Construction and immunogenicity prediction of Plasmodium *falciparum* CTL epitope minigene vaccine

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Abstract The minigenes encoding Plasmodium *falciparum* CTL epitopes restricted to human MHC class I molecular HLA-A2 and HLA-B51, which were both at high frequency among Chinese population, were constructed as mono-epitope CTL vaccines named pcDNA3.1/*tr* and pcDNA3.1/*sh*. The minigenes of the two epitopes were then tandem linked to form a dimeric CTL epitope minigene recombinant vaccine. After DNA transfection, the epitope minigenes were expressed respectively in two human cell lines, each bearing one MHC class I molecule named CIR/HLA-A2.1 and K562/HLA-B51. The intracellular expression of the CTL epitope minigenes not only enhanced the stability of HLA-A2.1 and HLA-B51 molecules but also increased the assemblage of MHC class I molecules on cell surfaces, which testified the specific process and presentation of those endogenous expressed epitopes. For the cells transfected with the dimeric minigene encoding two tandem linked epitopes, the expression and presentation of each epitope were also detected on cell membranes that bore different MHC class I molecules. It meant that the adjacency of the two CTL epitopes did not interfere with the specific process and presentation of each epitope. Compared with the ordinary CTL studies that inoculated synthesized epitope peptides with peripheral blood cells, this work aimed to process the epitopes directly inside HLA class I allele specific human cells, and thus theoretically imitated the same procedure *in vivo.* It was also an economical way to predict the immunogenicity of CTL epitopes at an early stage especially in laboratories with limited financial resource.

Keywords: Plasmodium *falciparum*, $CD8^+$ cytotoxic T cell, MHC class I molecule.

Despite the best efforts of hundreds of researchers and decades of work, malaria remains a scourge that affects thirty to fifty millions of people each year in tropic and subtropical countries and causes as many as three millions of people to die each year. Since the increasing incidence of parasite resistance to anti-malaria drugs and of mosquito tolerance to insecticides, malaria vaccine development becomes one of the most important strategies in controlling the disease.

$CD8^+$ cytotoxic T cell (CTL) plays a key role in host's protective immunity against Plasmodium *falciparum* during the pre-erythrocyte stage[1,2]. An effective CTL response blocks the life cycle of the parasites, avoids the appearance of clinical syndromes and reduces the pathologic injury of the disease. Specific CTL response, therefore, is a critical rule to evaluate the effectiveness of malaria vaccine. The main direction of CTL vaccine development has been to use vectors as virus, bacteria or naked DNA to process recombinant antigen[3—5]. However, the vaccine

constructed with mono recombinant antigen has hardly induced satisfactory immune response against malaria[2]. Some studies, therefore, have been focusing on developing recombinant malaria vaccine including multiple antigen or epitopes[6].

From the recent work on MHC class I molecules, researchers estimated that CTL epitopes restricted to five groups of common MHC class I could probably cover 80%—90% populations[7,8]. Since there is no animal model for the study of Plasmodium *falciparum* CTL epitopes, most people have been using rodent, which has far genetic connection with human being and is not the right host for the specific Plasmodium strain[9,10]. Those disadvantages obstruct the view to close observe human CTL vaccines.

It has been demonstrated that there are two constructions of MHC class I heavy chain molecules, folding and unfolding, existing in a system of dynamic equilibrium[11,12]. Without binding with a specific peptide (epitope), the heavy chain of MHC class I molecule is usually unfolding. The number of folding protein is obviously increased as soon as the MHC class I heavy chain binds to a specific epitope. Since the folding conformation could be recognized and bound by a construction-dependent monoclonal antibody, it is possible to evaluate the presentation of a specific CTL epitope with the antibody. In this study, we constructed mono and dimeric minigenes encoding malaria CTL epitope and transfected them into human cell lines bearing specific HLA class I alleles with high frequency among Chinese population. We further evaluated the endogenous process and presentation of those minigenes with experiments including peptide-MHC class I molecule stability and peptide-MHC class I molecule-assembling assay on cell surfaces. This work aimed to establish an *in vitro* system that mimics precisely the endogenous CTL epitope processing in human being and to evaluate the possibility of predicting the immunogenicity of CTL epitopes in a less complex and expensive way.

1 Materials and methods

1.1 Construction of minigene vaccines

The minigenes were designed with universal codon and incorporated with a Kozak sequence at upstream of the ATG initiation codon, a double-stop condon at the downstream primer. A short peptide GAGAA precedes upstream epitope sequence. The oligonucleotides of the genes were synthesized as partially complimentary single chain (Sango Inco.). The double-stranded DNA with endonuclease restricted sides, *Bam*H I and *Xba*l I, at each end was produced by PCR and inserted into a mammalian cell expression vector, pcDNA3.1/Hygro+(Invitrogen), to generate pcDNA3.1/*tr* and pcDNA3.1/*sh*. The minigene *tr* on pcDNA3.1/*tr* and *sh* on pcDNA3.1/*sh* were tandem linked together to produce pcDNA3.1/*ts* by using a pair of isocaudamer, *Cla* I and *Bst*B I, on the synthetic sequences and *Eco*R I on the vector, which has been described previously[13]. The plasmids were purified with Qiagen kits (Chatsworth, CA).

1.2 Peptide synthesis and production of rabbit polyclonal antibodies

Peptide TR and SH were synthesized by Institute of Pharmacology and Toxicology, Academy

of Military Medical Sciences. The sequences are shown in table 1. The synthetic peptides were coupling to a carrier protein, keyhole limpet hemocyanin (KLH) with *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). Preparation of rabbit antiserum against the synthesis peptides followed the protocols in textbook[14]. Serum IgG was purified by DEAE-Gel-Affi-Chromatography.

Table 1 DNA sequences and deduced amino acids of three minigenes

Recombinant & presenting HLA	DNA sequence & epitope peptide sequence
tr	ACCATGGCCTTCGAAGGAGCTGGAGCTGGA**CACCTGGGCAACGTGAAGTACCTGGTG**ATCGATTAATAA
HLA-A2.1	M A F E G A G A A **H L G N V K Y L V** I D
sh	ACCATGGCCTTCGAAGGAGCTGGAGCTGCC**ATGCCCCTGGAGACCCAGCTGGCCATC**ATCGATTAATAA
HLA-B51	M A F E G A G A A **M P L E T Q L A I** I D
ts	ACCATGGCCTTCGAAGGAGCTGGAGCTGGA**CACCTGGGCAACGTGAAGTACCTGGTG**ATCGAAGGAGCT
HLA-2.1+HLA-B51	M A F E G A G A A **H L G N V K Y L V** I E G A
	GGAGCTGCC**ATGCCCCTGGAGACCCAGCTGGCCATC**ATCGATTAATAA
	G A A **M P L E T Q L A I** I D

The Plasmodium *falciparum* sequences are shown in boldface.

1.3 Cell lines and transfection

C1R/A2 cells were derived from an Epstein-Barr virus-transformed B-cell line C1R[15] transfected with HLA-A2.1 gene, which were kindly provided by Dr. Michael Edidi (Hopkins University, USA). K562 cells, a human chronic myelogenous leukemia cell line (HLA class I negative) with a transfected HLA-B51 gene, were kindly provided by Dr. Elisabeth Weiss (Müchen University, Germany). Both HLA-B51 and HLA-A2.1 cells mentioned above were transfected with the plasmid containing minigenes *tr*, *sh*, *ts* and empty vector respectively by using the kit of Lipofectin (Gibco/Life Technologies Ltd.), following the manufacturer's instruction. The stable transfectants were selected by hygromycin two weeks after the transfection. The cells were tested for minigene transcription and expression by RT-PCR and HLA molecules stabilization assay on cell surfaces.

1.4 RT-PCR

RNA was isolated from the epitope gene transfected cell with the TRIZOL Reagent (Gibco/Life Technologies Ltd.). Aliquots of 3 μg of RNA were ethanol precipitated and resupended in 11 μL of H_2O with or without the addition of RNAase to 2.5 μg/mL. Reverse transcriptase PCR was carried out with the primer 1: forward 5′-GAGCTGGACACCTGGGC-AACGT-3′, and reverse 5′-TGGAGCTGCCATGCCCCTGGAGA-3′ for pcDNA3.1/*tr* and pcDNA3.1TS, and primer 2: forward 5′-GGAAAGGACAGTGGGAGTGGCA-3′, reverse 5′-TGGAGCTGCCATGCCCCTGGAGA-3′ for pcDNA3.1/*sh* using the superscript reverse transcription kit (Gibco/Life Technologies Ltd.) following the manufacturer's instructions.

1.5 Measurement of MHC-peptide complex stability at 37℃[16]

C1R/A2.1 and K562/HLA-B51 cells transfected with different minigenes at a concentration of 2 million cells were incubated in 10% FCS RPMI1640 with 10 μg/mL brefeldin A (BFA) to block new surface expression of Class I molecules, and divided into separated tubes for various incubation periods at 37℃. The cells reacted with W6/32, an anti-HLA-A, B, C conformation-specific mAb[17], followed by FITC-coupled second antibody and analyzed by FACScan (Becton Dickinson, Mountain View, CA). The results were expressed as percentage of positive cells.

1.6 Immunoprecipitation and Western-blotting

The immunoprecipitation was done with Cellular Labeling and Immunoprecipitation kit (Boehringer), following the manufacturer's instructions. Briefly, cell surface proteins were biotinylated, then MHC Class I complexes on cell surfaces were immunoprecipitated with anti-HLA-A, B, C conformation-dependent mAb, W6/32. The eluted samples were analyzed by electrophoresis on 12% polyacrylamide gel and by Western blot with ECL Western blotting analysis system (Amersham Pharmacia Biotech) following the protocols. The positive bands of Class I heavy chain localized on X-ray film were semi-quantified by light density scan. Simultaneously, precipitated products were separated by electrophoresis in a Tris-Trcine system and analyzed by Western-blot with anti-peptide polyclonal antibodies, anti-SH and anti-TR, to evaluate the co-precipitation of CTL epitope minigenes band with MHC Class I molecules.

2 Result

2.1 Recombinant vaccine constructs

Table 1 shows the DNA and amino acid sequences of the minigenes constructed. The minigene *tr* was from the thrombospondin-related anonymous protein, which was restricted to HLA-2.1 molecule[18]. The minigene was *sh* from the sporozite hepatocyte-binding antigen, which showed a high affinity with the HLA-B51 molecule[1]. The two minigenes were tandem linked to form the third minigene *ts*, which had the oligonucleotides encoding a five amino acid peptide, GAGAA, in between of the two epitopes to make the conformation more flexible. All of those clones were confirmed by DNA sequencing and restrict endonuclease mapping.

2.2 RT-PCR detected the minigenes expression

RT-PCR amplification of the total RNA from the cell lines K562/HLA-B51 and C1R/HLA-A2.1, which were transfected with recombinant plasmids pcDNA3.1/*sh*, pcDNA3.1/*tr*, pcDNA3.1/*ts* or empty vector pcDNA3.1, produced respectively the products in expected size (fig. 1).

2.3 Stability of peptide-MHC class I on cell surfaces

The remaining cell surface peptide-MHC class I complexes of K562/HLA-B51 and C1R/HLA-A2.1 transfected with minigenes were detected by W6/32 at different time points after the transporting of new endogenous peptides to cell surfaces had been blocked by BFA.

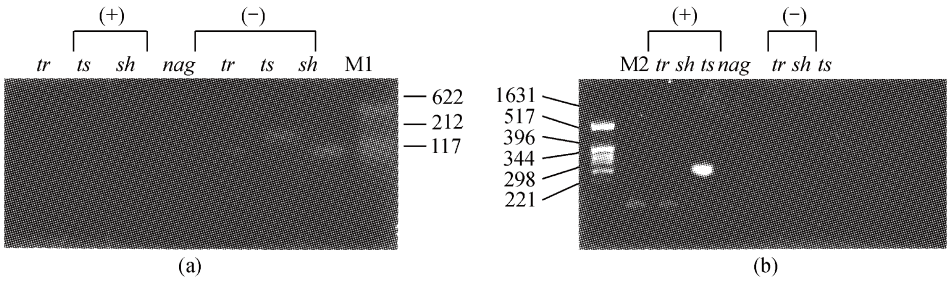

Fig. 1. Detection of minigenes RNA in transfected cell lines. RT-PCR was carried out on total RNA from K562/HLA-B51 (a) and C1R/HLA-2.1 (b) cells transfected with minigenes, with (lanes +) or without (lanes −) pretreatment of RNaseA. The amplification products *tr* (173 base pairs), *sh* (173 base pairs) and *ts* (215 base pairs) are shown in both figures. nag is negative control with monk cells. M1 and M2 are DNA molecular marker pBR322/*Msp* I and pBR322/*Bst*N I. Products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide.

The results (fig. 2) showed that minigene *tr* containing Plasmodium *falciparum* epitope was restricted to HLA-A2.1 stabilized C1R/HLA-A2.1 cell surfaces MHC class I complexes. Minigene *sh* containing HLA-B51 restricted Plasmodium *falciparum* epitope promoted K562/HLA-B51 cell surfaces MHC class I complexes stabilization. Minigene *ts* encoding the two epitopes mentioned above stabilized the MHC class I complexes on both cell line surfaces. Because the dissociation of peptides from MHC is a linear process, the stability of the peptide-MHC complexes was expressed as the time required for 50% of the molecules to decay (DT_{50}). Fig. 2 shows that C1R/HLA-A2.1 was transfected with minigenes *tr* and *ts*, K562/HLA-B51 transfected with minigenes *sh* and *ts*, displayed $DT_{50} > 4$ h; C1R/HLA-A2.1 transfected with minigenes *sh* and empty vector, K562/HLA-B51 transfected with minigenes *tr* and empty vector, displayed $DT_{50} < 3$ h.

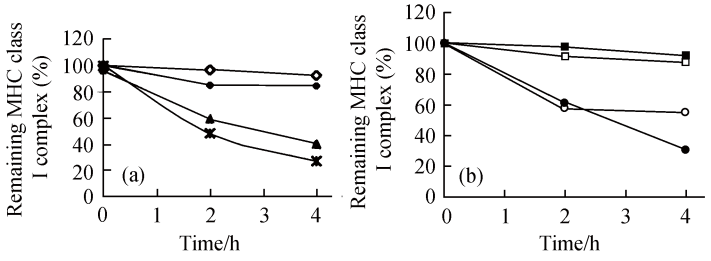

Fig. 2. Stability of peptide-MHC class I complex on cell surfaces. Cells were transfected with different minigenes. Residual surface expression at various time points after BFA blocking was analyzed by flow cytometry using mAb W6/32. (a) k562 transfected group: ◇, K562/*ts*; ●, K562/*sh*; ▲, K562/*tr*; ✳, K562. (b) C1R transfected group: ○, CIR; □, CIR/*tr*; ■, CIR/*ts*; ●, CIR/*sh*.

2.4 Cell surfaces peptide-MHC class I assembly assay

In order to demonstrate minigenes encoding peptide expression at protein levels and involved in cell surfaces peptide-MHC class I assembly, cell surfaces peptide-MHC class I complexes of K562/HLA-B51 and C1R/HLA-A2.1 transfected with different minigenes were immunoprecipitated by W6/32 mAb. Class I heavy chain bands localized on X-ray film were semi-quantified by light density scan.

The co-precipitation of peptides and heavy chains was detected by Western blotting assay with anti-peptide antibodies. The results showed that minigenes *sh* and *ts* transfections increased heavy chain amounts on K562/HLA-B51 cell surfaces; minigenes *tr* and *ts* transfections increased heavy chain amounts on C1R/HLA-A2.1 cell surfaces (fig. 3(a), (b)). Those co-precipitated products were specifically recognized by polyclonal antibodies from serum of rabbit immunized with the peptide TR and SH, respectively, in Western-blotting assays as shown in fig. 3(c) and (d).

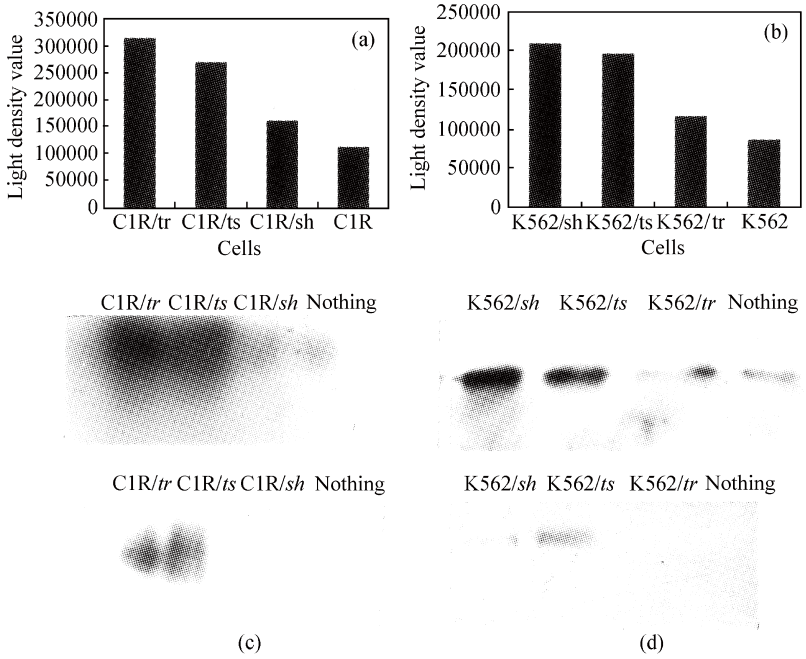

Fig. 3. Minigenes encoding peptides co-precipitated with MHC class I molecules and affected class I molecule assembling. The cells from C1R/HLA-A2.1 transfected group (a) and K562/HLA-B51 transfected group (b) were immunoprecipitated with the conformation-dependent mAb W6/32, and run on 10% polyacrylamide SDS gels. The heavy chains appearing on X-ray films were scanned using a densitometer. The above immunoprecipitated products were electrophoresed on 15% polyacrylamide SDS gels in tricine-tris buffer, detected by Western-blotting assay with anti-peptides antibodies TRAb (c) and SHAb (d).

3 Discussion

Antibody response was considered to be the most important immune response in protecting host against malaria when people started to search for an effective vaccine. Much work had aimed to induce specific humoral response since then. Since no satisfactory immune protection could be obtained with those vaccines that induced high antibody level, it has been accepted that a protective malaria vaccine must induce both humoral and cellular immune responses including the activation of $CD8^+$ cytotoxic T cells. The two CTL epitopes selected for this work, TR and SH, were both from an African strain of P. *falciparum*. The epitope TR was from a thrombospondin-related anonymous protein, TRAP, which was HLA-A2 restricted and recognized by CTL[18]. The epitope SH was from the sporozoite hepatocyte-binding antigen (SHEBA) and showed a high affinity with HLA-B51[1] but not positive in CTL recognition. Since the frequency of MHC class I

allele HLA-A2.1 and HLA-B51 is 27.6% among Chinese population, to choose those CTL epitopes in a CTL vaccine would theoretically increase the coverage of the vaccine in China (see table 1). We constructed three Plasmodium *falciparum* CTL epitope minigene plasmids: two were monomers including different epitopes, and one was a dimmer having the two epitopes tandem linked. By transfecting the genes into human cells that were specifically expressing HLA-A2.1 or HLA-B51 molecules, we characterized the expression and endogenous presentation of the epitopes *in vitro*.

Peptide-MHC class I molecule affinity has been used as one of the most important parameters for evaluating the immunogenicity of an MHC class I restricted peptide for several years. From the experiments in either human or mouse, it has been demonstrated that most of the peptides that showed high affinities with MHC class I molecules were strong immunogenicity[19,20]. The substance of those affinities is a dynamic equilibrium between association and dissociation of a trimolecular complex: peptides, MHC class I molecule and β_2m. For the purpose of evaluating the immunogenicity of the peptide, people inferred that the dissociating rate of the trimolecular was more critical than other parameters[16]. Compared with the experiment inoculating synthesized peptides with cultured cells, our experiments tested endogenous expression and antigen presentation of peptides by observing peptide-MHC class I molecule stability and peptide-MHC class I molecule assembling on cell surfaces, which was obviously closer to antigen processing *in vivo.* With those methods we have first shown that after the transfection of the two mono CTL epitope minigenes, either *tr* or *sh* could increase the MHC class I molecule stability on cell surface. The results of immunoprecipitation and Western blot further confirmed the specificity of the peptides that bound with MHC class I molecules of the transfected cells. Although a final conclusion for the available CTL epitope vaccines must rely on the detection of specific $CD8^+$ T cells in human peripheral blood directly, the methods established here would be the first step to predict the immunogenicity of a CTL epitope *in vitro*.

With the mammalian cell expressing system and the assays established for CTL epitope characterization, we further analyzed the cells transfected with the minigene encoding two CTL epitopes tandem linked, and found that the close adjacency of the two genes did not affect the specific processing and presentation of each epitope within a related cell line that expressed only one HLA class I allele. It has been reported that the flanking sequence of a peptide played a critical role in the antigen processing and presentation[21,22]. According to other groups work on maintaining free folding angle for peptide [21], we inserted a short peptide, which had five amino acids (GAGAA) that has homology with neither human being nor Plasmodium parasite, in between of the two CTL epitopes. Our results have shown that the insertion of the flanking sequence did not affect the endogenous processing and presentation of the epitopes within the cells. This observation was in agreement with Dell's who found in animal model that the immunogenicity of a recombinant vaccine was not affected by the mini hydrophobic peptide added at flank [21]. Meanwhile, the cell model for endogenous processing of peptides described

here was useful in observing the effect of flanking sequences.

The characterization of the dimeric CTL epitope minigene vaccine provided encouraging data for further work in developing a multi-epitope CTL vaccine that presumably covers as much population as possible. By using the human cell lines expressing specific HLA class I molecule and by testing the stability and assembling of endogenous peptide-MHC class I molecule, an early evaluation of the CTL epitope peptide was feasible and reliable. Since there is no animal model available for studying human CTL epitope vaccine and considering the time, labor and outlay consuming for a large-scale CTL epitope search in human being, the methods described here would be an economical and effective way for laboratories to develop CTL vaccine.

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