, epitope, hepatitis B virus, mutation

Introduction

Hepatitis B virus (HBV) is extensively epidemic in the world. Its infection can result in acute and chronic hepatitis, and greatly enhance the risk of liver cirrhosis and hepatocellular carcinoma [1]. Evidence suggests that a vigorous, multi-targeted cytotoxic T lymphocyte (CTL) response occurs during acute hepatitis B (AHB) [2–6], while the HBV-specific CTL response is very low or undetectable during chronic infection. Moreover, several reports have shown that CTL epitopes mutation abrogate recognition of HBV by prototype CTLs [7,8], which favour chronic infection by allowing viruses to evade host immunity [9,10]. These researches indicate that HBV-specific CTLs play a major role in the control and clearance of viral infection [11]. Thus, it is especially critical to identify dominant epitopes that will elicit strong CTL responses in HBV-infected patients. Although a number of epitopes in HBV antigens have been identified in European and northern American HBV patients [2,4,12–15], it remains unclear whether mutant epitopes still elicit CTL response *in vivo*. Theoretically, the T cell receptor (TCR) repertoire is large enough [16] that every viral mutation should be recognized by a specific TCR, thus allowing the host immune system to mount a novel response to mutant peptide-bearing viruses and control their replication. In addition, HBV has been classified into eight genotypes, A to H, according to the divergence of $\geq 8\%$ in the complete genome nucleotide sequence [17–20]. Clinical investigation has indicated that genotypes B and C are the most frequent in China [21,22], while other genotypes are more prevalent in Europe and North America [23–25]. Distinct distribution of the genotype may cause the sequence mutation of some originally identified epitopes in China. However, so far there has been a paucity of reports regarding epitope discrepancies in HBV-infected patients in China.

In this report we examined the prevalence of the HBV epitope, HBs antigen (HBsAg)183–191 (FLLTRILTI), which is a major human leucocyte antigen-A2 (HLA-A2)-restricted epitope [26]. Our results show that approximately 16·5% of HBV-infected patients have a mutation with an arginine to lysine switch at the fifth amino acid residue in the HBsAg183–191 (FLLTRILTI) epitope, K187. Importantly, subsequent identification showed K187 was still an HLA-A2-restricted epitope. Our findings may aid the design of more efficient HBV-specific epitope vaccines to boost anti-viral immune responses in chronic hepatitis B (CHB) patients.

Materials and methods

Patients

Nine HLA-A2-positive patients (three with AHB and six with CHB) were enrolled for analysis of HBV peptide-specific CTLs. All the patients met the diagnosis standards of AHB and CHB, as described previously [27,28]. Briefly, patients who display a serum HBsAg conversion within 6 months following onset of HBV infection are classified as AHB, while patients who have viral persistence for more than 6 months and typical manifestations of hepatitis or abnormal hepatic function are classified as CHB. Two HBV-uninfected healthy HLA-A2-positive individuals were used as controls for tetramer staining. One hundred and three samples with viral titres $>10^7$ copies/ml were chosen to investigate epitope sequence prevalence. Individuals with concurrent HCV, HDV, HGV or HIV infections or autoimmune liver disease were excluded from the study. Our protocol was approved by the Ethics Committee of Beijing 302 Hospital and written informed consent was obtained from each subject.

Tissue typing and peptide synthesis

The HLA-A2 haplotype was determined with fluorescein isothiocyanate (FITC)-conjugated HLA-A_2 monoantibody staining. The K187 mutant peptide and the H-K^d-restricted epitope, HBcAg87–95 (SYVNTNMGL) [29], were synthesized at GL Biochem Ltd (Shanghai, China). Peptide purity was >98%, as confirmed by reverse phase high-performance liquid chromatography (HPLC) and mass spectrometry.

Amplification and sequencing of the epitope-encoding region of HBV

To amplify the nucleotide sequence that encodes the HBsAg183–191 epitope, we designed the following primers: 5′-CCTAGGACCCCTGCTCGTGTTACAG-3′ and 5′-CCCTACGAACCACTGAACAAATGGCAC-3′. The HBV genome was isolated from infected patients using virus genome extraction kits (TaKaRa Biotechnology (Dalian) Co. Ltd, Dalian, China), and stored at −80°C prior to use as a polymerase chain reaction (PCR) template. Amplification was performed as follows: 5 min denaturing at 95°C, 30 cycles with 30 s denaturing at 94°C, 30 s annealing at 58°C and 48 s extension at 72°C. The amplified fragment was extracted from the gel and inserted into the vector using a T-A cloning method. At least three clones per sample were sequenced with a 3730 sequencer in Beijing (Sunbiotech Co. Ltd, Beijing, China).

Refolding of HLA-A2 molecules with peptide

HLA-A2 heavy and light chains and human β2 microglobulin (β2m) were overexpressed in *Escherichia coli*. The inclusion bodies of each protein were purified and dissolved in 8 M urea; 4 μg peptide was added to 500 ml refolding buffer (100 mM Tris-HCl (pH 8·0), 400 mM L-arginine-HCl, 2 mM Na ethylenediamine tetraacetic acid (NaEDTA), 0·5 mM oxidized glutathione and 5 mM reduced glutathione) followed by 15 mg β2m at 4°C. After 30 min, 15 mg HLA-A2 heavy chain was added gradually and the refolding buffer was stirred. The refolded protein was concentrated in a pressurized chamber and run over a Superdex 75 column.

Peptide binding assay

To determine whether the synthesized peptide could bind to HLA-A2 molecules, we measured peptide-induced HLA-A2 up-regulation on T2 cells [30,31]. In brief, T2 cells were incubated with 50 μM of the candidate peptides and 3 μg/ml β2m in serum-free RPMI-1640 medium for 18 h at 37°C in a 5% CO_2 incubator. Surface expression of HLA-A2 on the T2 cells was quantified using FITC-conjugated anti-HLA-A2 monoclonal antibody and analysed by flow cytometry. The fluorescence index (FI) was calculated as follows: FI = (mean fluorescence with a given peptide − mean fluorescence with no peptide)/(mean fluorescence with no peptide). Peptides with an FI greater than 1 were regarded as high-affinity epitopes.

Tetramer production and staining

Tetrameric HLA-A2 peptide complexes (tetramers) were constructed as described previously [31,32]. Briefly, HLA-A2 heavy chain, with a Bir A site, and human β2m were expressed in bacteria and the purified inclusion bodies were denatured in 8 M urea buffer. The proteins were refolded with the peptide *in vitro*, as described above. The HLA-A2-peptide–β2m complexes were then purified through a Superdex 75 column and biotinylated with Bir A enzyme. The biotinylated complexes were purified further by gel filtration, and tetramerization was accomplished by mixing biotinylated HLA-A2-peptide–β2m complex and phycoerythrin (PE)-conjugated streptavidin at a molar ratio of 4 : 1. Peptide-induced lymphocyte cultures were incubated with PE-conjugated tetramers for 30 min at 4°C, and incubated with FITC-labelled anti-human CD8 monoclonal antibody (mAb) for an additional 30 min at 4°C. After washing with PBS, stained cells were fixed with 0·5% paraformaldehyde and analysed by flow cytometry [33–35].

Immunization of HLA-A2 transgenic mice

HLA-A2/K^b transgenic mice [36], a generous gift from Dr Cao Xuetao (Shanghai Second Military Medical University), were bred and maintained in specific pathogen-free facilities. Expression of HLA-A2 on the cellular membrane was assessed by flow cytometry using FITC-labelled HLA-A2-

specific monoclonal antibody (eBioscience Co. Ltd, San Diego, CA, USA). Six to 8-week-old mice were immunized three times intramuscularly every 2 weeks with a mixture of 100 μg peptide with incomplete Freund's adjuvant (IFA), as described previously [37,38]. Seven days after the last immunization, mouse splenocytes were isolated and specific CTL responses were analysed by enzyme-linked immunospot (ELISPOT) assays.

Analysis of interferon (IFN)-γ-producing T cells by ELISPOT

ELISPOT was performed using a commercially available human or mice interferon-γ (IFN-γ) ELISPOT kit (e-Bioscience Co. Ltd). Briefly, 96-well polyvinylidene fluoride (PVDF)-treated microtitre plates were coated with anti-IFN-γ mAb at 4°C overnight. Then 2×10^5 cells, which were stimulated with 10 μg/ml peptide for 24 h in 24-well culture plates, were added to each well of the ELISPOT plate. After 16–24 h incubation the cells were discarded and the assay was performed according to the manufacturer's instructions. Wells were imaged with an ELISPOT reader, and the spots were counted with an automated system, after setting parameters for size, intensity and gradient with Saizhi ELISPOT analysis software. A response was considered positive if the mean number of spot-forming cells (SFCs) in triplicate sample wells exceeded background. Assay results were displayed as SFC per 1×10^6 cells.

Results

Prevalence of the HBsAg183–191 epitope sequence in CHB patients

Because the variability and distribution of HBV differs among global populations, the epitope sequence identified in Caucasian patients may be mutated in Chinese patients. In order to investigate the prevalence and mutant state of the HBsAg183–191 epitope in Chinese patients, we enrolled 103 subjects randomly from more than 500 CHB patients. The HBV genome was amplified by PCR, DNA fragments containing the HBsAg183–191 epitope sequence were cloned and at least three clones from each sample were confirmed further by direct DNA sequencing. Of the 103 samples, 17 patients (16·5%) had a mutant epitope in whom arginine was substituted with a lysine (Table 1). The results indicate that a considerable percentage of Chinese patients harbour HBV with the K187 mutant HBsAg183–191 sequence.

Table 1. Frequency of the HBs antigen (HBsAg)183–191 sequence in patients with chronic hepatitis B virus (HBV) infection.

Item	K187 mutant peptide	R187 peptide	Total sample
Number of samples	17	86	103

The HBV genome was extracted from 103 chronic hepatitis B patients and the HBsAg183–191 sequence was amplified by polymerase chain reaction. The fragment was cloned into a pMD 18-T vector and three clones were sequenced. Of the 103 samples, 17 had the K187 mutant peptide sequence and 86 had the R187 peptide sequence.

HLA-A2 heavy chain can refold with β2m in the presence of K187 mutant peptide

Major histocompatibility complex (MHC) class I-restricted epitopes form a complex with MHC-I molecules within the cellular endoplasmic reticulum (ER). The complex is then displayed on the cellular membrane for peptide presentation. Individual MHC-I molecules are not stable *in vitro* unless they have bound a suitable epitope. In the presence of an epitope, the heavy chain of MHC-I can refold with β2m [39]. To determine whether the K187 mutant peptide still has the property of an HLA-A2-restricted epitope, we performed a refolding assay with the K187 mutant peptide, HLA-A2 and β2m at a molar ratio of 1 : 4·5 : 18. The refolding efficiency was determined by gel filtration analysis. As shown in Fig. 1a, HLA-A2 molecules/peptide complexes formed *in vitro*. This was confirmed further by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1b). These results indicate that the K187 peptide retains the capacity to bind HLA-A2, encouraging us to investigate its characteristics further.

K187 mutant peptide up-regulates expression of HLA-A2 molecules on T2 cells

The affinity of peptides for MHC-I is associated closely with their immunogenicity [40]. High-affinity peptides are

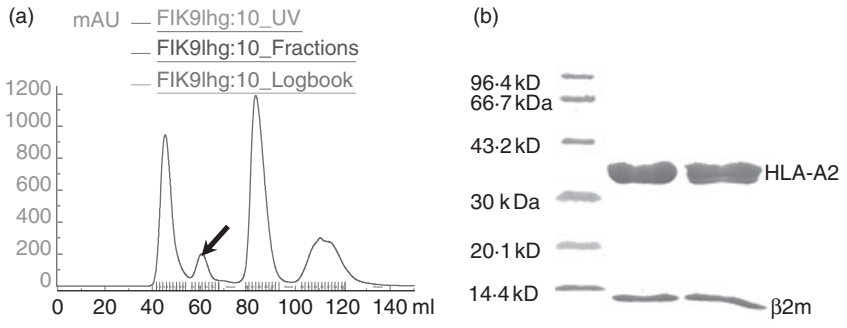

Fig. 1. Refolding assay using the K187 mutant peptide. (a) Human leucocyte antigen-A2 folded with β2m in the presence of K187 mutant peptide at 4°C. The folded complex was run over a Superdex 75 column. The second peak represents the refolded product (see arrow). (b) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis assay using the complex represented by the second peak.

Table 2. Binding affinity of K187 or R187 to human leucocyte antigen-A2 molecule on T2 cells.

Name of sample	Mean fluorescence value	FI value
β2m control well	723	0
HBcAg87–95 peptide control well	863	0·193
K187 mutant peptide well	1862	1·575
R187 mutant peptide well	1903	1·632

Mean fluorescence value of T2 cells stained with the fluorescein isothiocyanate-human leucocyte antigen-A2 (FITC-HLA-A2) monoclonal antibody and fluorescence index (FI) value. The FI value was obtained according to the following formula: FI = (mean FITC fluorescence of the given peptide – mean FITC fluorescence without peptide)/mean FITC fluorescence without peptide.

immunogenic, while low-affinity peptides are not. Affinity for MHC-I is often evaluated with the competitive binding inhibition assay [17]. It is also monitored by detection of peptide-induced up-regulation of HLA-A2 molecules on transporter protein for antigenic peptide (TAP)-deficient T2 cells [41]. To measure the affinity of K187 for HLA-A2, we adopted a protocol in which affinity is expressed as a fluorescence index (FI) value, as described previously [30,31,42]. As shown in Table 2, the FI of K187 mutant peptide reached 1·575, while the FI of the control peptide, HBcAg87–95, reached only 0·193. Simultaneously, we performed the experiment with the R187 epitope and identified its FI value as 1·632. The histograms of three peptides were overlaid with the negative control curve, using WinMDI version 2·9 software (Fig. 2). Both K187 mutant peptide and R187 epitope showed a higher fluorescence intensity than the control peptide, HBcAg87–95, and no peptide samples. These results indicate that K187 has an almost equally high affinity for HLA-A2 as R187, and possesses the property of a $CD8^+$ CTL epitope.

K187 mutant peptide elicits specific $CD8^+$ CTL responses in HLA-A2/K^b transgenic mice

It is critical to show that $CD8^+$ CTL epitopes can elicit a specific CTL response efficiently *in vivo*. To address this, we immunized HLA-A2/K^b transgenic mice with K187 mutant peptide or the control peptide, HBcAg87–95. After three immunizations, splenocytes were isolated and specific CTL responses were analysed by ELISPOT. As shown in Fig. 3a, K187 mutant peptide mounted a peptide-specific CTL efficiently in HLA-A2/K^b mice, while the control peptide did not. The results indicate that the K187 mutant peptide is presented to $CD8^+$ T cells *in vivo*.

K187-specific CTLs are detected in AHB patients, but not in CHB patients

Based on our results, we expected the K187 mutant peptide to elicit specific CTLs in HBV-infected patients. To confirm this further, we stained the peripheral blood mononuclear cells (PBMCs) with tetramer in HLA-A2-positive AHB and CHB patients. Approximately 3·93% of the total $CD8^+$ T cells were specific for K187 in the AHB patients (Fig. 4). ELISPOT analysis supported the result (Fig. 3b). Through PCR amplification and direct cDNA sequencing, we confirmed that the HBsAg183–191 sequence in these patients is the K187 mutant peptide, FLLTKILTI. Our findings indicate that patients who recover successfully from an acute self-limiting HBV infection develop strong K187 epitope-specific CTL responses.

R187 epitope-specific CTLs can cross-react with K187 epitope

To address cross-reactivity between the above-mentioned two epitopes, we enrolled two HLA-A2 restricted patients who recovered from acute HBV infection and were found with HBV-infected R187 epitopes. PBMCs of the patients were isolated and stimulated with R187 and K187 epitopes, respectively. Secretion of IFN-γ by R187-specific CTLs were detected with ELISPOT assay. As shown in Fig. 5, both R187 and K187 epitopes can stimulate the R187 epitope-specific CTLs to secrete IFN-γ. The data indicate that R187 and K187 epitopes can cross-react.

Discussion

Epitopes in HBV antigens are important for viral clearance in patients. Many epitopes have been identified within the HBV core [43,44], envelope [17], polymerase [4] and X proteins [20] of infected Caucasian individuals. As the HBV

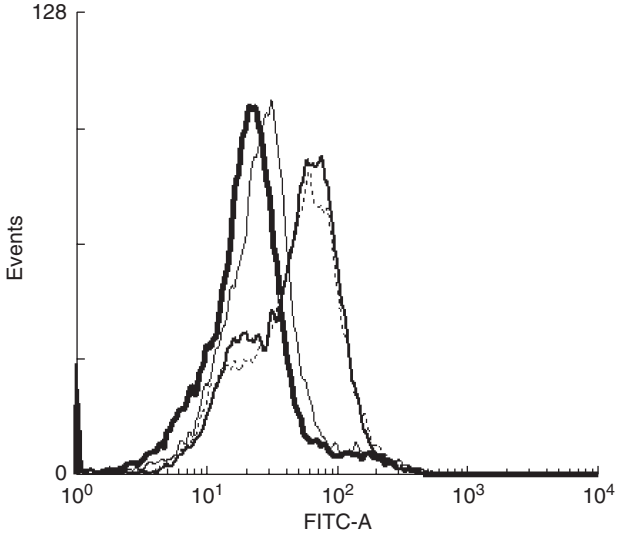

Fig. 2. Human leucocyte antigen-A2 expressing on T2 cells: thick-line histogram represents T2 cells incubated with β2m protein alone; thin-line histogram represents T2 cells incubated with HBcAg87–95 control peptide and β2m; broken-line histogram represents T2 cells incubated with K187 mutant peptide and β2m; narrow-brush histogram represents T2 cells incubated with R187 peptide.

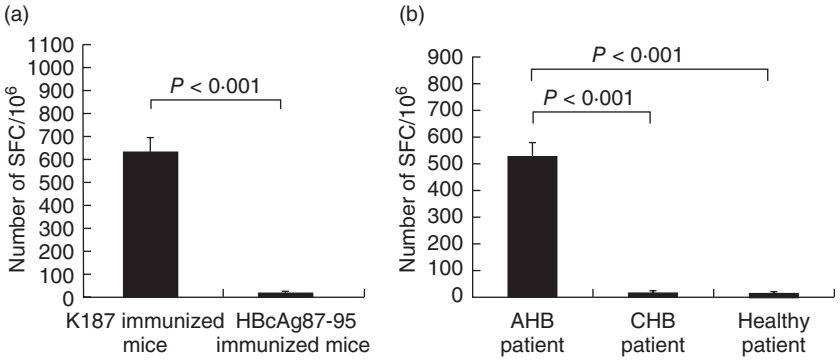

Fig. 3. Detection of hepatitis B virus-specific cytotoxic T lymphocytes (CTLs) by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. (a) Mice were immunized three times with 100 μg K187 mutant or HBcAg87–95 control peptide. Seven days following the third injection, splenocytes were isolated and peptide-specific IFN-γ cells were quantified by ELISPOT. (b) K187-specific CTLs were detected in representative acute hepatitis B and chronic hepatitis B patients or healthy subjects. Peripheral blood mononuclear cells (PBMCs) from each individual were isolated, stimulated with 10 μg/ml peptide, and IFN-γ responses were detected by ELISPOT assay.

genome is variable, HBV epitope sequences often generate mutations. However, very little research has focused upon the prevalence of these mutations and their effect on CTL generation, particularly in China. Here, we found that approximately 16·5% of HBV-infected Chinese patients bore a mutant HBsAg183–191 epitope in which the original arginine (R187) was substituted with a lysine (K187 mutant peptide). In addition, the mutated K187 epitope can cross-react with R187-specific CTLs, and elicits a protective anti-HBV CTL response in Chinese patients with HBV infection.

In this study, we found that the mutant sequence of the HBsAg183–191 epitope, K187, can bind effectively to HLA-A2 molecules. Our results were not consistent with other reports illustrating that mutant HBV epitopes have a reduced ability to bind HLA-A2 molecules [11]. The short peak of the refolding complex in Fig. 1 did not suggest that HLA-A2 and β2m have a lower folding efficiency in the presence of K187. A parallel experiment using the HBsAg183–191 epitope confirmed this result (data not shown). Instead, the short peak was likely to be caused by the hydrophobicity of the peptide, which decreased its water solubility. FI values were also very similar between the K187 mutant and R187 peptides (data not shown). These results can be explained by previous findings that interior amino acid residues in HBV epitopes are required for TCR interaction [45]. Their mutation does not affect the binding ability of epitope to MHC-I molecule [46,47].

Previous studies have shown that substitution of internal amino acid residues in some epitopes of HBV disrupts the ability of the virus to be recognized by TCRs [11,12,48]. In this study, we found that K187-specific CTLs can be primed in HLA-A2/Kb transgenic mice and in patients with acute HBV infection, indicating that the mutant peptide complexes efficiently with MHC-I molecules and interacts with the TCR. Our study is the first to confirm that the K187 mutant epitope can induce CTLs *in vivo*.

Some epitope mutations favour virus escape from the host immune response [14,49]. However, our findings suggest

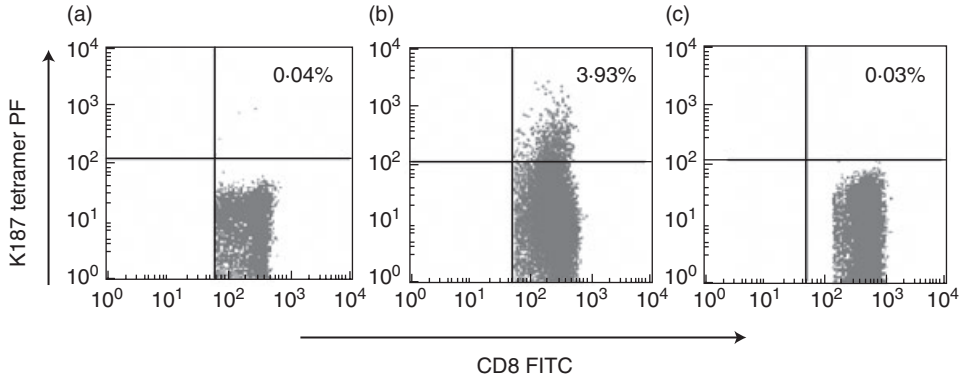

Fig. 4. Tetramer staining of K187-specific cytotoxic T lymphocytes. Peripheral blood mononuclear cells (PBMCs) were prepared from the blood of uninfected healthy human leucocyte antigen-A2-positive person (a), acute hepatitis B (b) and chronic hepatitis B patients (c) by Ficoll density gradient centrifugation and stained for 15 min with fluorescein isothiocyanate-labelled anti-human CD8 monoclonal antibody and phycoerythrin-conjugated K187 mutant peptide tetramer.

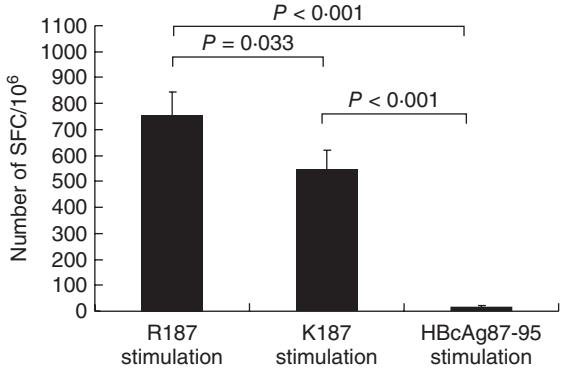

Fig. 5. Cross-reaction between the K187 epitope and R187 epitope-specific cytotoxic T lymphocyte (CTL): R187-specific CTLs in a human leucocyte antigen-A2-restricted acute hepatitis B patient were stimulated with K187 and R187 epitope, respectively, in enzyme-linked immunospot (ELISPOT) plates for 24 h. The epitope-specific CTLs were counted with ELISPOT reader.

that this may be not the case for the K187 mutation, as this epitope still induced HBV-specific CTLs *in vivo*. Alternatively, these HBsAg183–191-specific CTLs may still respond to mutant epitope by different TCRs which have a different Vβ chain [48,50]. In addition, many HBV epitopes are found in the surface, core and polymerase proteins [4,17–19]. These epitopes also elicit CTLs *in vivo* and can contribute to the elimination of the K187 mutant HBV. However, because our investigation does not show other mutations at the 187 position of the HBsAg183–191 epitope, we do not know whether or not other mutations at this position cause escape from the host immune response. Riedl *et al*. have reported that mutant epitopes can disrupt the immune tolerance of prototype epitopes, even with only one amino acid substitution [51]. Our findings confirm further that R187-specific CTLs can cross-react with the K187 epitope. In future, according to the research by Riedl *et al*., the K187 mutant epitope is a good choice to use to disrupt the immune tolerance of the R187 epitope-bearing HBV to cure CHB patients.

Overall, our results show that the substitution of K187 for R187 in the HBsAg183–191 sequence affects neither epitope binding to HLA-A2 nor CTL recognition of the epitope. Indeed, K187 is an efficient HLA-A2-restricted immunogenic peptide capable of inducing an anti-HBV-specific CTL response in AHB patients. Our findings will aid the design of alternative therapies for CHB patients in China.

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