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• *BASIC RESEARCH* •

Procedure for preparing peptide-major histocompatibility complex tetramers for direct quantification of antigen-specific cytotoxic T lymphocytes

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Abstract

AIM: To establish a simplified method for generating peptide-major histocompatibility complex (MHC) class I tetramers.

METHODS: cDNAs encoding the extracellular domain of human lymphocyte antigen (HLA)-A*0201 heavy chain (A2) and β_2-microglobulin (β_2m) from total RNA extracted from leukocytes of HLA-A2$^+$ donors were cloned into separate expression vectors by reverse transcription-polymerase chain reaction. The recombinant A2 and β_2m proteins were expressed in *Escherichia coli* strain BL21(DE3) and recovered from the inclusion body fraction. Soluble A2 proteins loaded with specific antigen peptides were refolded by dilution from the heavy chain in the presence of light chain β_2m and HLA-A2-restricted peptide antigens. The refolded A2 monomers were biotinylated with a commercial biotinylation enzyme (BirA) and purified by low pressure anion exchange chromatography on a Q-Sepharose (fast flow) column. The tetramers were then formed by mixing A2 monomers with streptavidin-PE in a molar ratio of 4:1. Flow cytometry was used to confirm the expected tetramer staining of CD8$^+$ T cells.

RESULTS: Recombinant genes for HLA-A*0201 heavy chain (A2) fused to a BirA substrate peptide (A2-BSP) and mature β_2m from HLA-A2$^+$ donor leukocytes were successfully cloned and highly expressed in *E. coli*. Two soluble monomeric A2-peptide complexes were reconstituted from A2-BSP in the presence of β_2m and peptides loaded with either human cytomegalovirus pp65$_{495\text{-}503}$ peptide (NLVPMVATV, NLV; designated as A2-NLV) or influenza virus matrix protein Mp58–66 peptide (GILGFVFTL, GIL; designated as A2-GIL). Refolded A2-NLV or A2-GIL monomers were biotinylated and highly purified by single step anion exchange column chromatography. The tetramers were then formed by mixing the biotinylated A2-NLV or A2-GIL monomers with streptavidin-PE, leading to more than 80% multiplication as revealed by SDS-PAGE under non-reducing, unboiled conditions. Flow cytometry revealed that these tetramers could specifically bind to CD8$^+$ T cells from a HLA-A2$^+$ donor, but failed to bind to those from a HLA-A2$^-$ donor.

CONCLUSION: The procedure is simple and efficient for generating peptide-MHC tetramers.

Key words: Major histocompatibility complex; HLA-A2; Tetramers; Cytomegalovirus; Immune responses; Cytotoxic T lymphocytes

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INTRODUCTION

Cytotoxic T lymphocytes (CTLs), or CD8$^+$ T cells, play a critical role in the clearance of viral infections and the eradication of tumors[1]. A better understanding of cellular immunity against viruses and/or tumor cells requires careful analysis of the responding CD8$^+$ T cells, particularly in terms of their numbers and effector functions[2]. Because specific CD8$^+$ T cells are often vastly outnumbered by irrelevant T cells, quantification of antigen-specific T cells often requires *in vitro* culture and re-stimulation, which may introduce bias in the results[2]. The standard method for deriving specific CTL frequency information is limiting dilution analysis (LDA)[2]. However, accumulating evidence indicates that this technique may significantly underestimate the number of specific CTLs, because it does not detect cells that lack proliferative potential[2,3]. This limitation has been overcome by the introduction of peptide-major histocompatibility complex (MHC) class I tetrameric complex technology[3-6], which initiates a profound revolution in the field of cellular immunology[7-10]. Peptide-MHC tetramer-based assays have enabled direct flow cytometric quantification[3-6], phenoty-

ping[3,8-11] and even functional analysis[8,11] of antigen-specific $CD8^+$ T cells. Thus, peptide-MHC tetramer technology is a powerful tool for evaluating the fundamental aspects of T-cell immunity.

Though tetramer technology for direct quantification of the frequency and/or function of antigen-specific CTLs has been generally accepted, wide application of tetramer strategies is limited by the complex procedures necessary for tetramer production[3]. Moreover, the expression level of recombinant human MHC class I heavy chain in *Escherichia coli* is usually minimal due to the low translation frequency of human proteins in *E. coli*[12]. To address these problems, we sought to develop a simplified and efficient method for production of peptide-MHC class I tetramers. We constructed prokaryotic expression vectors for recombinant human lymphocyte antigen (HLA)-A2 heavy chain and β_2-microglobulin (β_2m) proteins, in which nucleotide residues in the translation initiation region (TIR) were substituted to the preferred codons for *E. coli*[13] and decreased the G/C content in this region[14]. The recombinant A2 and β_2m proteins were overexpressed in *E. coli* in the form of inclusion bodies, thus facilitating their high purity isolation by simple washing and centrifugation. Further simple procedures allowed us to establish an efficient, streamlined procedure for the preparation of HLA-A2 tetramers. This improved procedure should facilitate the general application of tetramer technology in both basic research and clinical applications.

MATERIALS AND METHODS

Materials

E. coli strain DH5α was stored in our laboratory. *E. coli* strain BL21(DE3) and plasmid pET-3c were purchased from Novagen (Madison, WI, USA). *Nde*I, *Bam*HI, T4 DNA ligase and high fidelity DeepVent Taq polymerase were purchased from New England Biolabs (Beverly, MA, USA). The TRIzol reagent and ThermoScript reverse transcription-polymerase chain reaction (RT-PCR) system were obtained from Invitrogen (Carlsbad, CA, USA). Q-Sepharose (fast flow) was obtained from Amersham (Uppsala, Sweden). Mouse anti-human monoclonal antibodies CD3-FITC, CD8-CyChrome and HLA-A2-FITC were purchased from PharMingen (San Diego, CA, USA). Streptavidin R-phycoerythrin (PE) conjugate (streptavidin-PE) was purchased from Molecular Probes (Eugene, OR, USA). Protein molecular mass markers were obtained from Sigma (St. Louis, MO, USA). The biotinylation enzyme, BirA, was purchased from Avidity (www.avidity.com). The NLVPMVATV (NLV) peptide derived from pp65 ($pp65_{495-503}$) of HCMV[11], and the GILGFVFTL (GIL) peptide derived from the matrix protein (Mp_{58-66}) of the influenza A virus[15], were synthesized by BioAsia (Shanghai, China) and purified to >98%. All other chemicals were from Sigma and were analytically pure.

Cloning of HLA-A*0201 heavy chain and β_2m cDNAs

Heparinized human peripheral blood was collected from three HLA-A2 positive (identified by anti-human HLA-A2-FITC staining and flow cytometric analysis) donors by venipuncture. Total RNA was extracted from freshly isolated PBMCs using the TRIzol reagent. cDNAs were synthesized from the isolated RNA using the ThermoScript RT-PCR system according to the recommended procedure. PCR amplification of the resultant cDNA was performed in a total volume of 50 μL containing high fidelity DeepVent Taq polymerase. For amplification of β_2m, PCR was performed with an initial denaturation for 2 min at 94 ℃, followed by 35 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 1 min, and a final extension for 10 min at 72 ℃. The primers (5'-ATA TCC ATA TGT CTC GCT CCG TGG CCT TAG-3' and 5'-AAC TAG GGA TCC TTA CAT GTC TCG ATC CCA C-3') were designed to amplify the entire coding sequence of human β_2m cDNA. For amplification of the mature HLA-A*0201 heavy chain, PCR was performed as above except that the extension time at 72 ℃ was 1.5 min and the primers (5'-TAT ACA TAT GGG CTC TCA CTC CAT GAG GTA TTT C-3' and 5'-AAC CAG GGA TCC TAC ACT TTA CAA GCT GTG AGA G-3') were designed to amplify the entire coding sequence of mature A2. The resultant PCR products were cloned into the *Nde*I–*Bam*HI sites of the pET-3c vector. Randomly selected clones were *Nde*I/*Bam*HI digested and screened for the presence of a correctly sized insert. Several independent clones were submitted for DNA sequencing of the HLA-A*0201 heavy chain cDNA using the dye-labeled deoxy-terminator protocol on a 377 automated DNA sequencer (Applied Biosystems). The cloned β_2m cDNA was also confirmed by DNA sequencing analysis.

Construction of expression vector for mature β_2m

To create a β_2m expression construction with optimized codon usage and G/C content, the DNA fragment for mature β_2m was PCR amplified from the cloned β_2m cDNA using specific primers (5'-AT ATC CAT ATG ATT CAA CGT ACT CCA AAA ATT CAA GTT TAC TCA CGT CAT CC-3' and 5'-CGA CTG GAT CCT TAC ATG TCT CGA TCC CAC TTA AC-3'). The underlined codons were optimized for expression in *E. coli*[13] by synonymous substitutions from ATC, CAG, AAG, CAG to ATT, CAA, AAA and CAA, respectively. These alterations also reduced the G/C content in the TIR. The resulting PCR product was inserted into the *Nde*I-*Bam*HI sites of the pET-3c vector, and a positive clone with a correct sized insert was confirmed by DNA sequencing. This recombinant plasmid was designated as pET-β_2m.

Construction of expression vector for extracellular domain of HLA-A2 heavy chain fused with BirA substrate peptide (BSP)

To construct an expression vector in which the HLA-A2 heavy chain was fused with BSP, the DNA fragment encoding a Gly-Ser linker and a BSP (LHHILDAQKMVWNHR) was fused to the 3' end of the cDNA encoding the extracellular domain of the HLA-A2 heavy chain (1-275) by PCR amplification from cloned HLA-A2 heavy chain cDNA with specific primers (5'- ATA CAT ATG GGT TCT CAT TCT ATG CGT TAT TTT TTT ACA TCT GTT TCC CGG CCC GGC CGC-3' and the 3' primer 5'-GCG CGG ATC CTT AAC GAT GAT TCC ACA CCA TTT TCT GTG CAT CCA GAA TAT GAT GCA GAG AGC CCG GCT CCC ATC TCA GGG T-3'). The underlined codons were

optimized for expression in *E. coli*[13], and reduced the G/C content without any changes in amino acid sequence. These codons were changed from GGC, CAC, TCC, AGG, TTC, TTC, TCC and GTG, respectively. The resultant PCR product was *Nde*I/*Bam*HI digested and subcloned into plasmid pET-3c. Clones with correct sized inserts were verified by direct sequencing, and the recombinant plasmid was designated as pET-A2-BSP.

Expression and isolation of recombinant A2-BSP and β_2m proteins

BL21(DE3) competent cells were transformed with either pET-β_2m or pET-A2-BSP, single colonies were used to inoculate 100 mL of LB medium, and cultures were incubated at 37 ℃ overnight until cells reached the stationary phase. Each stationary culture was diluted 10-fold with fresh LB medium (to 1 L) and incubated at 37 ℃ for a further 2 h, 0.4 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG) was added, and cells were incubated for an additional 4 h. Cells were collected by centrifugation, and insoluble protein aggregates (inclusion bodies) were purified essentially as described[16]. In brief, each cell pellet was re-suspended in 50 mmol/L Tris-HCl buffer (pH 8.0) containing 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 10 mmol/L dithiothreitol (DTT), and then sonicated on ice. The insoluble pellet was collected by centrifugation and washed with washing buffer (50 mmol/L Tris-HCl pH 8.0, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT and 5 g/L Triton X-100), followed by three more rounds of sonication, centrifugation and collection. The pellet was washed three more times with washing buffer without Triton X-100 and the isolated inclusion body pellet was dissolved in 20 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0, containing 8 mol/L urea, 10 mmol/L EDTA, and 0.1 mmol/L DTT). The insoluble material was pelleted by centrifugation and removed. The protein concentration of the remaining solution was determined by measuring $A_{280\ nm}$ and $A_{260\ nm}$, and calculated according to the empirical formula ($1.45\times A_{280\ nm}-0.74\times A_{260\ nm}$ = protein concentration in mg/mL). The protein solution was immediately frozen at -70 ℃.

Refolding of monomeric HLA-A2

The HLA-A2 monomers were refolded essentially as described by Garboczi and Wiley[17], with slight modifications. Briefly, 2 mg of peptide (NLV or GIL peptide) dissolved in DMSO was added in drops to 200 mL of pre-chilled refolding buffer (100 mmol/L Tris-HCl pH 8.0, containing 400 mmol/L L-arginine, 2 mmol/L EDTA, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione and 0.2 mmol/L PMSF). Then, 6 mg A2-BSP in 1 mL injection buffer containing 3 mol/L guanidine–HCl pH 4.2, 10 mmol/L sodium acetate and 10 mmol/L EDTA was forcefully injected to the stirring reaction through a 26-gauge needle as close to the stir bar as possible. Five micrograms of β_2m was injected similarly, and the refolding mixture was incubated at 10 ℃ for 3 d. At the end of the incubation, 200 mL of the refolding mixture was concentrated to 5 mL with an ultrafiltration apparatus (Amicon, Millipore, Bedford, MA, USA) containing a 10 ku molecular mass cut-off membrane, and dialyzed against 10 mmol/L Tris-HCl buffer (pH 8.0, containing 0.2 mmol/L PMSF). The refolded monomeric HLA-A2 was centrifuged to eliminate precipitates, and then biotinylated.

Biotinylation and purification of monomeric HLA-A2

The refolded HLA-A2 was enzymatically biotinylated by incubation with BirA according to the procedure provided by the supplier (Avidity Co.). The biotinylated HLA-A2 was dialyzed against 10 mmol/L Tris-HCl buffer (pH 8.0, containing 0.2 mmol/L PMSF) and loaded onto an anion-exchanger Q-Sepharose column (2 cm×8 cm) equilibrated with the same buffer. The column was eluted with a 0-300 mmol/L NaCl linear gradient, and 1.5 mL fraction was collected. Fractions exhibiting both HLA-A2 heavy chain and β_2m bands upon SDS-PAGE analysis were pooled and concentrated to 300 μL. The buffer of the biotinylated HLA-A2 was changed to 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) containing 0.2 mmol/L PMSF and 2 mmol/L EDTA through ultrafiltration.

Tetramerization of soluble HLA-A2 monomers

HLA-A2 tetramers were formed by mixing the biotinylated proteins with streptavidin-PE at a molar ratio of 4:1. Tetramers were analyzed by SDS-PAGE of samples prepared without boiling, in a loading buffer that contained no reducing reagent[10].

Tetramer staining and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were separated from 3 mL whole blood of two volunteers (over 40 years) by centrifugation over a Ficoll-Hypaque density gradient (Lymphoprep; NYCOMED, Norway). For staining, 1×10^6 cells suspended in 50 μL PBS containing 20 mL/L fetal calf serum and 1 g/L sodium azide were incubated on ice for 1 h with 10 μL of CD3-FITC, 10 μL of CD8-CyChrome and 1 μg of HLA-A2 tetramer. After being washed, the cells were fixed in 300 μL of 10 g/L paraformaldehyde and detected on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). For each sample, 50 000 events were collected and analyzed with the CELLQuest software (Becton Dickinson). The results were expressed as the percentages of tetramer-binding cells in the total T cell population.

RESULTS

Cloning of cDNA for HLA-A*0201 heavy chain and β_2m

The cDNA encoding the mature HLA-A2 heavy chain was RT-PCR amplified from total RNA of three HLA-A2 positive donors (identified as such by flow cytometry after anti-HLA-A2-FITC staining). The DNA fragments with the expected length (1 100 bp) were inserted into pET-3c, and eight independently transformed DH5a clones were identified to have the correct insert (Figure 1). DNA sequencing of these clones showed that six clones from donors 1 and 2 contained the cDNA for HLA-A*0201 heavy chain (designated pET-A2), while those from donor 3 contained the cDNA for HLA-A*0207 heavy chain. The cDNA sequence for HLA-A*0201 was submitted to GenBank (accession number AY191309).

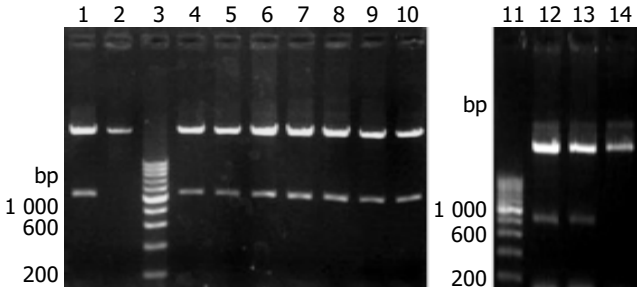

Figure 1 Identification of clones with correct sized inserts, using *Nde*I/*Bam*HI double digestion. Lanes 1 and 4-8: pET-A2 clones from donors 1 and 2; lanes 2 and 14: pET-3c; lanes 3 and 11: 200-bp DNA ladder; lanes 9 and 10: pET-A2 clones from donor 3; lanes 12 and 13: pET-A2-BSP.

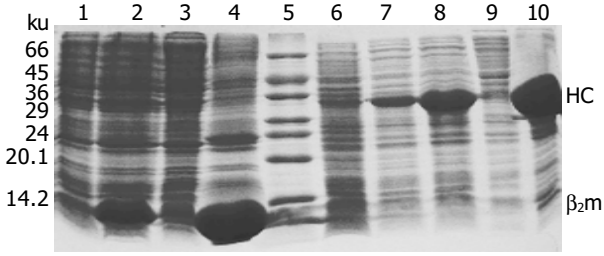

Figure 2 SDS-PAGE (150 g/L) analyses of recombinant A2-BSP and β_2m proteins expressed in *E. coli* strain BL21(DE3). Lane 1: BL21 (pET-β_2m) before IPTG induction; lane 2: BL21 (pET-β_2m)+IPTG; lane 3: supernatant of BL21 (pET-β_2m) lysate; lane 4: washed inclusion body of β_2m; lane 5: MW marker; lane 6: BL21 (pET-3c)+IPTG; lane 7: BL21 (pET-A2-BSP) before IPTG induction; lane 8: BL21 (pET-A2-BSP)+IPTG; lane 9: supernatant of BL21 (pET-A2-BSP) lysate; lane 10: washed inclusion body of A2-BSP. HC: heavy chain.

Similarly, the cDNA encoding β_2m was cloned from the total RNA of one donor and inserted into pET-3c (data not shown). The sequence was verified by DNA sequencing and found to be identical to the published one (submitted to GenBank, accession number AY187687).

Construction of expression vectors for recombinant A2-BSP and β_2m proteins

According to Altman's strategy[3], the DNA fragment encoding a Gly-Ser linker and a BSP (LHHILDAQKMVWNHR) was fused to the 3' end of the DNA fragment encoding the extracellular domain of HLA-A*0201 heavy chain (residues from 1 to 275) by PCR amplification of plasmid pET-A2. In order to increase the expression level in *E. coli*, our primers introduced synonymous substitutions at the 5' region, intended to reduce the G/C content of the TIR and to optimize codons for the bias usage of *E. coli*[13,14]. The amplified DNA fragment (900 bp) was inserted into pET-3c. Two clones with the correct insert were confirmed by DNA sequencing, and the generated expression vector was designated as pET-A2-BSP (Figure 1).

The expression vector for mature β_2m was similarly constructed by PCR amplification using cloned β_2m cDNA as template. The codons at the 5' region were optimized for better expression of β_2m in *E. coli*. The sequence of the insert was verified by DNA sequencing, and the vector was designated as pET-β_2m.

Refolding and biotinylation of monomeric HLA-A2

The expression vectors pET-A2-BSP and pET-β_2m were transformed into *E. coli* BL21(DE3). The cells showed leaky expression of the two proteins, and the expression levels increased dramatically after induction with IPTG (Figure 2). The molecular mass of the recombinant β_2m was approximately 12 ku, which was consistent with that of native human β_2m, while the engineered A2 heavy chain-BSP fusion protein (A2-BSP) had the expected molecular mass of 33 ku. The expression levels of these two recombinant proteins accounted for more than 20% of the total cellular proteins (Figure 2). The yields were approximately 32 and 50 mg/L for A2-BSP and β_2m, respectively. Western blotting showed that the recombinant β_2m could react with antibodies against human native β_2m (data not shown). HLA-A2 heavy chain expression was not analyzed by Western blotting, because

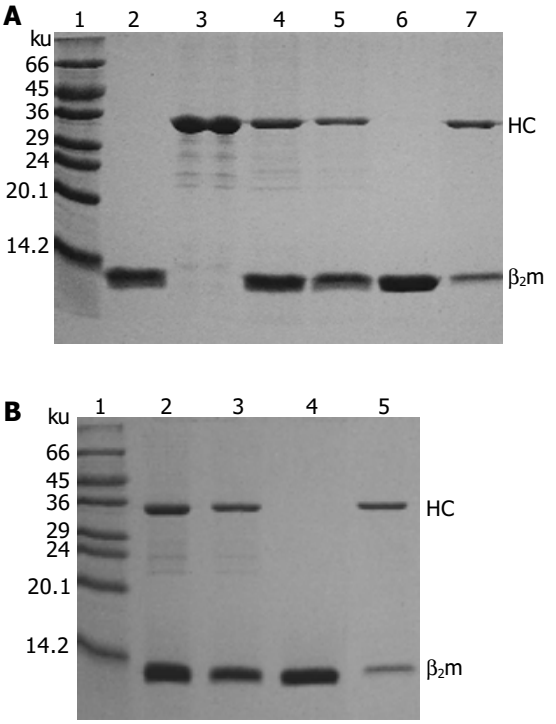

Figure 3 Analyses of refolded A2-GIL (**A**) and A2-NLV (**B**) monomers after purification and biotinylation with SDS-PAGE (150 g/L). In (**A**) lane 1: protein MW marker; lane 2: solubilized β_2m; lane 3: solubilized A2-BSP; lane 4: refolded A2-GIL monomer; lane 5: biotinylated A2-GIL monomer; lane 6: peak I (β_2m) (Figure 4); lane 7: peak II (purified A2-GIL). In (**B**) lane 1: protein MW marker; lane 2: refolded A2-NLV monomer; lane 3: biotinylated A2-NLV monomer; lane 4: peak I (β_2m); lane 5: peak II (purified A2-NLV). HC: heavy chain.

no suitable antibody was available. Both A2-BSP and β_2m were largely expressed in the insoluble fraction (inclusion bodies), which facilitated purification through simple washing and centrifugation (Figure 3, lanes 2 and 3).

Monomeric HLA-A2 was refolded by dilution of A2-BSP and β_2m with refolding buffer in the presence of HLA-A*0201-restricted antigenic peptides. Two peptides were used to reconstitute the HLA-A2 complex. One was the immunodominant NLVPMVATV (NLV) peptide, which was derived from pp65 (pp65$_{495\text{-}503}$) of HCMV[11] and used to

reconstitute the HLA-A2-NLV (hereafter referred to as A2-NLV) complex. The other was the predominant CTL epitope GILGFVFTL (GIL) peptide derived from the matrix protein (Mp_{58-66}) of influenza A virus[15], which was used to reconstitute the HLA-A2-GIL (referred to as A2-GIL) complex. The yield of refolding was about 10-15%. The refolded A2-NLV and A2-GIL complexes appeared as two bands (A2-BSP and β_2m) on SDS-PAGE (Figure 3). After biotinylation, each complex was purified by single Q-Sepharose column chromatography. Typically, three peaks eluted from the column, as shown by monitoring of absorbance at 280 nm (Figure 4). Soluble β_2m eluted as the first peak. The second peak comprised the desired soluble HLA-A2 complex (A2-NLV or A2-GIL) consisting of a heavy chain and β_2m (Figure 3), which was of 95% purity as analyzed with the PhotoCapt Ver11.01 software (Vilber Lourmat, France). The fractions in this peak were pooled and concentrated by ultrafiltration. The third peak contained non-protein materials with a much higher absorbance at 260 nm than at 280 nm. Refolding without peptide (negative control) yielded no stable HLA-A2 complex.

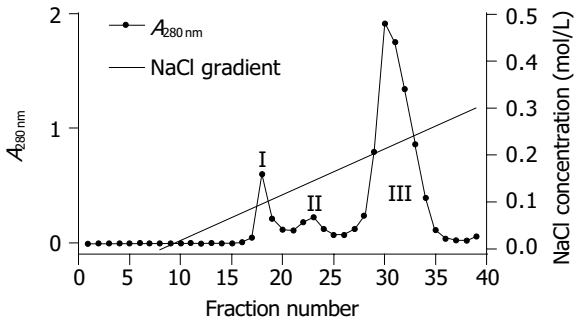

Figure 4 A typical elution profile of biotinylated HLA-A2 monomer from Q-Sepharose (fast flow) column (2 cm×8 cm).

Formation of HLA-A2 tetramers

HLA-A2 tetramers were formed by mixing the biotinylated A2-NLV or A2-GIL monomers with streptavidin-PE at a 4:1 molar ratio. SDS-PAGE analysis of the HLA-A2 tetramers could be used to estimate the extent of tetramer formation, because the complex formed between streptavidin and biotin was stable in the absence of boiling treatment and DTT[10]. As shown in Figure 5, the single main band observed in the lanes of monomeric A2-NLV or A2-GIL corresponded to the 33 ku heavy chain, as β_2m quickly migrated to the bottom of the gel. Software analysis (PhotoCapt Ver11.01) showed that the amount of heavy chain in the A2-NLV tetramer lane was about 10-20% of that in the lane containing monomeric HLA-A2, indicating that about 80% of the A2-NLV monomer formed multimers with streptavidin-PE. The A2-GIL tetramer showed a similar extent of multimer formation.

Staining of CTLs with HLA-A2 tetramers

Finally, the A2-NLV and A2-GIL tetramers were tested by flow cytometry for their abilities to identify HCMV- or influenza-specific $CD8^+$ T cells in freshly isolated PBMCs from HLA-A2 positive and negative donors. PBMCs were analyzed by three-color flow cytometry after A2-NLV and A2-GIL tetramer staining. Background staining was performed with streptavidin-PE instead of the tetramers.

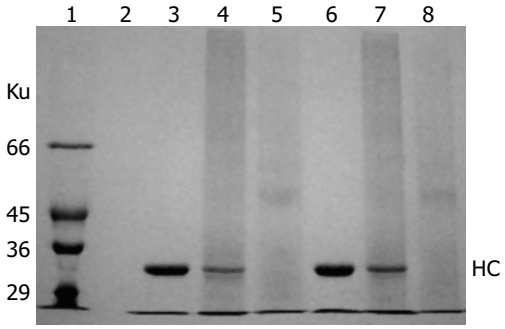

Figure 5 SDS-PAGE (75 g/L) analyses of A2-NLV and A2-GIL tetramers under non-reducing conditions without boiling. Lane 1: protein MW marker; lane 2: empty; lane 3: A2-NLV monomer (5 μg); lane 4: A2-NLV tetramer (5 μg A2-NLV monomer+8 μg streptavidin-PE); lanes 5 and 8: streptavidin-PE (8 μg); lane 6: A2-GIL monomer (5 μg); lane 7: A2-GIL tetramer (5 μg A2-GIL monomer+8 μg streptavidin-PE). HC: heavy chain.

Subpopulations of $CD8^+$ T cells specific for either $pp65_{495-503}$ or Mp_{58-66} epitopes were found in HLA-A2 positive donors by A2-NLV or A2-GIL tetramer staining. Of the total T cells, 0.19% were stained by the A2-NLV tetramer, while 0.17% bound to the A2-GIL tetramer (Figure 6). However, only 0.07% and 0.12% of T cells from the HLA-A2 negative donor were nonspecifically stained by the A2-NLV or A2-GIL tetramers, respectively, which was comparable to the control staining with streptavidin-PE (0.06%). This result suggests that these tetramers are quite specific for MHC-restricted T cells.

DISCUSSION

As the current method of limiting dilution assay (LDA) tends to underestimate CTL frequency[2,3], other methods have been sought for the quantification of antigen-specific $CD8^+$ T cells in immune responses. T cell antigen receptors (TCR) expressed on $CD8^+$ T cells can recognize peptide-MHC complexes, the specific ligands on the antigen-presenting cells (APCs). Therefore, direct staining of $CD8^+$ T cells with their soluble cognate ligands should be an ideal approach. However, the strategy initially failed because soluble monomeric peptide-MHC complexes have low affinities for the T-cell receptor (TCR)[1-3]. This obstacle has been overcome by Altman *et al.*[3]. Tetramer reagents have been shown to bind specifically to cognate T cells in numerous systems, allowing fast and direct quantification of antigen-specific T cells by flow cytometry[3,4,8,9,18-20]. Thus, tetramer-based assays have become a powerful new technology for detecting antigen-specific T cells[4,8,21]. However, the general application of this strategy is limited by the complex, labor intensive protocols required for preparing specific peptide-MHC tetramers.

It is difficult to prepare a large amount of MHC class I heavy chain, because the translation efficiency in *E. coli* is very

low. However, optimization of codons in the TIR has been shown to increase translation efficiency[14]. Sato *et al*.[12], found that the expression of wild type HLA-A*2402 heavy chain in *E. coli* is undetectable, whereas a synonymous mutant in which the TIR mammalian usage codons are replaced by those of *E. coli*, can be expressed at high levels. Lakey *et al*.[22], showed that the production of recombinant mycobacterial proteins increases 54-fold following selective replacement of low-usage *E. coli* codons in mycobacterial proteins with high-usage *E. coli* codons. In addition, the high G/C content in the TIR can block the expression of human proteins in *E. coli*[14]. The previous paper did not include a detailed description of the expression vector or *E. coli* system, instead of referring readers to the system used by Garboczi[17], in which no codon or G/C content changes were described. In the present study, recombinant proteins were expressed largely in the form of inclusion bodies, which greatly facilitated isolation and purification of the refolded peptide-MHC complexes, thus simplifying the tetramer preparation procedure. In addition to the difficulty in obtaining a large amount of MHC heavy chain, the practical application of tetramers is also limited by the complexity of the necessary preparation procedures. In this study, we reconstituted the monomeric HLA-A2 complexes based on the dilution strategy described by Garboczi[17]. After biotinylation, the HLA-A2 monomer could be highly purified by single step ion-exchange chromatography. It should be noted that the purification in the present study was carried out in a low pressure chromatography system requiring no HPLC facilities, whereas the previous protocol included three purification steps with high performance column chromatography[3].

The results of this study indicate that the generated reagents can be used for the detection of antigen-specific T cells. Our novel procedure for generating tetramers should be applicable for preparing other HLA-A2 tetramers or for generating tetramers of other human MHC class I alleles.

Tetramer technology is primarily used to determine the frequency of antigen-specific T cells[3-5], and has been extensively used in evaluating the dynamics of cytomegalovirus (CMV)-specific CTL responses in humans[5-7,9,11]. It is generally believed that adults over 40 years in developing countries are almost 100% CMV positive[9], and that the pp65$_{495\text{-}503}$ NLV peptide (NLVPMVATV) is the predominant CMV epitope presented by HLA-A2 molecules[11,23]. Quantification

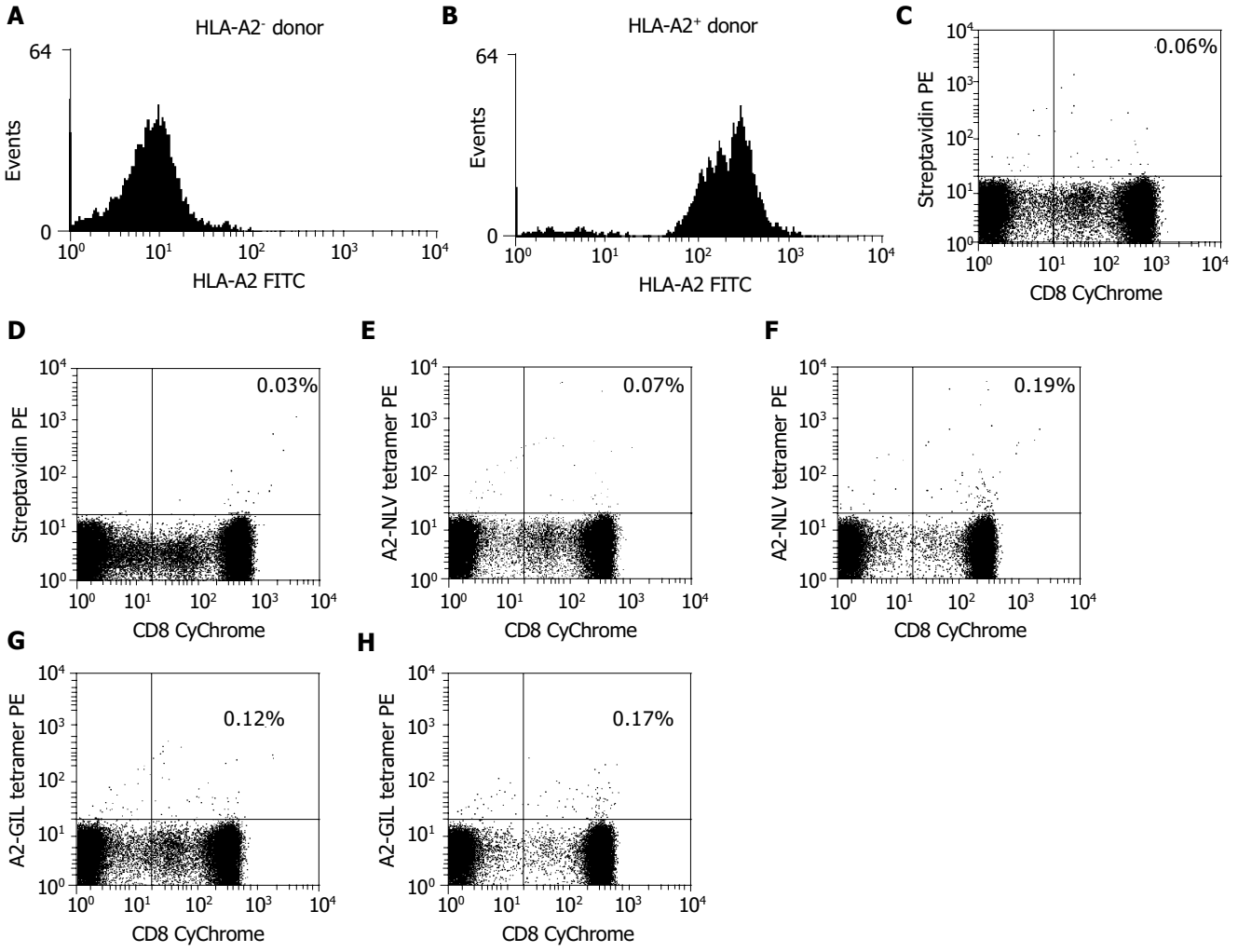

Figure 6 Flow cytometry of antigen-specific CD8+ T cells stained with A2-NLV tetramer (panels **E** and **F**) and A2-GIL tetramer (panels **G** and **H**) from both HLA-A2 positive (**B**) and negative (**A**) donors. Background staining was performed with streptavidin-PE instead of the tetramers (**C** and **D**).

of specific T cells in the peripheral blood of healthy CMV carriers using an A2-NLV tetramer demonstrated that the percentage of NLV-specific CTLs in CMV carriers is about 0.02-6.19% within the $CD8^+$ T cell population[24]. In our study, tetramer staining showed that there were 0.19% NLV-specific CTLs among total T cells from the peripheral blood of a randomly selected HLA-A2-positive volunteer over 40 years, confirming again that NLV dominates the HLA-A2-restricted cellular immune response against CMV[23]. There is substantial evidence that quantification of NLV-specific CTLs by tetramers is of clinical significance[5-7,9,11,24,25]. Despite antiviral therapy, CMV remains an important cause of morbidity and mortality after allogeneic stem cell transplantation (SCT)[26]. Through direct quantification of CMV-specific $CD8^+$ T cells using A2-NLV tetramers, several studies demonstrated that recovery of CMV-specific $CD8^+$ T cells after SCT is critical for protection against CMV disease[6,7,25]. Accordingly, enumeration of HLA-restricted, CMV-specific $CD8^+$ T cells by tetramer technology in grafts, and monitoring of these cells after SCT may constitute a rapid and sensitive tool for identifying SCT recipients at risk for developing CMV disease. Hence, tetramer technology is of value both in the development of novel transplant protocols and in clinical management of individual cases[5-7,9,25].

It is also possible to combine tetramer staining with other flow cytometry-based assays to gain more phenotypic or even functional information on antigen-specific CTLs[3,8,10,12]. For example, tetramer staining can be combined with intracellular detection of cytokines (e.g. interferon-γ), chemokines (e.g. macrophage inflammatory protein 1α), and cytotoxins (e.g. perforin/granzymes) following *in vitro* antigen/mitogen stimulation to assess the functional status of tetramer-positive T cells[27,28]. Moreover, tetramers can be used in the isolation of antigen-specific cells for characterization and even expansion of immunotherapeutic use[11,25,29]. Conjugation of a specific tetramer with antibodies against a tumor-associated antigen has been used to redirect CMV-specific CTLs to kill tumor cells by binding to the surface of tumor cells and sensitizing them to lysis by tetramer-specific CTLs, suggesting the potential therapeutic application of tetramers[30]. Thus, tetramer technology has opened new routes for further study of T-cell responses and tumor immunotherapy.

In summary, we have established a simplified and efficient procedure for generating peptide-MHC tetramers, a highly specific and very useful reagent with a number of important applications in cellular immune response studies. The present method may provide an alternative method for the preparation of MHC class I tetramers, and promote the use of this exciting technology.

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