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Identification and Characterization of HLA-A*0301 epitopes in HIV-1 gag proteins using a Novel Approach

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Abstract

Identification of CTL epitopes correlated to immune protection is important for the development of vaccines that enhance T cell-mediated immune responses. The correlation of positively selected amino acids (PS) of HIV-1 with host HLA alleles can identify regions containing potential T cell epitopes. However, the specific epitopes have to be identified and characterized using overlapping peptides through T cell functional assays. In this study we used a new approach to identify and characterize potential epitopes in the gag region containing PS mutations that significantly correlated with HLA-A*0301. The iTopia Epitope Discovery System was used to rapidly screen a panel of peptides overlapping the regions containing PS mutations and the peptides identified were assessed for relative affinity and complex stability. The potential epitopes were then validated by interferon gamma (IFN- γ) ELISpot assays with patient PBMCs. Using this approach we identified/confirmed the predicted HLA-A*0301 epitopes in two regions of gag containing PS mutations V7I and K403R, one previously reported and the other novel. Five of the seven peptides that bound to A*0301 contained the K403R mutation and corresponded to the documented LARNCRAPRK-A3 supertype epitope. Two epitope variants, RASVLSGGK and RASILSGGK containing the V7I mutation, were identified using the iTopia Epitope Discovery System, however only the consensus variant (RAK9C) was confirmed using the ELISpot assay and it represents a novel A*0301 epitope.

Keywords

HIV-1; T-cell Epitopes; iTopia Epitope Discovery System; Gag

1. Introduction

The most effective method of controlling infectious disease is through vaccination, however, a comprehensive understanding of HIV-1 immunopathogenesis and specific T-cell efficacy is

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required for HIV-1 vaccine development (Westrop et al., 2009). CTLs play an important role in host defense against HIV-1 by recognizing and eliminating infected cells (Cellini et al., 2008; Kitano et al., 2008; Westrop et al., 2009; Bangham, 2009). Identification of CTL epitopes correlated to immune protection is important for the development of vaccines that enhance T cell-mediated immune responses.

One of the most important immune targets in many studies is the HIV-1 Gag protein (Westrop et al., 2009). It is one of the most conserved viral proteins in HIV-1 and it is known to induce protective T-cell responses in animal models and in humans, making it a good candidate for development of an anti-HIV vaccine (Cellini et al., 2008; Kiepiela et al., 2004; Zuniga et al., 2006). Identification of CTL epitopes in this region is important and will help to understand host and virus interactions and contribute to vaccine development (Bangham, 2009). Many theoretical and empirical techniques have been used for epitope identification including predictive algorithms, defective cell lines, shotgun methods and peptide elution techniques (Shingler et al., 2008). These methods can be inaccurate and labor intensive given the large number of antigens and their variations and the diversity of Human Leukocyte Antigens (HLA) (MHC) (Frahm et al., 2006; Shingler et al., 2008). The iTopia Epitope Discovery System can be used to rapidly screen, identify and characterize T cell epitopes. The cell free system in combination with traditional ELISpot assays can accelerate T cell epitope identification and vaccine research and development.

Our previous study of HIV-1 *gag* in a large HIV-1-infected cohort (N=431) identified three positively selected (PS) amino acids, V7I and I34L in the p17 region of *gag* (unpublished) and K403R in the p7 region of *gag* (Peters et al., 2008). The K403R mutation was found to be significantly associated with A*0301 after multiple test corrections in Peters et al., 2008 and following reanalysis of the raw data, the V7I and I34L mutations were also found to be associated with A*0301. The significant associations suggests that these regions may contain potential T cell epitopes for A*0301. In this study we utilized the cell free iTopia Epitope Discovery System and rapidly assessed peptide:MHC binding capacity, stability and affinity to A*0301 in this region. We confirmed the identified potential CD8+ T-cell epitopes of HLA-A*0301 using peripheral blood mononuclear cells (PBMC) from A*0301 positive patients in ELISpot assays.

2. Materials and Methods

2.1. Study population

Subjects involved in this study were HIV-1 positive, antiretroviral therapy-naïve women (at the time of sampling) followed longitudinally in a sex worker cohort established in 1985 in the Pumwani District of Nairobi, Kenya (Fowke et al., 1996). All subjects gave informed consent to participate, and the study has been approved by the Institutional Review Boards at the Universities of Manitoba and Nairobi.

2.2. HLA class I typing

Genomic DNA was isolated from all study participants. PCR amplification of HLA-A, -B and -C genes was carried out using gene specific primers. Products were sequenced using the ABI 3100 Genetic Analyzer® (Applied Biosystems, Foster City, CA). Sequence outputs were genotyped with CodonExpress software developed based on a taxonomy-based sequence analysis (TBSA) (Luo et al., 1999).

2.3. p7 and p17 sequencing and analysis

The HIV-1 *gag* gene was amplified from genomic DNA of all study subjects. When multiple dominant quasispecies were present, the PCR products were cloned and analyzed. p7 and p17

sequences were analyzed with Sequencher 4.5 and aligned with Mega[®] 3.0 (Kumar et al., 2004). Quasi, a selection-mapping algorithm, was used to identify the PS amino acids (Stewart et al., 2008) shown in Table 1. SPSS 11.0 was used to correlate the PS amino acids with HLA data. In cases where multiple sequences were obtained, all sequences were analyzed for the presence or absence of a PS residue, ensuring that each subject was counted only once (Peters et al., 2008). In a 2×2 crosstabulation where all expected counts are above 10, Pearson's chi-square test was used to determine associations between the presence of a specific HLA allele and positive selection at a given site (Peters et al., 2008). For any cell that contains an expected count below 10, the Fisher's Exact test was used (Peters et al., 2008).

The original data from Peters et al., 2008 was re-analyzed for association with A*0301 in this study to ensure that we did not miss any real associations due to false negatives as a result of multiple comparisons. The V7I and I34L were both found to be significant in this study even though they missed the adjusted p value cutoff in the previous study (Peters et al., 2008).

2.4. Peptides synthesis for in vitro cell free screening with iTopia Epitope Discovery System

A total of 50 overlapping peptides (JPT Peptide Technologies GmbH, Berlin, Germany) were designed in sequences of 9 amino acid residues overlapping by 8, to span the regions containing the PS mutations in p17 and p7 (Table 2). The library contained peptides with the consensus residue and a PS residue at various positions. Control peptides were supplied with the iTopia Epitope Discovery System Kit (Beckman Coulter Inc., San Diego, CA.). Peptides were suspended in dimethyl sulfoxide (Sigma, Poole, UK) to a concentration of 10mM and stored at -20°C.

2.5. Binding, affinity and complex stability assays of the iTopia Epitope Discovery System

HLA-A*0301 specific iTopia kits were purchased from Beckman Coulter Inc. (San Diego, CA) and included microtitre plates coated with the A*0301 biotinylated MHC monomer folded correctly via β_2 microglobulin (β_2 M) and a placeholder peptide. These iTopia kits include the monomer-coated plates, assay buffers, anti-HLA-ABC-FITC conjugated monoclonal antibody, β_2 M and a positive control peptide (Shingler et al., 2008).

The iTopia system combines 3 assays to identify and characterize epitopes: Peptide Binding Assay, Affinity Assay and Off-Rate Assay (complex stability assay). The Peptide Binding Assay evaluated the ability of all 50 peptides to bind A*0301 under optimal conditions (11 μ M peptide incubated with β_2 M, anti-MHC mAb and plate bound MHC heavy chain at 21°C for 18 hours) (Shingler et al., 2008) using a fluorimeter (Spectra Max Gemini from Molecular Devices). Results were imported into the iTopia software and expressed as a percentage of the positive control (allele specific provided in the kit). Peptides showing binding of at least 30% of the positive control peptide were selected to be analyzed for affinity at a range of concentrations (10^{-4} through 10^{-9}), which is expressed as an ED50 value. ED50 represents the concentration at which the peptide is bound to the MHC at 50% maximum binding efficiency and is the mid-point of the sigmoidal dose response curve (Beckman Coulter Inc., 2003). Peptides were also analyzed for off-rate (complex stability) where $t_{1/2}$ represents the time taken for a reduction from optimal binding of 50% after a shift to dissociating conditions (Beckman Coulter Inc., 2003).

Peptide binding, affinity and off rate values were calculated using the iTopia software and Graphpad Prism[®]. ED50 was determined using a sigmoidal dose-response curve and $t_{1/2}$ was determined using a one phase exponential decay curve (Shingler et al., 2008).

2.6. ELISpot Peptides

Peptides identified by the iTopia Epitope Discovery system were synthesized (Sigma Genosys, Oakville, ON) for confirmation with interferon gamma (IFN- γ) ELISpot assays using A*0301 patient PBMCs.

2.7. Validation of Epitopes using ELISpot Assays

To determine whether peptides identified by the iTopia Epitope Discovery System were epitopes capable of stimulating T-cell responses, functional IFN- γ ELISpot assays were carried out as previously described (Semeniuk et al., 2009). Responses were considered positive if there were at least 50 SFU/million PBMC after background subtraction and the positive control was successful.

3. Results

3.1. Correlation analysis of positively selected amino acids in p17 and p7 with patient HLA alleles

Data from our previous study identified PS residues in the p17 and p7 regions of HIV-1 gag by quasi analysis. Re-analysis of A*0301 associations with three PS residues identified two more significant associations; V7I ($p = 0.0158$) and I34L ($p = 0.0096$) in the p17 region of gag (Table 1) and confirmed previously reported associations of K403R ($p = 0.0046$) in the p7 region of gag (Peters et al., 2008). These significant correlations suggest that these regions may contain epitopes of A*0301 exerting pressure on the virus to mutate. To explore these possibilities the iTopia Epitope Discovery System was used to screen 50 peptides spanning the three regions of interest.

3.2. HLA-A*0301 Peptide Binding analysis using the iTopia Epitope Discovery System

The Peptide Binding Assay is the first assay of the system that characterizes each peptide's ability to bind HLA-A*0301 relative to the positive control (Beckman Coulter Inc., 2003). All 50 overlapping 9-mer peptides designed to span the amino acid positions under selective pressure (V7I, I34L, and K403R) in the p17 and p7 region of HIV-1 gag were screened for their ability to bind HLA-A*0301 under optimal conditions (Table 2). Seven peptides bound A*0301 above the threshold of 30% of the positive control peptide (Figure 1). The K403R region of p7 contained 5 "hits" above 30%: LARNCRAPR (LR9s), AKNCRAPRK (AK9c), ARNCRAPRK (AK9s), KNCRAPRKK (KK9c), and RNCRAPRKK (RK9s) (Figure 1a). The first four peptides have been reported in the Los Alamos Database (Los Alamos Database, 2005-2006) and the last one (RK9s) may be novel. The V7I region of p17 contained two "hits" for A*0301, RASVLSGGK (RAK9c) and RASILSGGK (RAK9s) (Figure 1b). Neither peptide had been previously reported and appears to be novel. A few peptides within the I34L region had elevated binding to A*0301 (Table 2 and figure 3c) compared to the rest of the I34L peptides, however they did not make the 30% cutoff so additional testing was not carried out. All the peptides with binding above the 30% of the positive control peptide were evaluated for their binding affinity and off-rate using the iTopia System and are selected for epitope confirmation with IFN- γ ELISpot analysis using A*0301+ subject PBMC's.

3.3. Evaluation of binding Affinity and Off-rate stability of the identified peptides

The iTopia Epitope Discovery system was also used to analyze the affinity and off-rate of the identified peptides. The affinity of the peptides was measured by assessing the strength of the binding at various peptide concentrations relative to the positive control peptide (Figure 2) and expressed as the molar concentration giving half-maximal binding [ED50 (M)] (Table 3) (Shingler et al., 2008). The ED50 of the seven A*0301 binding peptides ranges from 1.91×10^{-05} to 8.43×10^{-05} .

The off-rate assay indicated the time it takes for half maximal dissociation of the peptide from the MHC complex, expressed as $t_{1/2}$ (Shingler et al., 2008). Most of the peptides have a $t_{1/2}$ less than 1 hour, however AKNCRAPRK (R402K of p7) had the highest stability with a $t_{1/2}$ of 17.954 hours (Figure 3 and Table 3).

3.4. Epitope Validation by IFN- γ ELISpot assay

A total of eleven A*0301 patients were tested for all seven peptides identified using the iTopia Epitope Discovery system. At least one positive response was detected for each identified peptide (Table 3), except the RAK9s peptide. We were able to identify and confirm 4 epitopes, LR9c, AK9c, AK9s, and KK9s, previously reported in the HIV database (Los Alamos Database, 2005-2006) in addition to a novel A*0301 epitope variant. The results showed that combining the cell free iTopia Epitope Discovery system for peptide screening with IFN- γ ELISpot assays using A*0301 subject PBMCs can efficiently identify and characterize T cell epitopes in regions containing PS mutations.

4. Discussion

Our previous studies demonstrated the utility of combining bioinformatical analysis with IFN- γ ELISpot assays in identifying and characterizing potential T cell epitopes in HIV gag regions containing PS mutations (Semeniuk et al., 2009; Peters et al., 2008). In this study we showed that using the iTopia Epitope Discovery system to screen overlapping peptides covering regions containing PS mutations can rapidly identify peptides binding to the specific HLA class I alleles and characterizes the binding properties of the identified peptides. This method can reduce the number of peptides that need to be analysed by ELISpot assays with precious patient samples. Epitopes identified by correlating HLA and PS residues are immunologically relevant because they are identified as the result of interactions between pathogens and host immune pressure.

The iTopia Epitope Discovery System is a rapid method used to screen overlapping peptides around regions of interest. The identified peptides can then be confirmed with functional IFN- γ ELISpot assays. It has been shown that in most chronic cases of HIV-1 infection, there is a lack of IL-2 production by CD4+ T-cells, thus proliferation may not be seen when the cells are stimulated with HIV antigen (Westrop et al., 2009). However, cytokines IFN- γ and TNF- α are not affected (Westrop et al., 2009) making IFN- γ ELISpot assays the best method for epitope detection.

One of the limitations of the iTopia Epitope Discovery system is the availability of alleles that can be used for epitope screening. Currently, eight HLA class I allele are available, therefore this cell free rapid peptide screening method is restricted. We chose to assess epitopes specific for one of these alleles, A*0301, following identifications of significant associations between PS mutations in HIV-1 gag with A*0301 by reanalyzing the PS data from our previous study (Peters et al., 2008).

Relative affinity is an important measure of MHC:peptide binding capability, however the off-rate or complex stability determines the time it takes for the MHC:peptide complex to reach the cell surface and interact with the T-cell receptor (TCR) (Shingler et al., 2008). By assessing both parameters we can eliminate the low affinity and low complex stability epitopes and enrich for high affinity and high complex stability epitopes (Shingler et al., 2008). This will increase the chances of identifying true CTL epitopes because the more stable the MHC:peptide interaction, the longer the peptide will be presented on the cell surface and higher density of presented peptide leading to increased T-cell response and possibly limited replicative ability of the virus (Westrop et al., 2009). Ranking peptides based on affinity (from high to low, RAK9c, RAK9s, AK9c, RK9s, LR9s, AK9s and KK9c) and off-rate (from high to low, AK9c, RAK9c, RAK9s, RK9s, AK9s, LR9s, KK9c) revealed some consistencies. The two novel

epitopes, RAK9c and RAK9s, rank in the top 3 for high affinity and high stability. The comparison between the consensus and selection variants of each pair of peptides in binding, affinity and off-rate showed that in some cases the consensus peptide appears to be a better epitope than the variant peptide containing the PS mutation, for example iTopia data suggests AK9c is better than AK9s. However, in other cases the variant peptide containing the PS mutation appears to be a better candidate epitope than the consensus, for example RK9s is predicted to be a stronger epitope than KK9c. Or both can appear equally capable of being an epitope, such as RAK9c and RAK9s, iTopia data shows that both are very good epitopes.

To confirm the epitopes identified by the iTopia System, functional IFN- γ ELISpot assays were carried out on a limited number of available samples. Of the eleven patients tested, we were able to obtain at least one response to peptides LR9s, AK9s, AK9c, RK9s, KK9c and the novel RAK9c and confirmed them as epitopes. There is sufficient ELISpot data to support that these are confirmed epitopes of A*0301, however more data is required to see if the strength of response corresponds to the ranking of the peptides based on the iTopia results. Future studies aim to expand the number of patients in the in vivo study group. We should also point out that the epitope assessment was based on ELISPOT, affinity and off-rate assays and has not considered the variability in intracellular antigen processing, which could be a worth well subject for further study.

This study has shown that the iTopia Epitope Discovery System is a useful tool in identifying epitopes, characterizing the affinity of the peptide for the MHC, and testing the stability of the peptide:MHC complex. Currently, the iTopia Epitope Discovery System is a system available to obtain detailed information about how long an epitope is presented and at what concentration it is required that is critical in vaccine development.

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Abbreviations

IFN- γ	(interferon gamma)
PS	(positively selected) amino acids
β_2 M	(β_2 microglobulin).

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Figure 1.

A*0301 Peptide Binding Data. (a) Peptide binding data for K403R. Five peptides (LR9s, AK9c, AK9s, RK9s and KK9c) made the 30% cut off and carried on in characterization and confirmation. (b) Peptide binding data for V7I peptides. Two peptides (RAK9c and RAK9s) made the 30% cut off and carried on in characterization and confirmation. (c) Peptide binding data for L34I. None of the peptides made the 30% cut off so no further characterization was carried out.

Figure 2.
The binding Affinities of the 7 peptides predicted to be A*0301 epitopes.

Figure 3.
The off-rates ($t_{1/2}$) of the 7 peptides predicted to be A*0301 epitopes.

Table 1

HLA association to HIV-1 mutations in Pumwani Sex Worker Cohort

Sub-region of gag	Mutation	HLA	Pearson's Chi Square p-value	Fisher's Exact p-value
p17	V7I	A*0301	0.00618	0.02
p17	I34L	A*0301	0.00614	0.01
p7	K403R	A*0301	0.00146	0

Table 2

iTopia A*0301 binding results for all 50 peptides tested

Mutation	Code	Peptide Sequence	% Peptide Binding ¹
V7I	MS9c	MDARASVLS	4.7
	MS9s	MDARASILS	5.0
	DG9c	DARASVLSG	1.4
	DG9s	DARASILSG	1.4
	AG9c	ARASVLSGG	0.0
	AG9s	ARASILSGG	0.6
	RAK9c	RASVLSGGK	96.5
	RAK9s	RASILSGGK	87.6
	AL9c	ASVLSGGKL	1.9
	AL9s	ASILSGGKL	2.4
	SD9c	SVLSGGKLD	0.0
	SD9s	SILSGGKLD	5.5
	VA9c	VLSGGKLDA	0.0
	IA9s	ILSGGKLDA	3.8
L34I	KL9c	KKKYRMKHL	4.5
	KL9s	KKKYRMKHI	3.7
	KV9c	KKYRMKHLV	19.8
	KV9s	KKYRMKHIV	4.8
	KW9c	KYRMKHLVW	7.5
	KW9s	KYRMKHIVW	3.8
	YA9c	YRMKHLVWA	0.0
	YA9s	YRMKHIVWA	0.0
	RS9c	RMKHLVWAS	15.0
	RS9s	RMKHIVWAS	22.4
	MR9c	MKHLVWASR	3.9
	MR9s	MKHIVWASR	2.6
	KE9c	KHLVWASRE	2.8
	KE9s	KHIVWASRE	3.3
	HL9c	HLVWASREL	3.1
	HL9s	HIVWASREL	3.5
	LE9c	LVWASRELE	4.3
	IE9s	IVWASRELE	3.8
K403R	CR9s	CGKEGHLAR	1.7
	CK9c	CGKEGHLAK	3.9
	GN9s	GKEGHLARN	2.9
	GN9c	GKEGHLAKN	3.1
	KC9s	KEGHLARNC	2.5

Mutation	Code	Peptide Sequence	% Peptide Binding ^I
	KC9c	K E G H L A K N C	2.3
	ER9s	E G H L A R N C R	1.2
	ER9c	E G H L A K N C R	0.5
	GA9s	G H L A R N C R A	1.7
	GA9c	G H L A K N C R A	1.8
	HP9s	H L A R N C R A P	2.6
	HP9c	H L A K N C R A P	3.1
	LR9s	L A R N C R A P R	62.5
	LR9c	L A K N C R A P R	7.3
	AK9s	A R N C R A P R K	73.5
	AK9c	A K N C R A P R K	80.2
	RK9s	R N C R A P R K K	62.1
	KK9c	K N C R A P R K K	37.9

^I expressed as a percent of the positive control peptide

Table 3

Summary of A*0301 peptide binding, affinity and off-rate data

Gag sub-region	Peptide Code	Peptide Sequence	% Peptide Binding ¹	Off-rate t _{1/2}	Affinity ED ₅₀	ELISpot Responses
p17	RAK9c	RASVLSGGK	96.5	1.040	1.74E-05	1/11
	RAK9s	RASILSGGK	87.6	0.5	1.91E-05	0/11
p7	LR9s	LARNCRAPR	62.5	0.31	2.40E-05	2/11
	AK9s	ARNCRAPRK	73.5	0.4	3.18E-05	1/11
	AK9c	AKNCRAPRK	80.2	17.95	2.03E-05	2/11
	RK9s	RNCRAPRKK	62.1	0.5	2.37E-05	1/11
	KK9c	KNCRAPRKK	37.9	0.29	8.43E-05	1/11