ELSEVIER

Journal of Immunological Methods 312 (2006) 148–156

JIM
Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

Improved preparation of class I HLA tetramers and their use in detecting CMV-specific CTL

Guang-Ping Ruan [a], Li Ma [a,*], Min-Jie Meng [a,*], Yong Zhu [c], Ze-Hong Chen [c], Ying Lin [b], Zheng-Qiang Wu [b], Xiao-Wei He [b], Ju-Fang Wang [b], Xiao-Ning Wang [b,*]

[a] *Institute of Molecular Immunology, Southern Medical University, Guangzhou 510515, China*
[b] *School of Biosciences and Bioengineering, South China University of Technology, Guangzhou 510641, China*
[c] *Time Biomedics Corporation, Ltd., Guangzhou 510730, China*

Received 8 December 2005; received in revised form 17 February 2006; accepted 15 March 2006
Available online 19 April 2006

Abstract

Different methods were used to prepare HLA tetramers and the yields of each method were compared. Our results indicate that preliminary refolding of the heavy chain (Hc) and light chain (β2m) yields more monomer than the typical conventional method with urea-solubilized Hc and β2m. We then used the corresponding tetramers to detect cytomegalovirus (CMV)-specific cytotoxic T lymphocytes (CTL). Increasing data suggest that the adoptive transfer of CMV-specific CTL constitutes an effective strategy against CMV infections. We designed a method that efficiently induces CMV-specific CTL to a higher frequency in vitro than is currently achieved. This method increased the percentage of CMV-specific CTL from below 1% to 20% of PBL, accounting for more than 40% of CD8+ T cells. Successful HLA tetramer preparation provides the basis for the subsequent detection of CMV-specific CTL in clinical applications.

Keywords: Class I HLA tetramers; CMV-specific CTL; Preparation; Detection

1. Introduction

In peripheral blood, the median percentage frequency of antigen-specific cytotoxic T lymphocytes (CTL) appears to be quite low, usually no more than 1% of peripheral blood leukocytes (PBL) in most people (Aubert et al., 2001). Therefore, it is very difficult to detect these crucial cells. Various observations indicate that cytomegalovirus (CMV)-specific CTL are extremely important in the control of CMV disease (Gratama and Cornelissen, 2003; Gibson et al., 2004). Whether the frequencies of CMV-specific CTL are high or low determines the incidence of CMV disease and patient

Abbreviations: ELISA, enzyme-linked immunosorbent assay; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; HLA, human leukocyte antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline, pH 7.4; PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; FACS, fluorescence-activated cell sorter; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PE, phycoerythrin; β2m, β2-microglobulin; TMB, 3,3′5,5′-tetramethylbenzidine.

* Corresponding authors. Tel.: +86 20 87114254; fax: +86 20 87547089 (X.-N. Wang). Tel.: +86 20 61648322; fax: +86 20 61648554 (L. Ma).
E-mail addresses: maryhmz@126.com (L. Ma), mminjie@263.net (M.-J. Meng), xnwang@21cn.net (X.-N. Wang).

0022-1759/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2006.03.002

mortality (Cwynarski et al., 2001). In the past, the detection of these specific CTL was very cumbersome and time-consuming. Methods such as limiting dilution analysis, chromium release assays, ELISPOT assays and intracellular cytokine assays typically measure proliferative responses or interferon γ production but do not allow further characterization of antigen-specific CTL (Bercovici et al., 2000; Whiteside, 2000). When Altman et al. (1996) developed human leukocyte antigen (HLA) tetrameric complexes in 1996, the detection of antigen-specific CTL entered a new era. In recent years, soluble major histocompatibility complexes (MHC) have received considerable attention as tools for the identification of antigen-specific T cells (Walter et al., 1998; Kalergis et al., 2000; Greten et al., 2002; Sato et al., 2002; Komatsu et al., 2003). This method permits the direct ex vivo visualization of T cells without in vitro amplification, thus providing a more accurate picture of the in vivo immune response. The tetramers bind antigen-specific CTL with high sensitivity and specificity, thus providing a valuable tool for the detection and enrichment of antigen-specific CTL. Although tetrameric HLA-peptide complexes only refold with their corresponding peptide and are restricted to specific MHC molecules, they form the basis for monitoring specific CTL responses to infectious agents and for evaluating vaccination strategies aimed at inducing CTL responses. The CMV pp65 peptide (sequence: NLVPMVATV) has been described elsewhere and has been shown to be immunodominant; it is presented in the context of HLA-A*0201 (Diamond et al., 1997).

In this study, we prepared tetramers with the CMV pp65 peptide epitope, which could then be used to detect CMV-specific CTL in peripheral blood from HLA-A*0201 subjects. pp65-specific CTL may represent an important effector population for the early control and limitation of CMV infection and disease (McLaughlin-Taylor et al., 1994; Wills et al., 1996). In the average person, however, the pool size of these specific T cells is very small. The median percentage frequency of cells doubly positive for tetramer-phycoerythrin (PE) and CD8-fluorescein isothiocyanate (FITC) conjugates is usually no more than 1% of PBL in most people. Therefore, our goal was to design a method that would efficiently generate CMV-specific CTL, using only simple equipment and reagents. We used the CMV-specific pp65 epitope peptide in combination with interleukin (IL)-2 to increase CMV-specific CTL from 1% to 20% of PBL, accounting for more than 40% of CD8+ T cells. This high frequency of cells permits the estimation of tetramer quality. The cells can be frozen in liquid nitrogen and thawed before use. Therefore, they are useful as positive control samples for each batch of tetramer that is produced.

2. Materials and methods

2.1. Induction and expression of proteins in Escherichia coli and washing of inclusion bodies

The bacterial strains expressing the HLA-A*0201 heavy chain (Hc) and light chain (β2-microglobulin, β2m) were kindly provided by Professor Xiaoning Xu of Oxford University (Oxford, UK). The strains were cultured overnight, then inoculated into low-salt LB medium at a 1:10 ratio. The cell transformants were incubated at 37 °C; at an optical density of $OD_{600}=0.5$, the cells were induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were harvested after 5-h induction. After centrifugation (15,000×*g* for 15 min), the cell pellets were mixed thoroughly with three suspension buffers at a concentration of 1 g per 10 ml; for every 1 g of wet cells, 10 mg of lysozyme were added. The three suspension buffers differed for each method: in the first method, PBS (pH 7.4) was used; in the second method, 50 mM Tris–HCl (pH 8.0), 1 mM EDTA was used; and in the third method, 20 mM Tris–HCl (pH 8.0), 5 mM EDTA was used. The resuspended cultures were frozen at −20 °C and thawed before sonication. The cells were lysed by sonication and then centrifuged at 25,000×*g* for 15 min. The supernatants and pellets were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The results indicate that the expressed proteins were almost entirely in the pellets. Therefore, we concluded that the proteins were expressed in the form of inclusion bodies. The inclusion bodies were washed using one of three methods (see below) and dissolved in 8 M urea buffer. The insoluble material was pelleted by centrifugation at 25,000×*g* for 20 min.

Urea-solubilized Hc and β2m were aliquoted according to protein concentration and frozen until use.

2.1.1. First wash method (Hc1 and β2m1), which constitutes the standard method of preparing tetramers

The inclusion bodies were washed three times with a "Triton wash" and once with "resuspension buffer". The washed inclusion bodies were dissolved in 8 M urea buffer. The 8 M urea buffer was identical in all three washing methods. The protein concentration was determined using the Bradford method (Beyotime Biotechnology, Haimen, China), according to manufacturer's instructions. The urea-solubilized Hc and β2m were frozen at −20 °C until use. The washes and buffers used

included: (a) Triton wash: 0.5% Triton X-100, 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.1% azide, 1 mM EDTA, 1 mM dithiothreitol; (b) resuspension buffer: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol; and (c) urea buffer: 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 100 mM β-mercaptoethanol, 8 M urea (freshly prepared).

2.1.2. Second wash method (Hc2 and β2m2)

The inclusion bodies were washed once with solutions A, B, C, D and E in sequence. Solution A: 20 mM Tris–HCl (pH 8.0), 2 mM EDTA; solution B: 20 mM Tris–HCl (pH 8.0), 10 mM EDTA, 2 mM β-mercaptoethanol, 0.1% Triton X-100; solution C: 20 mM Tris–HCl (pH 8.0), 2 M urea, 2 mM EDTA; solution D: 20 mM Tris–HCl (pH 8.0), 50% isopropanol, 2 mM EDTA; and solution E: 20 mM Tris–HCl (pH 8.0). The washed inclusion bodies were dissolved in 8 M urea buffer and protein concentrations were determined before freezing.

2.1.3. Third wash method (Hc3 and β2m3)

The inclusion bodies were washed twice with solutions A, B and C, and dissolved in 8 M urea buffer. Protein concentrations were determined before freezing. The solution components were as follows. Solution A: 20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 2% Triton X-100, 2 M urea; solution B: 20 mM Tris–HCl (pH 8.0), 2 M urea, 1 mM dithiothreitol; and solution C: 20 mM Tris–HCl (pH 8.0).

2.2. Preliminary refolding of Hc and β2m

Hc or β2m was added to the refolding buffer, according to the urea-solubilized protein concentration, and the final protein concentration was adjusted to 2 mg/ml. However, it should be noted that the final protein concentration may have been less than 2 mg/ml because some proteins may have precipitated when refolding overnight at 4 °C. The refolding buffer contained 100 mM Tris–HCl (pH 8.0), 400 mM L-arginine HCl, 5 mM reduced glutathione, 0.5 mM oxidized glutathione and 2 mM EDTA.

2.3. Preparation of the monomer

In 200 ml of refolding buffer, 3 mg of peptide (dissolved in 100 μl of DMSO) was added with vigorous stirring. After 20 min, 12 mg of β2m was added with vigorous mixing. After another 20 min, 12 mg of Hc was added and the mixture was stirred continuously for 48 h at 4 °C. The refolded mixture was filtered through 0.8 μm filter paper, then concentrated to a volume of less than 5 ml with an Amicon stir cell apparatus (membrane cutoff, 10 kDa). The buffer was then converted to biotinylation buffer using a disposable PD-10 column (Amersham Pharmacia Biotech). The concentrated mixture was biotinylated overnight at room temperature with BirA enzyme in the presence of biotin, ATP, pepstatin and leupeptin. The refolded molecules were purified by fast protein liquid chromatography on a Superdex 75 column (Amersham Biosciences). The column bed volume was 124 ml and it was preequilibrated with 250 ml of buffer (20 mM Tris–HCl [pH 8.0], 50 mM NaCl). According to its molecular weight, the monomer should correspond to the second peak fraction eluted from the column.

1. The first refolding method involved urea-solubilized Hc and β2m, and was equivalent to the usual method for the preparation of tetramers.
2. The second refolding method involved preliminarily refolded Hc and urea-solubilized β2m. The Hc was initially refolded overnight at 4 °C and the protein concentration was determined. Hc (12 mg) was then added to the refolding buffer according to the standard procedure.
3. The third refolding method involved initially refolded β2m and urea-solubilized Hc. The β2m was first refolded overnight at 4 °C and the protein concentration was determined. β2m (12 mg) was added to the refolding buffer according to the standard procedure.
4. The fourth refolding method involved the preliminary refolding of both Hc and β2m. Hc and β2m were both first refolded overnight at 4 °C and the protein concentrations were determined. Both Hc (12 mg) and β2m (12 mg) were added to the refolding buffer according to the standard procedure.

2.4. Monomer identification

Each protein fraction was collected for resolution by SDS-PAGE, enzyme-linked immunosorbent assay (ELISA) and dot-ELISA. The results all indicated that the monomer was the second peak fraction eluted from the column.

The monomer was concentrated by ultrafiltration with Amicon Ultra-15 centrifugal filter devices (Millipore) and 1 μl was spotted onto a nitrocellulose membrane. The membrane was incubated with the monoclonal antibody (mAb) W6/32, which recognizes an epitope on the heavy chain but only when the heavy chain is complexed with β2m (Garboczi et al., 1992). The

Table 1
Comparison of the purity achieved with the three methods of washing inclusion bodies

Washing method	Purity of target protein after induction (%)	Purity of target protein in inclusion bodies (%)	Purity of target protein after washing (%)	Purity of urea-solubilized target protein (%)	Purity of target protein after preliminary refolding (%)
Hc1	42±6	50±4	74±4	75±2	88±6
Hc2		54±6	77±3	78±2	88±2
Hc3		52±8	87±6	89±4	97±3
β2m1	49±5	52±4	85±4	87±1	90±1
β2m2		56±7	85±2	88±1	94±2
β2m3		57±5	91±6	92±2	95±5

Values are means±standard deviations (n=3). Results indicate that the inclusion bodies washed by the third method had the highest purity (Hc3, 87%; β2m3, 91%).

secondary antibody used in the reaction was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. Binding was visualized by incubation with a diamino-benzidine reagent.

An ELISA plate was coated with 5 μg/ml W6/32 overnight at 4 °C. The wells were blocked with 5% nonfat milk in PBST at 37 °C for 45 min. A control lane with no W6/32 was included. The wells were washed three times with PBST. An aliquot (100 μl) of 2% nonfat milk in PBST was then added to each well. An aliquot (10 μl) of each chromatographic peak was added to each well. The plate was incubated at 37 °C for 45 min and

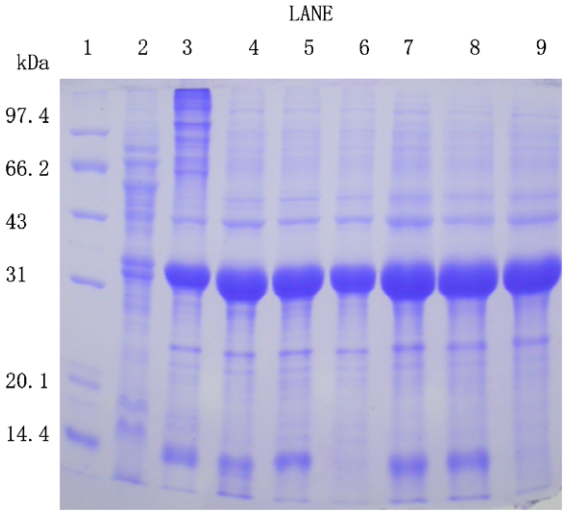

Fig. 1. Lane 1: standard proteins in kDa; lane 2: supernatant after sonication; lane 3: precipitate after sonication; lane 4: Hc1 after washing; lane 5: Hc2 after washing; lane 6: Hc3 after washing; lane 7: 8 M urea-solubilized Hc1; lane 8: 8 M urea-solubilized Hc2; lane 9: 8 M urea-solubilized Hc3. The gel was stained with Coomassie Blue R-250.

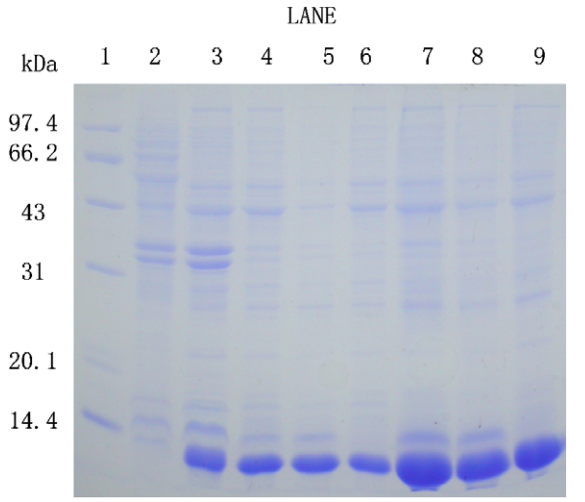

Fig. 2. Lane 1: standard proteins in kDa; lane 2: supernatant after sonication; lane 3: precipitate after sonication; lane 4: β2m1 after washing; lane 5: β2m2 after washing; lane 6: β2m3 after washing; lane 7: 8 M urea-solubilized β2m1; lane 8: 8 M urea-solubilized β2m2; lane 9: 8 M urea-solubilized β2m3. Proteins were detected with Coomassie Blue R-250.

then washed three times with PBST. A 1/50 dilution of mouse anti-human β2m-HRP conjugate in 2% nonfat milk was added and incubated at 37 °C for 45 min. After three washes with PBST, the reactions were developed using TMB (Sigma) and the ODs were recorded. Experimental readings, measured against a known positive control, were used to quantify the amount of refolded protein.

2.5. Preparation of tetramers

Tetrameric molecules were formed by the addition of PE-labeled ExtrAvidin (Sigma) at a 4:1 molar ratio. The

Table 2
Yield comparison for the three methods of washing inclusion bodies

Washing method	Yield after sonication (%)	Yield after washing (%)	Yield of urea-solubilized proteins (%)	Yield of proteins after preliminary refolding (%)
Hc1	62±5	65±9	23±4	76±10
Hc2	47±6	84±7	23.5±6.5	70±9
Hc3	38±4	89±2	25±1	77±2
β2m1	62±8	64±8	29.5±4.5	88±11
β2m2	61±13	90.5±2.5	26.5±4.5	60±6
β2m3	46±7	75±6	30±7	88±3

Values are means±standard deviations (n=3). Yields were calculated as amount of total protein divided by the amount of total protein in the previous step. Yields after sonication were calculated as the weight of inclusion bodies divided by the weight of wet cells.

Table 3
Yields from 1 l of low-salt LB medium, given as mean yields

Washing method	Wet cells (g)	Yield after sonication (g)	Yield after washing (g)	Yield of urea-solubilized proteins (mg)	Yield of proteins after preliminary refolding (mg)
Hc1	4	2.48	1.61	370	281
Hc2		1.88	1.58	371	260
Hc3		1.52	1.35	338	260
β2m1	3	1.86	1.19	351	309
β2m2		1.83	1.66	440	264
β2m3		1.38	1.04	312	275

final tetrameric complexes were stored at 4 °C in the dark until used for FACS analysis.

2.6. T2-cell peptide-binding assay

The T2 cells used were a HLA-A*0201+ TAP (transporter associated with antigen processing)-deficient cell line, in which HLA class I molecules are inefficiently loaded with endogenous peptides. When incubated with HLA-A*0201 high-affinity exogenous peptides, stable peptide-HLA-A*0201 complexes should form. Staining with FITC-conjugated W6/32, an antibody that recognizes the integral conformations of class I molecule, and its subsequent detection by flow cytometry, revealed an upregulation in the fluorescence index of the cells. Our data showed that NLVPMVATV, but not RPPIFIRRL, was the HLA-A*0201 high-affinity peptide. Previous reports have indicated that RPPIFIRRL is the HLA-B*0702 affinity peptide (Bachinsky et al., 2005). When cells were incubated with the NLVPMVATV affinity peptide, the fluorescence index increased from 53.82 to 114.11. However, when they were incubated with the RPPIFIRRL peptide, the fluorescence index showed no obvious change. The fluorescence index also showed no obvious change in the presence or absence of β2m. Therefore, as indicated in the T2-cell peptide-binding assay, β2m does not appear to be essential to the formation of integral class I HLA molecule structures.

2.7. Isolation and amplification of CMV-specific CTL

Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll gradient centrifugation from sequence-specific PCR-defined HLA-A*0201-positive donor peripheral blood. The cells were washed twice with RPMI-1640 medium before they were counted. Aliquots of PBMC (2×10^6) were resuspended in 100 μl of RPMI-1640 containing 10% fetal calf serum (FCS) and incubated with different concentrations of the NLVPMVATV peptide at 37 °C for 2 h. At the same time, parallel studies were performed with the RPPIFIRRL peptide or with no peptide as matched controls. Only a few peptide molecules were required because the volume was very small (100 μl). Then, 2 ml of RPMI-1640 containing 10% FCS were added to each sample and the samples were cultured in 24-well plates at 37 °C in a humidified 5% CO_2 incubator. On the second day, IL-2 was added to a final concentration of 100 units/ml. On the ninth day, 800 μl of the old medium was aspirated from each well and replaced with 1 ml of RPMI-1640 plus 10% FCS containing 100 units/ml of IL-2 and culture was continued. On the 15th and 18th days, an aliquot of the cells was aspirated to detect CMV-specific

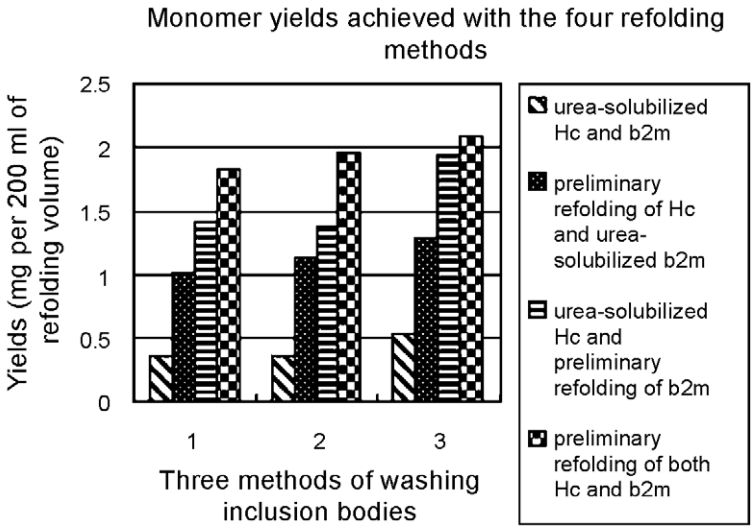

Fig. 3. Monomer yield in 200 ml of refolding buffer, prepared by four refolding methods (shown as mg per 200 ml of refolding volume).

CTL by fluorescence-activated cell sorter (FACS) analysis. After the samples had been aspirated, the contents of the wells were replenished with fresh complete medium containing 100 units/ml IL-2. The experiments were repeated at least three times with similar results.

2.8. FACS analysis

PBMC were aspirated from the wells and centrifuged at 300×*g* for 5 min. The supernatants were discarded and the cell pellets resuspended in 50 μl of cold FB (2% FCS and 0.1% sodium azide in PBS). Tetramer-PE and CD8-FITC conjugates were added at the appropriate dilutions. After incubation at 4 °C in the dark for 1 h, the cells were washed twice with cold FB and fixed in PBS containing 2% formaldehyde before they were subjected to FACS analysis. Data were acquired with a FACSCalibur flow cytometer and analyzed with CELL-Quest software (both from Becton Dickinson).

2.9. Statistical analysis

Statistical analysis (two-way analysis of variance [ANOVA]) was carried out using SPSS for Windows Version 10.0 software.

3. Results

3.1. Comparison of the three methods of washing inclusion bodies

3.1.1. Comparison of the purity achieved with the three methods of washing the inclusion bodies (Figs. 1 and 2; Table 1)

Hc and β2m were washed by one of three methods, then subjected to SDS-PAGE (Table 1). The results are shown in Figs. 1 and 2. The gels were scanned with BandScan software to determine the purity of the products. From the figures and table, it is clear that the inclusion bodies washed by the third method display the highest purity (Hc3, 87%; β2m3, 91%).

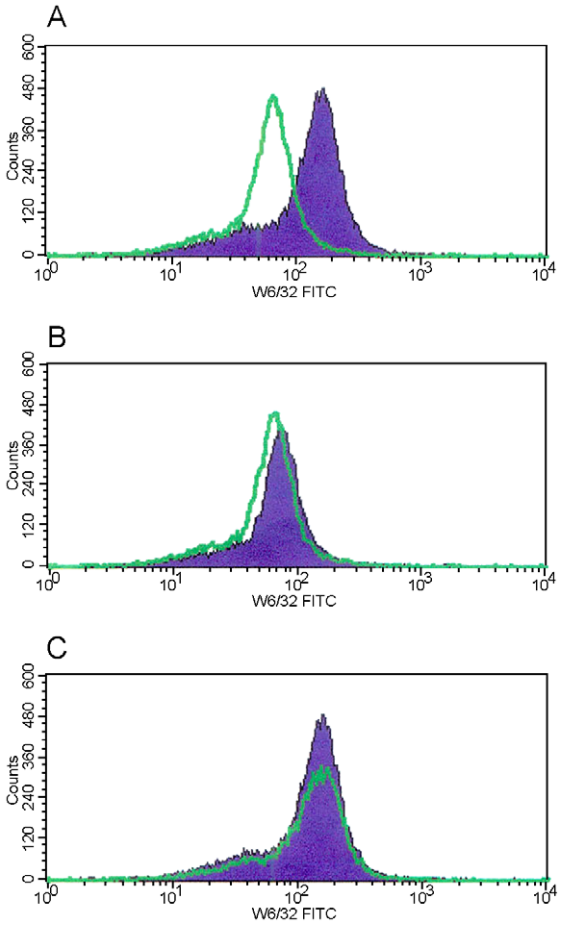

Fig. 4. (A) Bold line: T2 cells without peptide. Shaded profiles: T2 cells with NLVPMVATV peptide. (B) Bold line: T2 cells without peptide. Shaded profiles: T2 cells with RPPIFIRRL peptide. (C) Shaded profiles: T2 cells with NLVPMVATV peptide. Bold line: T2 cells with NLVPMVATV peptide and β2m. Results indicate an upregulation of the fluorescence index with no requirement for β2m.

Table 4
Statistical results

Tests of between-subjects effects
Dependent variable: yields

Source	Type III sum of squares	*df*	Mean square	*F*-value	*P*-value
Corrected model	4.199[a]	5	0.840	69.484	0.0001
Intercept	19.482	1	19.482	1611.934	0.0001
Refolding method	3.975	3	1.325	109.629	0.0001
Wash method	0.224	2	0.112	9.268	0.015
Error	7.252E−02	6	1.209E−02		
Total	23.754	12			
Corrected total	4.271	11			

R^2 = 0.983 (adjusted R^2 = 0.969).

3.1.2. Comparison of the yields obtained with the three methods of washing the inclusion bodies (Table 2)

Because once refolding only 12 mg each of Hc and β2m were required for each refolding reaction (200 ml of refolding buffer), the initially refolded proteins from 1 l of medium (260 mg) were sufficient to repeat the monomer preparation 22 times, thus ensuring an adequate monomer yield (Tables 2 and 3).

3.2. Comparison of monomer yields achieved with the four refolding methods

Our results (Fig. 3) demonstrate that, by using preliminarily refolded Hc and β2m to prepare the monomers, the yields obtained were higher than those obtained with conventional methods (urea-solubilized Hc and β2m).

The results of the statistical analysis are presented in Table 4 ($F=109.629$, $P=0.0001$) and indicate that the monomer yields for the four refolding methods were significantly different. The use of preliminarily refolded Hc and β2m to prepare the monomers resulted in the highest yield.

3.3. Monomer purification and identification

Concentrated, refolded molecules applied to the column produced four peaks. The results of SDS-PAGE, ELISA and dot-ELISA all indicate that the first peak consisted of aggregated heavy chain; the second peak consisted of monomer; the third peak consisted of monomeric β2m; and the fourth peak consisted of peptide.

3.4. T2-cell peptide-binding assay

When cells were incubated with the affinity peptide (NLVPMVATV), the fluorescence index showed an obvious increase (Fig. 4). When cells were incubated with the irrelevant control peptide (RPPIFIRRL), the fluorescence index showed no obvious change. The fluorescence index showed no change in the presence or absence of β2m. These results indicate an upregulation of the fluorescence index with no requirement for β2m.

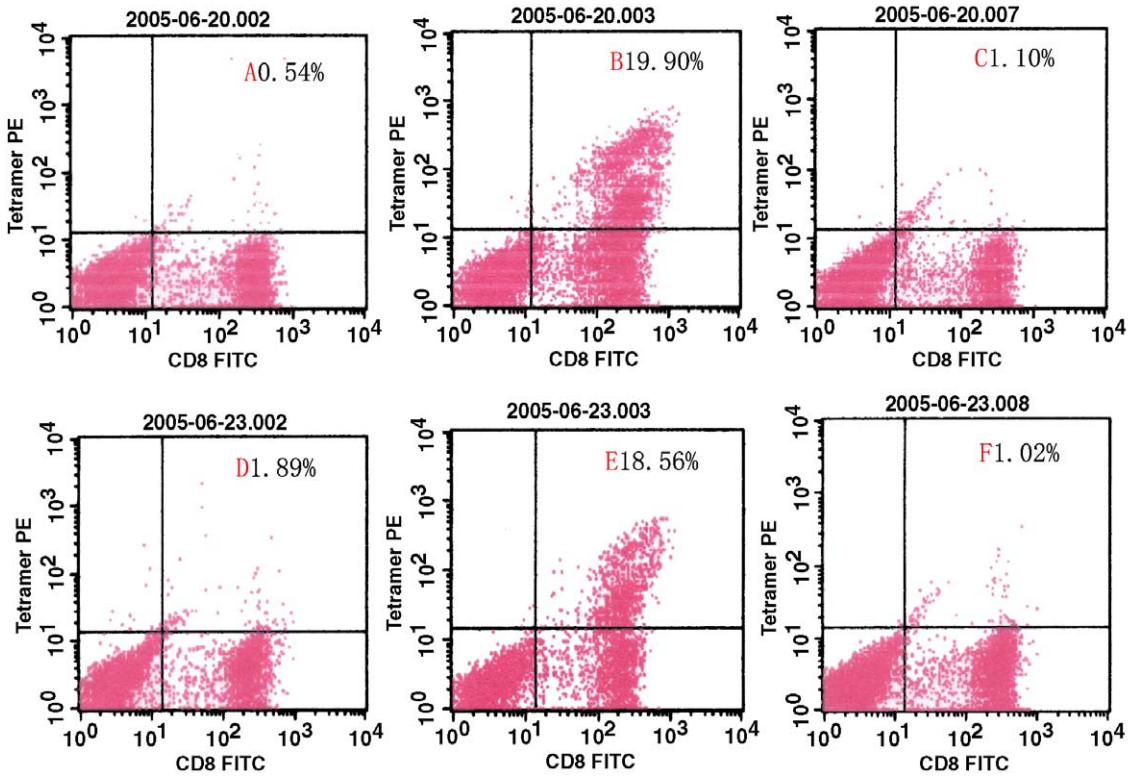

Fig. 5. Flow cytometry of PBMC. Panels A, B, C: Fifteen days after peptide stimulation. Panels D, E, F: Eighteen days after peptide stimulation. Panels A and D: Without the addition of peptide. Panels B and E: With NLVPMVATV peptide (50 μg/ml) stimulation for 2 h. Panel C: With RPPIFIRRL peptide (50 μg/ml) stimulation for 2 h. Panel F: With RPPIFIRRL peptide (100 μg/ml) stimulation for 2 h.

3.5. Detection of CMV-specific CTL

Taken together, these results indicate that the 50 μg/ml peptide concentration was optimal for the population expansion of CMV-specific CTL (Fig. 5). When a volume of 100 μl was used, only 5 μg of peptide was required to stimulate the cells for 2 h. The cells were then cultured for 15 days, at which time the percentage of CMV-specific CTL was highest, comprising 19.9% of PBL and accounting for 38% of CD8+ T cells. CMV-specific CTL were maintained at this high frequency beyond day 18, comprising 18.56% of PBL and accounting for 42% of CD8+ T cells. When the peptide concentration was then increased, the percentage frequency of CMV-specific CTL decreased, suggesting a possible immunological tolerance mechanism.

4. Discussion

The critical parameter in the course of tetramer production is the yield of monomer. We used initially refolded Hc and β2m to prepare the monomers, which achieved the greatest increase in monomer yield. Statistical discrepancies are apparent when this method is compared with conventional methods. For example, with conventional methods, 200 ml of refolding buffer yields 0.53 mg of monomer. Under identical conditions but using preliminarily refolded Hc and β2m to prepare the monomer, the monomer yield was 2.1 mg. To the best of our knowledge, this is a novel approach to the preparation of monomers and yields more monomer via a two-step refolding procedure. A possible explanation is that the initially refolded Hc and β2m are easier to refold with the peptide than are urea-solubilized Hc and β2m. The overexpression of Hc and β2m, at 200–300 mg/l (achieved in this study), coupled with a refolding yield of 2.1 mg of monomer per 12 mg of Hc and 12 mg of β2m, provides an abundant source of monomer from which tetramers can be produced for the subsequent detection of peptide-specific T cells. Moreover, the detection of antigen-specific CTL in PBMC (1×10^6) requires only 1 μg of monomer. Therefore, only 1 mg of monomer is necessary to assay 1000 samples.

Typically, the method used to estimate tetramer quality involves testing the ability of the tetramer to detect antigen-specific CTL. The pool size of antigen-specific CTL is very small in T cells (Lopez-Labrador et al., 2004; Tsai et al., 2004). Therefore, to produce positive control samples to test the quality of each batch of tetramer, a source is required in which the percentage of antigen-specific CTL exceeds 1% of PBL. In the past, a subject with the HLA-A*0201 haplotype and a CMV-specific CTL frequency higher than normal would be sought. The corresponding tetramers were then used to detect the subject's CMV-specific CTL in PBL to estimate tetramer quality. In our experiments, we efficiently induced the growth of CMV-specific CTL, expanding the population from less than 1% to approximately 20% of PBL. These cells can withstand freeze/thaw cycles and should be ideal positive control samples in the estimation of tetramer quality.

Acknowledgements

This work was supported by grants from NSFC & the Research Grant Council of Hong Kong Joint Research Fund (30170872, 30418003), 973 projects (2001CB510008, 2003CB514113) & 863 projects (2001AA215121, 2003AA215050).

References

Altman, J.D., Moss, P.A., Goulder, P.J., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., Davis, M.M., 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science 274, 94.

Aubert, G., Hassan-Walker, A.F., Madrigal, J.A., Emery, V.C., Morte, C., Grace, S., Koh, M.B., Potter, M., Prentice, H.G., Dodi, I.A., Travers, P.J., 2001. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. J. Infect. Dis. 184, 955.

Bachinsky, M.M., Guillen, D.E., Patel, S.R., Singleton, J., Chen, C., Soltis, D.A., Tussey, L.G., 2005. Mapping and binding analysis of peptides derived from the tumor-associated antigen surviving for eight HLA alleles. Cancer Immun. 5, 6.

Bercovici, N., Duffour, M.T., Agrawal, S., Salcedo, M., Abastado, J.P., 2000. New methods for assessing T-cell responses. Clin. Diagn. Lab. Immunol. 7, 859.

Cwynarski, K., Ainsworth, J., Cobbold, M., Wagner, S., Mahendra, P., Apperley, J., Goldman, J., Craddock, C., Moss, P.A., 2001. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. Blood 97, 1232.

Diamond, D.J., York, J., Sun, J.Y., Wright, C.L., Forman, S.J., 1997. Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. Blood 90, 1751.

Garboczi, D.N., Hung, D.T., Wiley, D.C., 1992. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. Proc. Natl. Acad. Sci. U. S. A. 89, 3429.

Gibson, L., Piccinini, G., Lilleri, D., Revello, M.G., Wang, Z., Markel, S., Diamond, D.J., Luzuriaga, K., 2004. Human cytomegalovirus proteins pp65 and immediate early protein 1 are common targets for CD8+ T cell responses in children with congenital or postnatal human cytomegalovirus infection. J. Immunol. 172, 2256.

Gratama, J.W., Cornelissen, J.J., 2003. Diagnostic potential of tetramer-based monitoring of cytomegalovirus-specific CD8+ T lymphocytes in allogeneic stem cell transplantation. Clin. Immunol. 106, 29.

Greten, T.F., Korangy, F., Neumann, G., Wedemeyer, H., Schlote, K., Heller, A., Scheffer, S., Pardoll, D.M., Garbe, A.I., Schneck, J.P., Manns, M.P., 2002. Peptide-b2-microglobulin-MHC fusion molecules bind antigen-specific T cells and can be used for multivalent MHC-Ig complexes. J. Immunol. Methods 271, 125.

Kalergis, A.M., Goyarts, E.C., Palmieri, E., Honda, S., Zhang, W., Nathenson, S.G., 2000. A simplified procedure for the preparation of MHC/peptide tetramers: chemical biotinylation of an unpaired cysteine engineered at the C-terminus of MHC-I. J. Immunol. Methods 234, 61.

Komatsu, H., Sierro, S.V., Cuero, A., Klenerman, P., 2003. Population analysis of antiviral T cell responses using MHC class I-peptide tetramers. Clin. Exp. Immunol. 134, 9.

Lopez-Labrador, F.X., He, X.S., Berenguer, M., Cheung, R.C., Wright, T.L., Greenberg, H.B., 2004. The use of class-I HLA tetramers for the detection of hepatitis C virus NS3-specific CD8(+) T cells in patients with chronic infection. J. Immunol. Methods 287, 91.

McLaughlin-Taylor, E., Pande, H., Forman, S.J., Tanamachi, B., Li, C. R., Zaia, J.A., Greenberg, P.D., Riddell, S.R., 1994. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J. Med. Virol. 43, 103.

Sato, Y., Sahara, H., Tsukahara, T., Kondo, M., Hirohashi, Y., Nabeta, Y., Kawaguchi, S., Ikeda, H., Torigoe, T., Ichimiya, S., Tamura, Y., Wada, T., Yamashita, T., Goto, M., Takasu, H., Sato, N., 2002. Improved generation of HLA class I/peptide tetramers. J. Immunol. Methods 271, 177.

Tsai, S.L., Lee, T.H., Chien, R.N., Liao, S.K., Lin, C.L., Kuo, G.C., Liaw, Y.F., 2004. A method to increase tetramer staining efficiency of CD8+ T cells with MHC-peptide complexes: therapeutic applications in monitoring cytotoxic T lymphocyte activity during hepatitis B and C treatment. J. Immunol. Methods 285, 71.

Walter, J.B., Garboczi, D.N., Fan, Q.R., Zhou, X., Walker, B.D., Eisen, H.N., 1998. A mutant human beta2-microglobulin can be used to generate diverse multimeric class I peptide complexes as specific probes for T cell receptors. J. Immunol. Methods 214, 41.

Whiteside, T.L., 2000. Monitoring of antigen-specific cytolytic T lymphocytes in cancer patients receiving immunotherapy. Clin. Diagn. Lab. Immunol. 7, 327.

Wills, M.R., Carmichael, A.J., Mynard, K., Jin, X., Weekes, M.P., Plachter, B., Sissons, J.G., 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J. Virol. 70, 7569.