In vitro and in vivo identification of a novel cytotoxic T lymphocyte epitope from Rv3425 of Mycobacterium tuberculosis

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ABSTRACT

The identification of novel cytotoxic T lymphocyte (CTL) epitopes is important to analysis of the involvement of CD8+ T cells in Mycobacterium tuberculosis infection as well as to the development of peptide vaccines. In this study, a novel CTL epitope from region of difference 11 encoded antigen Rv3425 was identified. Epitopes were predicted by the reversal immunology approach. Rv3425-p118 (LIASNV AGV) was identified as having relatively strong binding affinity and stability towards the HLA-A*0201 molecule. Peripheral blood mononuclear cells pulsed by this peptide were able to release interferon-γ in healthy donors (HLA-A*02+ purified protein derivative+). In cytotoxicity assays in vitro and in vivo, Rv3425-p118 induced CTLs to specifically lyse the target cells. Therefore, this epitope could provide a subunit component for designing vaccines against Mycobacterium tuberculosis.

Key words: Cytotoxic T lymphocyte, epitope, Mycobacterium tuberculosis, Rv3425.

Tuberculosis continues to be a major global health problem. Although there has been considerable success in addressing this problem, the number of new cases continues to grow, approaching 10 million in 2010; in developing countries, the problem is particularly serious (1). The attenuated Mycobacterium bovis strain (BCG), which has been the only TB vaccine used in almost a century, confers highly variable protection (2). The increasing emergence of multi-drug resistant and extensively drug resistant strains of M. tuberculosis and the lack of an effective vaccine against the infectious form of TB have made TB control even more problematic (3, 4).

Cellular immunity is essential for fighting infections caused by intracellular pathogens, including M. tuberculosis (5). Epitope-specific M. tuberculosis reactive CD8+ T cells are reportedly present in very large numbers in the circulation of PPD (purified protein derivative) positive individuals and patients with active TB, which indicates the importance of CD8+ T cells in immunity to M. tuberculosis (6, 7). It also emphasizes that vaccines that activate CD8+ T cell subsets could be effective tools for controlling M. tuberculosis. The bottleneck for the development of these peptide vaccines is identification of CTL epitopes. Many major histocompatibility complex class I-restricted
CTL epitopes continue to be identified from antigens of *M. tuberculosis* (5, 8–10). Genome comparative analysis has revealed that several genomic regions of *M. tuberculosis* are deleted in BCG and other mycobacteria. These deleted regions (RDs) have been predicted to encode over 100 proteins. To overcome cross-reaction between BCG vaccine and *M. tuberculosis*, great efforts have been made to identify antigens that are missing in BCG but present in *M. tuberculosis*, such as those encoded by the genes in the RD region (11). Rv3425, an RD-11 region encoded protein, is a novel immunodominant antigen in *M. tuberculosis* that induces humoral and cellular immune responses in mice. In the BCG genome, the nucleotide sequence for the C-terminal fragment of Rv3425 is absent; however, its N-terminal fragment is conserved as a fusion protein with the N-terminal fragment of Rv3429. This fusion protein is annotated as BCG3495 in the BCG Pasteur genome. Rv3425 is expressed in *M. tuberculosis* H37Rv during exponential growth in vitro, and recognized by patients with both pulmonary and extra-pulmonary TB (12, 13). Therefore, we deduced that Rv3425 might contain CD8+ T cell epitopes.

Because of the high prevalence of the HLA-A2 super-type in Chinese subjects, we performed reversal immunology approach to search for potential HLA-A*02*-restricted epitopes in Rv3425. We identified novel cytotoxic T lymphocyte epitopes by *in vitro* and *in vivo* assays.

**MATERIALS AND METHODS**

**Human subjects**

Peripheral heparinized venous blood samples were obtained from seven healthy donors (HLA-A*02*+ PPD+). The ethics committee of Zhengzhou University approved this sample collection.

**Cell line**

A human TAP-deficient T2 cell line (14) transfected with HLA-A*0201* was cultured in IMDM supplemented with 10% FBS in an incubator with a humidified atmosphere containing 5% CO2. The T2 cell line was provided by Professor Yu-Zhang Wu (Third Military Medical University, China).

**Peptide prediction and synthesis**

The internet services of BIMAS, SYFPEITHI and NetCTL (15–17) were all used for epitope prediction. Native peptides with prediction scores ranked in the top three by at least two prediction tools were selected. A standard solid phase Fmoc strategy was used for 9-mer predicted peptides synthesis. The products were purified to more than 95% purity by RP-HPLC and their sequences confirmed by ESI-MS. As a positive control for the HLA-A*0201* binding assay, COX-2_321 (ILIGETIKI) was used (18). The IAβ-restricted HBV core antigen-derived Th epitope (sequence 128–140: TPPAYRPPNAPIL) was used as a T helper epitope in the *in vivo* assay (19, 20).

**Transgenic mice**

HLA-A2.1/Kb transgenic mice were kindly supplied by Professor Xue-Tao Cao (Second Military Medical University, China) (21). The mice were bred and maintained in specific pathogen-free facilities. In the present experiment, mice at 8 to 12 weeks of age were used.

**T2 binding affinity**

To determine the binding properties of the candidate peptides toward the HLA-A*0201* molecule, up-regulation of peptide-induced HLA-A*0201* molecule on T2 cells was examined according to a previously described protocol (22). Briefly, T2 cells were incubated with peptides (50 μg/mL) in serum-free IMDM containing 3 μg/mL human β2-M (Sigma, St Louis, MO, USA) at 37°C for 18 hr. The cells were then washed three times, the cells were then treated with fluorescein isothiocyanate-labeled goat IgG anti-mouse immunoglobulin (Sigma). The cells were then harvested and analyzed by a FACSCalibur flow cytometer (Becton Dickinson, Sparks, MD, USA). The FI was calculated by the following formula: FI = (MFI sample - MFI background)/MFI background, where MFI background represents the value without the peptide.

**Peptide/HLA-A*0201* complex stability assay**

T2 cells were incubated with peptides (50 μg/mL) in serum-free IMDM medium containing 3 μg/mL β2-M at 37°C for 18 hr. The cells were then washed to remove free peptides and incubated with 10 μg/mL brefeldin-A for 2, 4 and 6 hr. The cells were then washed twice, stained and analyzed by flow cytometer. The DC50 was defined as an estimate of the time required for 50% reduction of the MFI value recorded at time 0.

**In vitro expansion of peptide-specific CD8+ T cells**

Induction of CTLs in vitro was performed in accordance with the procedures previously described (23). PBMCs were separated from the peripheral blood of HLA-A*02*+ PPD+ healthy donors by Ficoll-Hypaque density gradient centrifugation. These cells were then stimulated every 7 days with 9-mer peptides (10 μg/mL) and β2-M.
(3 μg/mL) in IMDM medium supplemented with 10% FBS. On the third day of the first stimulation and after each subsequent stimulation, human recombinant IL-2 (50 U/mL) was added. The cells were employed for further experiments on day 21.

**In vivo expansion of peptide-specific CD8+ T cells**

Three groups of HLA-A2.1/Kb transgenic mice (four mice in each group) were immunized at the base of the tail with 100 μg of various peptides and 140 μg of the Th epitope prepared in IFA on days 0, 5 and 10 (24, 25). One group of mice receiving IFA containing PBS and another Th peptide were used as negative controls. On day 11, spleen lymphocytes (5 × 10^7 cells in 10 mL) were separated and re-stimulated with peptide (10 μg/mL) in vitro. On day 7 after re-stimulation, the specific cytotoxicity assay was employed.

**Enzyme-linked immunospot assay for interferon-γ**

A commercial kit (human IFN-γ precoated ELISPOT kit, Dakewe Biotech, Shenzhen, China) was used for ELISPOT assay. Effector cells (1 × 10^5) and stimulator cells (peptide-pulsed T2 cells, 1 × 10^5) were co-cultured in 96-well microplates coated with antibody specific for human IFN-γ (26). After incubation at 37°C for 16 hr, the cells were removed and the plates processed. The number of spots was determined automatically using a computer-assisted spot analyzer (Dakewe Biotech).

**Cytotoxic activity of peptide-specific T cells**

A non-radioactive method for assaying cytotoxic T lymphocytes was employed. It was based on measurement of the LDH released from target cells at graded E:T ratios (12.5:1, 25:1 and 50:1, CTLs from the PBMCs of healthy donors; 20:1, 40:1 and 80:1, CTLs from the spleen lymphocytes of transgenic mice) (26). Briefly, T2 cells loaded with 10 μg/mL peptide at 37°C for 4 hr were used as target cells. The target cells (1 × 10^4/well) were then cocultured with various numbers of effector cells at 37°C for 5 hr. The percentage of specific lysis of the target cells was determined according to the following equation: percentage of specific lysis = ([experimental release - effector spontaneous release - target spontaneous release]/[target maximum release - target spontaneous release]) × 100.

**Statistical analysis**

All data are expressed as means ± S.D. Significance was analyzed by one way analysis of variance. P < 0.05 was considered significant. All statistical analyses were performed with SPSS 10.0.

**RESULTS**

**Prediction and synthesis of candidate peptides**

First, we selected three candidate peptides (p118, p43, and p29) derived from Rv3425 protein with optimal predicted scores (prediction score ranked in the top three by at least two prediction tools) towards the HLA-A*0201 molecule for further study (Table 1). Rv3425-p118 peptide is involved in the N-terminal fragment of Rv3425 and conserved in the virulent strain of *M. bovis* and BCG. We confirmed the molecular weights of the peptides by ESI-MS (Table 2).

**Relative affinities and stabilization capacities of the synthetic peptides**

To evaluate the binding affinities of these peptides to the HLA-A*0201 molecule and the stability of the peptide/HLA-A*0201 complexes in vitro, we used binding affinity and stability assays. We labeled the affinities and stabilization capacities of the nonamer peptides as FI and DC_{50}, respectively (Table 2). Of the three candidates, Rv3425-p118 (LIAASNAGV) showed the highest binding affinity and stability (DC_{50} > 2 hr) towards HLA-A*0201. We therefore chose this peptide for further study.

**Rv3425-p118 elicits interferon-γ production by T cells from purified protein derivative positive healthy donors**

We tested by an ELISPOT assay whether peptide-specific CTLs can cause release of IFN-γ from expanded PBMCs from HLA-A*02+ PPD+ individuals when they have been challenged with the peptides in vitro. We stimulated PBMCs from seven donors with Rv3425-p118, Rv3425-p43 and Rv3425-p29. We used PBS and PHA as negative and positive controls, respectively. Rv3425-p118 induced the PBMCs of two donors (D1 and D5) to produce IFN-γ (Fig. 1). PHA, a positive control, elicited IFN-γ production by PBMCs from all seven donors; PBS did not elicit IFN-γ production by any of them (partial data not shown).

**In vitro cytotoxic activity of peptide-specific T cells**

In addition to performing the IFN-γ release assay, we carried out an LDH release assay to test the cytotoxic activities of the CTLs induced by Rv3425-p118. We investigated whether Rv3425-p118 triggers specific and functional CTL
A novel CTL epitope of Rv3425

Table 1. Prediction of HLA-A*0201 restricted epitopes from Rv3425 by NetCTL, SYFPEITHI, and BIMAS

| Peptide | Sequence   | Scores (Rank) |            |            |
|---------|------------|---------------|------------|------------|
|         |            | NetCTL   | SYFPEITHI | BIMAS     |
| p118    | LIASNVAGV  | 1.0900 (1)  | 29 (1)    | 37.393 (4) |
| p43     | SLEDELDEL  | 1.0680 (2)  | 28 (2)    | 6.317 (10) |
| p29     | QLRELAYSV  | 0.9259 (3)  | 26 (3)    | 21.672 (6) |

Table 2. ESI-MS data and the HLA-A*0201 binding affinity and stability of the candidate peptides

| Protein | Peptide | ESI-MS [M + H]+ | Calculated | Observed  | FI       | DC50     |
|---------|---------|-----------------|------------|-----------|----------|----------|
| Rv3425  | p118    | 843.1           | 843.2      | 0.79      | >2 hr    |
|         | p43     | 1062.1          | 1062.2     | 0.64      | <2 hr    |
|         | p29     | 1078.2          | 1078.3     | 0.32      | <2 hr    |
| COX-2   | p321a   | 999.6           | 1000.3     | 1.16      | >4 hr    |

DC50, half-life of dissociation of peptide/HLA-A*0201 complexes; FI, (MFI of the peptide - MFI background)/MFI background.

aPositive peptide.

In vivo induction of peptide-specific cytotoxic T lymphocytes in HLA-A2.1/Kb transgenic mice

We proved that CTLs induced by Rv3425-p118 do lyse target cells in vitro. However, the in vivo environment is more complex. It is more important to determine whether the peptide can be naturally processed, presented, and then induce peptide-specific CTLs in vivo. Therefore, we used HLA-A2.1/Kb transgenic mice as in vivo animal model. As shown in Fig. 3, the percentage of specific lysis of Rv3425-p118 increased to 13.7% at an E:T ratio of 80:1.

Although the mice were immunized with the epitope, their body weights were not significantly different from those of the control group mice, which indicates that Rv3425-p118 might have low toxicity (data not shown). All these in vivo results suggest that Rv3425-p118 can be naturally processed and presented and induce potent peptide-specific CTL responses in vivo.

DISCUSSION

Traditionally, identification of T cell epitopes has required synthesis of overlapping peptides that span the entire length of a protein, followed by experimental assays such as in vitro intracellular cytokine staining for each peptide to assess T cell activation. This method is economically viable only for single proteins or pathogens that consist of several proteins. Therefore, researchers have developed alternative computational approaches for predicting T cell epitopes that have significantly decreased the experimental burden previously associated with epitope identification. In practice, using only one computational algorithm to
predict CTL epitopes may lead to large numbers of false positives and false negatives. More recently, comprehensive validation using data from several different types of prediction software has proved accurate and has considerably reduced the cost of epitope discovery. However, because the different prediction tools are based on different computational algorithms, their predictions can be different. If we do not choose with care, we can miss the immunodominant epitopes. Therefore, in this study we chose three prediction tools based on different computational algorithms to achieve results that are more accurate.

One of the approaches to finding a new generation of effective and safe vaccines is epitope-based DNA vaccination, which enables focusing of the immune response on important and highly conserved epitopes (27). This approach provides the opportunity to use specific epitopes to shift the immune system toward a Th1- or Th2-mediated immune response and eliminate unwanted responses. In addition, CTL epitope-based immunization has the advantage of eliciting an immune response only against the protective epitope and avoidance of epitope drift in M. tuberculosis infections (28). Studies of M. tuberculosis in humans have shown that induction of broad T-cell-mediated immunity to M. tuberculosis and type 1 cytokines, including IL-2, IFN-γ and TNF-α, could be essential to TB vaccine design (29). Vaccines based on CTL epitopes represent a logical approach to generating effective cellular immunity in both the prophylactic and therapeutic settings because multiple epitopes can be incorporated into the vaccine design with the goal of inducing broadly reactive responses by multiple CTL clones directed against different epitopes. Multi-CTL epitope DNA vaccines have been reported to induce broad CTL responses against HIV, HBV, severe acute respiratory syndrome coronavirus and others (30–32). Whereas epitope-based vaccines are limited with respect to HLA polymorphism and population coverage, the use of supertype-restricted epitopes provides a means to address this problem (33).

In conclusion, we have identified a novel HLA-A2-restricted T cell epitope derived from Rv3425. Our results show that Rv3425-p118 (LIASNVAGV) could serve as a candidate for a peptide vaccine against M. tuberculosis.

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DISCLOSURE

There are no conflicts of interest.

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