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Detection of Abacavir-Induced Structural Alterations in Human Leukocyte Antigen-B*57:01 Using Phage Display

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The interaction of human leukocyte antigen (HLA) with specific drugs is associated with delayed-type hypersensitivity reactions, which cause severe cutaneous toxicity. Such interactions induce structural alterations in HLA complexes *via* several different mechanisms such as the hapten theory, p-i concept, and altered peptide repertoire model, leading to the activation of cytotoxic T cells. To date, comprehensive detection of such structural alterations in preclinical studies has been difficult. Here, we evaluated structural alterations in HLA complexes focusing on the interaction between the HLA-B*57:01 allele and abacavir (an anti-human immunodeficiency virus drug), representing a model of abacavir hypersensitivity syndrome induced by changes in the peptide repertoire on the HLA molecule. We employed a phage display method using a commercially available antibody library to screen specific phage antibodies able to recognize HLA-B*57:01. The affinity of selected phage antibodies increased because of structural alterations in HLA-B*57:01 following exposure to abacavir, indicating that specific phage antibodies can identify drug-mediated structural changes in HLA complexes. We also identified an unreported structural change in HLA-B*57:01 using the phage display method, whereby abacavir increased the expression of peptide-deficient HLA-B*57:01 on the cell surface. These results suggest that phage display technology is a useful method for detecting structural changes in HLA complexes. This technology represents a potential novel strategy for predicting HLA-associated hypersensitivity reactions by drugs in pre-clinical studies.

Key words human leukocyte antigen; adverse drug reaction; phage display

INTRODUCTION

T cell-mediated delayed-type hypersensitivity reactions to drugs range from rash to life-threatening severe cutaneous adverse reactions such as Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug reaction with eosinophilia and systemic symptoms (DRESS).[1] Such reactions are difficult to predict based on the results of preclinical studies, and they represent a barrier to drug development. Genome-wide association studies have shown that some drug hypersensitivity reactions are related to specific human leukocyte antigen (HLA) allotypes[2,3]; such as HLA-B*57:01 in abacavir-induced hypersensitivity reactions including skin rash[4]; HLA-B*15:02 in carbamazepine-induced SJS/TEN[5]; HLA-A*31:01 in carbamazepine-induced DRESS[6]; and HLA-B*58:01 in allopurinol-induced SJS/TEN.[7] Hypersensitivity reactions to abacavir, an inhibitor of human immunodeficiency virus (HIV) reverse transcriptase, have been widely investigated in association with HLA-B*57:01 allotype; only patients who possess this allotype develop hypersensitivity reactions following abacavir exposure.[4,8]

T cells stimulation *via* the T cell receptor (TCR) occurs *via* several mechanisms. The hapten theory assumes that drugs covalently bind to intracellular proteins or peptides, leading to the presentation of haptenized peptides to T cells.[9] A second hypothesis is the pharmacological interaction with immune receptor (p-i) concept, which suggests that drugs or their metabolites non-covalently bind to HLA or other immune receptors.[10] In the case of carbamazepine reacting with T cells, the drug forms interactions between specific TCR clonotypes and the HLA-B*15:02 molecule.[11] If these drug–immune receptor interactions have sufficient affinity, they may elicit an immune response. The third hypothesis, the altered repertoire model, suggests that drugs or their metabolites can non-covalently bind to the pocket of the peptide-binding groove in certain HLA molecules, resulting in the presentation of a new repertoire of endogenous self-peptides on the HLA molecule.[12] Recent studies have shown that abacavir non-covalently binds to the F-pocket of the HLA-B*57:01 peptide-binding groove, and modifies the peptide repertoire.[12,13] In this case, peptides with a small aliphatic residue, such as Leu or Ile, at the C-terminal end are favored over peptides with Trp or Phe, which are normally presented by unmodified HLA-B*57:01 molecules. This change in peptide repertoire activates $CD8^+$ T cells.[14]

Recent studies have shown that drugs can directly interact with HLA molecules, forming an antigenic structure that is able to induce a T-cell response.[14] Therefore, interactions between drugs and HLA have been evaluated to predict the risk of hypersensitivity reactions. For example, the use of a HLA library containing different allotypes has been proposed to screen new drugs for their ability to bind HLA and for

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their potential to induce severe drug hypersensitivity reactions has been proposed.[15] *In silico* docking simulations have been used to accurately predict the binding of abacavir, carbamazepine, and allopurinol to HLA-B*57:01, HLA-B*15:02, and HLA-B*58:01, respectively.[11,16,17] These assays determine whether a test compound can bind to HLA; however, it is difficult to demonstrate that the compound alters the HLA structure and affects T-cell recognition. Furthermore, such assays do not consider intracellular processes, such as drug metabolism and transport.

Therefore, we have developed a method to evaluate the ability of a drug to alter the structure of HLA and be recognized as a *neo*-antigen by T cells. For this purpose, we utilized phage display technology[18] to identify human TCR-like monoclonal antibody domains with specificity for the structurally altered HLA. This method involves the generation of phage clone libraries. Each phage clone carries genes that encode an antibody (a single chain Fv or a Fab fragment) as a fusion protein on their surface. Therefore, it is possible to select different phage clones by assessing whether they bind a target, and to isolate clones with the desired specificity. Phage display technology has been used to isolate various TCR-like antibodies against cancer–peptide/HLA complexes, with no cross-reactivity to normal tissues.[19,20]

In this study, we report the discovery of phage clones that recognize structural changes in HLA-B*57:01. We characterized the ability of phage clones to bind HLA-B*57:01, and their binding affinity was enhanced in the presence of abacavir. Our findings show that phage display may be a useful tool for evaluating the ability of test compounds to induce structural changes in HLA.

MATERIALS AND METHODS

Reagents Abacavir sulfate (Carbosynth, Berkshire, U.K.) was dissolved in MilliQ water. Carbamazepine (Wako, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO). For fluorescence-activated cell sorting (FACS) analysis, fluorescein isothiocyanate (FITC) anti-HLA-A,B,C (W6/32) antibody (Biolegend, San Diego, CA, U.S.A.), anti-M13 antibody (E1; Abcam, Cambridge, U.K.), anti-B17 antibody (0196HA; One LAMBDA, Canoga Park, CA, U.S.A.), and anti-HLA class I heavy chain antibody (HC10; Nordic-MUbio, Susteren, the Netherlands) were used.

Cell Culture HeLa cells, purchased from RIKEN Cell Bank (Tsukuba, Japan), were maintained in minimum essential medium (MEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, U.S.A.) plus antibiotic–antimycotic mixed solution (Nacalai Tesque) and non-essential amino acids solution (Nacalai Tesque). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Construction of an HLA Expression Vector The HLA-B*57:01 expression vector (pcDNA-HLA-B*57:01) was constructed as follows: HLA-B*57:01 cDNA was amplified from immortalized human B cells (ECACC, Salisbury, U.K.) and inserted into the pcDNA3.1D vector (Life Technologies) with 3× FLAG tags at the end of the C-terminus.[21] β_2-Microglobulin (β_2m), amplified from isolated human hepatocytes, was combined with HLA-B*57:01 by fusing a 2A peptide sequence. The HLA-B*15:01 expression vector (pcDNA-HLA-B*15:01) was constructed as follows: HLA-B*15:01 cDNA (RIKEN, Tsukuba, Japan) was amplified and 3× FLAG tags–2A peptide-human β_2m were inserted at the C-terminal end. The resultant HLA-FLAG-2A-β_2m plasmid was inserted into the pcDNA3.1D vector. To construct HLA-B*15:01 (pcDNA-HLA-B*15:01), HLA-B*27:05 (pcDNA-HLA-B*27:05), and HLA-B*35:01 (pcDNA-HLA-B*35:01) expression vectors, each gene amplified from the corresponding cDNA (all purchased from RIKEN) was replaced with the HLA-B*57:01 gene in pcDNA-HLA-B*57:01 using the In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA, U.S.A.). HLA-B*57:03, HLA-B*15:02, HLA-B*27:09, and HLA-B*35:05 expression vectors were generated by site-directed mutagenesis of pcDNA-HLA-B*57:01 (412G→A, 419C→A), pcDNA-HLA-B*15:01 (259G→A, 261G→C, 353C→T, 355C→A, 369C→T, 409C→T, 538T→C, 539G→T), pcDNA-HLA-B*27:05 (418G→C), and pcDNA-HLA-B*35:01 (351T→C, 353A→C, 363G→C, 369T→C), respectively.

Introduction of HLA Expression Vector and Drug Exposure HeLa cells were plated in flat-bottomed six-well plates (2.5×10^5 cells/well). After 24 h, cells were transfected with the HLA expression vector using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 24 h transfection, the cells were treated with 100 μM abacavir for 24 h.

HLA Immobilization on Magnetic Beads HeLa cells expressing introduced HLA were lysed on ice in lysis buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), and protease inhibitor cocktail (Roche Diagnostic, Indianapolis, U.S.A.). The concentration of protein in lysis buffer was measured using a BCA™ protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), according to the manufacturer's instructions. Then, 1 mg protein in 500 μL of lysis buffer was incubated with 10 μL of Anti-FLAG® M2 Magnetic Beads (Sigma-Aldrich, St. Louis, MO, U.S.A.) overnight at 4°C with gentle agitation. After incubation, magnetic beads were washed on ice with lysis buffer.

Phage Display Selection The Human Domain Antibody Library (DAb Library; Source BioScience, Nottingham, U.K.), which contains 3×10^9 independent heavy chain variable region (V_H) clones, was amplified for selection as previously described.[22] First, phages were pre-incubated with 50 μL of Anti-FLAG® M2 Magnetic Beads in phosphate buffered saline (PBS) (pH 7.4) including 1% bovine serum albumin (BSA, Nacalai Tesque) with gentle agitation for 90 min at 4°C (first negative selection). The supernatant (unbound phage pool) was reacted with HLA-immobilized magnetic beads under gentle agitation for 90 min at 4°C (second negative selection or drug-free positive selection). In METHOD-1, the supernatant was reacted with "abacavir-exposed HLA"-immobilized beads with gentle agitation for 90 min at 4°C (positive selection). After washing the beads with PBS containing 0.05% Tween 20 (Biorad, Hercules, CA, U.S.A.) and 1% BSA, phage-binding antibodies were eluted with 0.1 M glycine–HCl (pH 3.0). In METHOD-2, after the drug-free positive selection, phage-binding antibodies were eluted in the same manner as in METHOD-1. Eluted phage antibodies were used to infect TG1 *Escherichia coli*. The TG1 cells were then cultured on agar plates consisting of 2× TY medium plus ampicillin (100 μg/mL, Nacalai Tesque). Cells were removed from

plates by scraping, and infected with KM13 helper phage, then secreted polyclonal phage antibodies were purified from TG1 cells by PEG precipitation.[22] Concentration of phage antibodies was estimated by measuring absorption at 260 nm according to the following a formula: phage/mL = $OD_{260} \times 22.14 \times 10^{10}$. These binding, elution, and infection steps were repeated three or four times. After the final round of affinity panning, several colonies of TG1 cells were selected and the DNA sequence of the V_H domain was confirmed. Monoclonal phage purified from a single TG1 clone was stored in PBS containing 15% glycerol at −80°C.

Flow Cytometric Analysis HeLa cells were treated with 0.1 M glycine–HCl (pH 3.0) for 5 min as necessary. After washing with ice-cold PBS, HeLa cells were dissociated using Accutase™ (Nacalai Tesque). To evaluate the recognition of phage antibodies, 6×10^5 cells were incubated with 1×10^{11} phages in 500 μL PBS containing 1% FBS for 2 h at 4°C, with gentle agitation. After washing, the cells were stained with an anti-M13 antibody (E1) tagged using a Dylight® 488 Fast conjugation kit (Abcam) on ice for 30 min. To evaluate HLA complexes on the cell surface, $1–3 \times 10^5$ cells were immunostained with FITC anti-HLA A,B,C antibody (W6/32) or anti-B17 antibody tagged using a Dylight® 488 Fast conjugation kit. To capture cell-surface peptide-deficient HLA, $1–3 \times 10^5$ cells were immunostained with a mouse anti-HLA class I heavy chain antibody (HC10) and labeled with a secondary antibody conjugated to an Alexa Flour dye (Life Technologies). Data were acquired on an EC800 cell analyzer (SONY, Tokyo, Japan).

RESULTS

Selection of Phage Antibodies with Specificity for HLA-B*57:01 Structurally Modified by Abacavir The complex structure of HLA-B*57:01 is altered by specific binding to abacavir.[12] To detect the structurally modified HLA-B*57:01 complex *in vitro*, specific phage antibodies were selected. These antibodies can recognize the HLA complex modified by abacavir from the DAb Library, which contains M13 phage clones expressing various V_H of human antibody[22] (Fig. 1). The HLA-B*57:01 complex was acquired from the lysates of HeLa cells expressing introduced HLA-B*57:01, with or without abacavir (100 μM) (termed "structurally modified HLA-B*57:01" or "drug-free HLA-B*57:01," respectively), and then immobilized onto magnetic beads. First, the library phages were exposed to empty beads (without HLA-B*57:01 complexes) (to a final concentration of 2×10^{11} phages/mL), to remove phage clones non-specifically bound to the beads (first negative selection). Next, we exposed the unbound phage pools to the "drug-free HLA-B*57:01 complex"-immobilized beads to remove phage clones that recognize the HLA-B*57:01 complex, regardless of abacavir exposure (second negative selection). Finally, the unbound fraction was mixed with the "structurally modified HLA-B*57:01"-immobilized beads, and the bound compartment was collected as the desired phage antibodies (positive selection). A series of affinity panning processes, consisting of these three steps was defined as METHOD-1. By repeating the METHOD-1 cycle four times, clones of the phage antibody with specificity for the "structurally modified HLA-B*57:01" were selected. The sequence of the V_H regions expressed in 25 randomly selected phage clones were determined, and two kinds of phage clones with different DNA sequences were concentrated; #1 (16 out of 25; 64%) and #2 (8 out of 25; 32%) (Table 1). Compared with the amino acid sequences consisting of complementarity determining regions (CDRs), they were almost different, while partial similarity was observed in the CDR1.

Affinity of Selected Phage Antibodies to HLA-B*57:01 Structurally Altered by Abacavir To evaluate the ability of these selected phage antibody clones #1 and #2 to recognize the "structurally modified HLA-B*57:01" by abacavir,

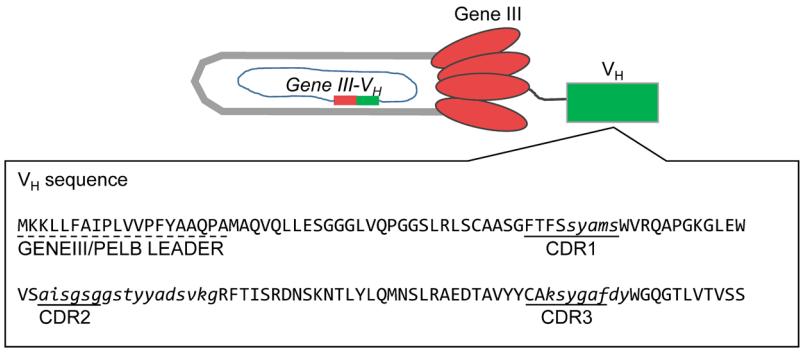

Fig. 1. Structure of a M13 Phage Expressing a Human Antibody Heavy Chain Variable Region (V_H)

V_H is genetically fused to *gene III* of M13 phage. The amino acid sequence of the V_H region is written below. Lowercase italics represent variable amino acid residues in the human antibodies. Underlined (solid) letters are variable amino acid residues in the DAb library. (Color figure can be accessed in the online version.)

Table 1. Phage Antibodies Concentrated by Affinity Panning METHOD-1 Using Structurally Modified HLA-B*57:01

	Frequency (%) $N = 25$	CDR1	CDR2	CDR3
#1	64	DKFNHYIMG	AITDAG	GPHDLGTKDSGSSTQFTY
#2	32	YKVIHYIMG	AIYDPS	GRVGNDSQIKY
Others	4			

they were incubated with HeLa cells expressing introduced HLA-B*57:01, with or without abacavir. Binding was then detected using an anti-M13 antibody by flow cytometry. Both phage clones were able to bind to HeLa cells expressing the "structurally modified HLA-B*57:01" (Fig. 2a), as shown by the higher signal intensity compared with the "drug-free HLA-B*57:01."

Unexpectedly, these clones had low binding potential for the "drug-free HLA-B*57:01." We then aimed to confirm whether the increased affinity of phage binding was dependent on structural changes in the HLA-B*57:01 complex induced by abacavir. To address this, the selected phage antibodies were incubated with HeLa cells expressing introduced HLA-B*57:03. This allotype was used as a negative control as it is not bound by abacavir, and does not carry a risk of drug-induced hypersensitivity syndrome.[23] As observed with HLA-B*57:01, a low level of binding was observed; however, in contrast to HLA-B*57:01, the affinity to HLA-B*57:03 complexes was not changed following exposure to abacavir (Fig. 2a). A control phage clone that recognizes a specific nuclear protein concentrated in another independent experiment did not demonstrate specificity to HLA-B*57:01 or B*57:03, regardless of exposure to abacavir (Fig. 2a). These results indicated that the structure of HLA was changed by the drug, and these changes could be detected by utilizing the biotechnological phage display method.

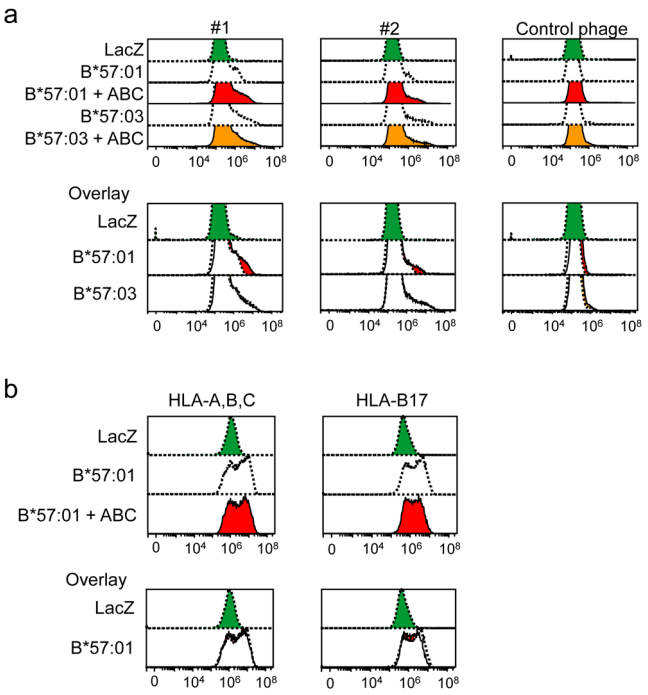

Fig. 2. Flow Cytometric Evaluation of the Affinity of Phage Antibodies to HLA-B*57:01 Structurally Altered by Abacavir

(a) HLA-B*57:01-, HLA-B*57:03-, and lacZ-introduced HeLa cells cultured with abacavir (ABC, 100 μM, solid lines) or without ABC (dotted lines) for 24 h were incubated with phage antibodies (#1 and #2, and the control phage). Binding phages were detected with an anti-M13 antibody using flow cytometry. Overlaid images of the data with or without ABC are also displayed. (b) HLA-B*57:01- or lacZ-introduced HeLa cells cultured with ABC (100 μM, solid lines) or without ABC (dotted lines) for 24 h were incubated with anti-HLA A, B, C antibody (W6/32) (HLA-A,B,C) or anti-B17 antibody (0196HA) (HLA-B17), and the binding antibody was then detected using flow cytometry. Overlaid images of the data with or without ABC are also displayed. (Color figure can be accessed in the online version.)

Since the affinity of the concentrated phage antibodies to HLA-B*57:01 was enhanced by abacavir, this suggests that any other antibodies that recognize HLA-B*57:01 might display enhanced (or weakened) affinity to the "structurally modified HLA-B*57:01" in response to abacavir. Here, to clarify whether all anti-HLA antibodies alter their affinity to HLA-B*57:01 by abacavir exposure, we used two commercially available antibodies; anti-HLA A,B,C antibody (W6/32, reacting with HLA class I α_2 and α_3 domain[24]) and anti-B17 antibody (0196 HA, reacting with HLA-B57 and HLA-B58 α_1 domain[25]), both of which recognize HLA-B57. However, exposure to abacavir did not change the affinity of these two commercial antibodies to HLA-B*57:01 (Fig. 2b). These results indicated that these commercial antibodies were not useful for evaluating structural alterations in HLA complexes, and phage display technology may provide unique antibody clones capable of discriminating structural alterations in HLA complexes.

Simplifying Selection to Concentrate Phage Antibodies to Discriminate HLA-B*57:01 Structurally Altered by Abacavir As described, the two concentrated phage antibodies were able to recognize HLA-B*57:01 structurally modified by abacavir; unexpectedly, they were able to partially bind "drug-free HLA-B*57:01" (Fig. 2a). These observations suggest that, to collect phage antibodies able to discriminate between the "structurally modified HLA-B*57:01" and the "drug-free HLA-B*57:01," the positive selection in METHOD-1 is not necessary. In fact, selecting clones of phage antibodies binding to "drug-free HLA-B*57:01" is sufficient. Therefore, we attempted to simplify the affinity panning method as follows; phages from the DAb library were exposed to empty beads at a final concentration of 2×10^{11} phages/mL (first negative selection), and the unbound fraction was exposed to the "drug-free HLA-B*57:01"-immobilized beads. Then, the bound compartment was collected as the desired phage clones (drug-free positive selection). A series

Table 2. Phage Antibodies Concentrated by Affinity Panning METHOD-2 Using Drug-Free HLA-B*57:01

	Frequency (%)		CDR1	CDR2	CDR3
	Trial 1 $N=24$	Trial 2 $N=40$			
#1	46	5	DKFNHYIMG	AITDAG	GPHDLGTKDSGSSTQFTY
#2	4	8	YKVIHYIMG	AIYDPS	GRVGNDSQIKY
#3	0	5	FKVTDYDMG	AIYGRD	TPTPDPEEFEY
#4	0	10	FRVNHKTMS	SIANRG	SKNSHAKALKS
Others	50	73			

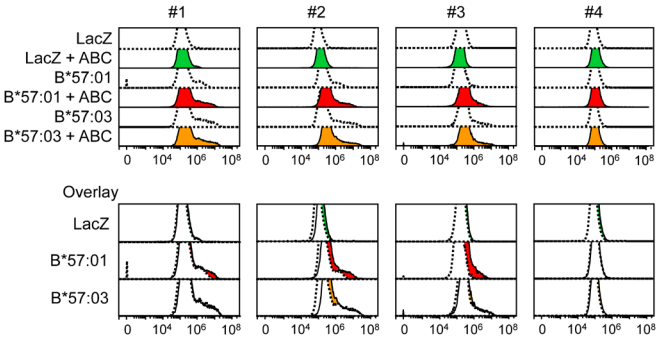

Fig. 3. Simplifying the Selection Method to Concentrate Phage Antibodies That Can Discriminate Structurally Altered HLA-B*57:01 by Abacavir

HLA-B*57:01-, HLA-B*57:03- or lacZ-introduced HeLa cells cultured with abacavir (ABC, 100 μM, solid lines) or without ABC (dotted lines) for 24 h were incubated with phage antibodies (#1 to #4 that we selected). Binding phages were detected with an anti-M13 antibody using flow cytometry. Overlaid images of the data with or without ABC are also displayed. (Color figure can be accessed in the online version.)

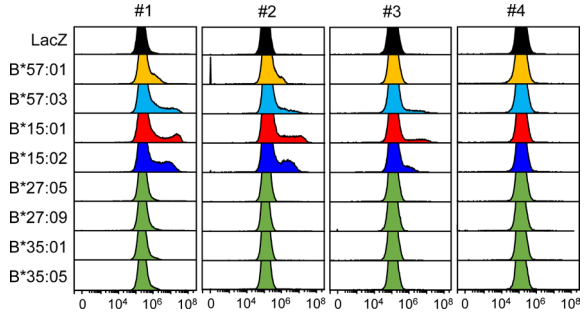

Fig. 4. Binding Characteristics of Phage Antibodies Able to Recognize HLA-B Complexes

HLA-B- or lacZ-introduced HeLa cells were incubated with phage antibodies (#1 to #4). Binding phages were detected with an anti-M13 antibody using flow cytometry. (Color figure can be accessed in the online version.)

Table 3. Phage Antibodies Concentrated by Affinity Panning METHOD-1 and METHOD-2 Using HLA-B*57:03

	Frequency (%)			CDR1	CDR2	CDR3
	Method 1	Method 2				
	$N=20$	Trial 1 $N=24$	Trial 2 $N=48$			
#1	15	8	10	DKFNHYIMG	AITDAG	GPHDLGTKDSGSSTQFTY
#2	20	0	17	YKVIHYIMG	AIYDPS	GRVGNDSQIKY
#3	20	4	10	FKVTDYDMG	AIYGRD	TPTPDPEEFEY
#4	5	0	8	FRVNHKTMS	SIANRG	SKNSHAKALKS
Others	40	88	54			

Table 4. Phage Antibodies Concentrated by Affinity Panning METHOD-2 Using HLA-B*15:01/02

	Frequency (%)		CDR1	CDR2	CDR3
	B*15:01 $N=44$	B*15:02 $N=42$			
#1	7	5	DKFNHYIMG	AITDAG	GPHDLGTKDSGSSTQFTY
#2	0	2	YKVIHYIMG	AIYDPS	GRVGNDSQIKY
#3	45	55	FKVTDYDMG	AIYGRD	TPTPDPEEFEY
#4	0	0	FRVNHKTMS	SIANRG	SKNSHAKALKS
Others	48	38			

of affinity panning processes consisting of these two steps was defined as METHOD-2. Consequently, by repeating the METHOD-2 cycle three–four times, we were able to concentrate the same two phage clones #1 and #2 (Table 2).

Since we deduced that the same or similar phage antibodies could be concentrated from the affinity panning cycles when using either HLA-B*57:03 or HLA-B*57:01, affinity panning METHOD-1 and METHOD-2 was conducted using HLA-B*57:03. As a result, phage clone #1 was concentrated in all tests performed with good reproducibility, while clone #2 was concentrated by METHOD-1 and after the second trial of METHOD-2 (Table 3). In addition, two further phage clones were concentrated from at least two affinity panning trials; clone #3 and clone #4. The binding affinity of these phage antibodies to HeLa cells expressing HLA-B*57:01 or HLA-B*57:03 was evaluated by flow cytometry (Fig. 3). Phage antibody #3 bound to the "structurally modified HLA-B*57:01," while affinity to the "drug-free HLA-B*57:01" was weak. This phage clone also bound to HLA-B*57:03, regardless of exposure to abacavir. Conversely, phage antibody #4 could not bind to HLA-B*57:01 or 03, regardless of abacavir exposure, indicating that this antibody might be concentrated non-specifically from the repeated affinity panning cycles.

Binding Characteristics of Phage Antibodies with Specificity for HLA-B Complexes As described above, repeated affinity panning using HLA-B57 allotypes resulted in very similar phage antibodies (Tables 2, 3). Therefore, we predicted that other phage antibodies would be concentrated when using other HLA-B allotypes. For example, phage anti-

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B*57:01 GSHSMRYFYTAMSRPGRGEPRFIAVGYVDDTQFVRFDSDAASPRMAPRAPWIEQEGPEYW
B*57:03 ----------------------------------------------------------------
B*15:01 ----------------------------------------------------------------
B*15:02 ----------------------------------------------------------------
        1       10        20        30        40        50        60
B*27:05 --------H-SV----------T-------L-----------EE--------------
B*27:09 --------H-SV----------T-------L-----------EE--------------
B*35:01 -------------------------------------------TE--------------
B*35:05 -------------------------------------------TE--------------


B*57:01 DGETRNMKASAQTYRENLRIALRYYNQSEAGSHIIQVMYGCDVGPDGRLLRGHDQSAYDG……
B*57:03 ----------------------------------------------------N-Y----
B*15:01 -R--QIS-TNT-----S--NLRG----------TL-R----------------------
B*15:02 -RN-QIS-TNT-----S--NLRG-----------R---------------Y-------
        61       70        80        90        100       110       120
B*27:05 -R--QIC--K---D--D--TL-----------TL-N---------------YH-D----
B*27:09 -R--QIC--K---D--D--TL-----------TL-N---------------YH-H----
B*35:01 -RN-QIF-TNT-----S--NLRG-----------R-----L-----------------
B*35:05 -RN-QIF-TNT-----S--NLRG----------TL-S-----L-----------------
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Fig. 5. Sequence Alignments of HLA-B*57:01 with Other HLA-B Subtypes

Sequence alignments of HLA-B*57:01 (1–120 amino acids) with HLA-B*57:03, HLA-B*15:01, HLA-B*15:02, HLA-B*27:05, HLA-B*27:09, HLA-B*35:01, and HLA-B*35:05 are displayed, respectively. (Color figure can be accessed in the online version.)

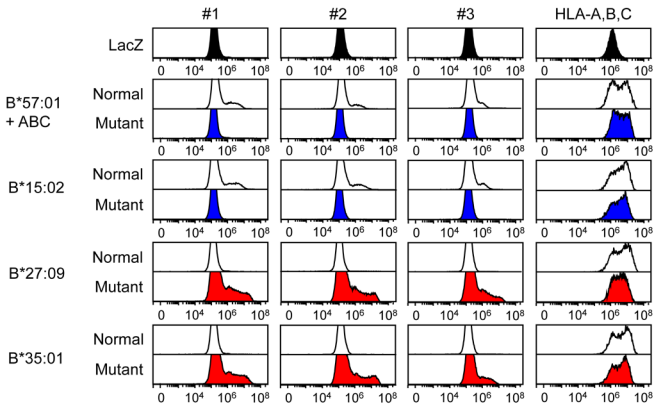

Fig. 6. Identification of Crucial Regions in HLA-B57 and HLA-B15 for Binding to Selected Phage Antibodies

Each HLA-B (Normal), artificially mutated HLA-B (Mutant), or lacZ-introduced HeLa cells cultured with abacavir (ABC, 100 μM) or without ABC for 24 h were incubated with phage antibodies (#1 to #3). Binding phages were detected with an anti-M13 antibody using flow cytometry. The cells were also incubated with anti-HLA A,B,C antibody (W6/32) (HLA-A,B,C). (Color figure can be accessed in the online version.)

bodies with binding potential to HLA-B*15:02, an allotype related to carbamazepine-induced SJS/TEN, were selected by repeating the affinity panning METHOD-2. After repeating the METHOD-2 cycle using HLA-B*15:01- or HLA-B*15:02-immobilized beads three–four times, phage clones #1, #2, and #3, which were concentrated when using HLA-B57, were also concentrated (Table 4). Thus, we evaluated the binding affinity of these phage antibodies to multiple HLA-B allotypes by flow cytometry to investigate how broadly they could recognize HLA-B allotypes. The results showed that these three phage clones only bound HLA-B57 and HLA-B15, but could not recognize HLA-B27 or HLA-B35 (Fig. 4). These findings suggest that the concentrated phage clones have some cross-reactivity among multiple HLA-B allotypes.

Identifying Regions in HLA-B57 and HLA-B15 Crucial for Binding the Selected Phage Antibodies Based on the observations that selected phage antibodies targeted to HLA-B57 allotypes were able to bind HLA-B15 allotypes (Fig. 4), we speculated the existence of common regions and structures in HLA molecules important for binding to these phage antibodies among these allotypes. Therefore, we compared the amino acid sequences of several HLA-B heavy chains, and identified two unique amino acids (Met45 and Ala46) that exist only in HLA-B57 and HLA-B15 (Fig. 5). To evaluate whether these amino acids were crucial for binding of the selected phage antibodies, we examined their affinity to artificially mutated HLA-B complexes with Met45Thr and Ala46Glu. We found that substituting these two amino acids prevented the binding of phage antibodies to HLA-B57 and HLA-B15 (Fig. 6). Conversely, the phage clones #1, #2, and #3 acquired binding potential to the artificially mutated HLA-B27 and HLA-B35 following the introduction of Met45 and Ala46 in the heavy chain region (Fig. 6). These findings indicated that Met45 and Ala46 in HLA-B57 and HLA-B15 are crucial for the binding of the selected phage antibodies. Mutagenesis of these two residues may have induced partial structural alteration of HLA-B*57:01, resulting in preventing the recognition of selected phage antibodies.

Identifying Novel Structural Alterations of HLA-B*57:01 Induced by Abacavir The presence of Met45 and Ala46 of HLA-B heavy chains within the peptide binding groove of HLA-B complexes[26] (Fig. 7a) suggested that the selected antibodies could not bind to HLA-B if a peptide is normally presented on the HLA complex. Therefore, we speculated that these phage antibodies might recognize peptide-deficient HLA-B57 and HLA-B15, which do not present any antigen peptide on these HLA complexes. To confirm this, we used acidification to elute any components within the peptide binding groove in the HLA-B*57:01 complexes, and then exposed the selected antibodies to the HLA complexes. Consequently, the binding affinity of these antibodies to the HLA complexes was substantially increased (Fig. 7b), implying that the selected antibodies recognized peptide-deficient HLA-B*57:01.

As described, the selected phage antibodies bound strongly to HLA-B*57:01 following exposure to abacavir (Fig. 2a). Since these phage antibodies recognize peptide-deficient HLA-B*57:01, we hypothesized that abacavir exposure increased the expression of peptide-deficient HLA-B*57:01 on HeLa cells. To evaluate the amount of peptide-deficient

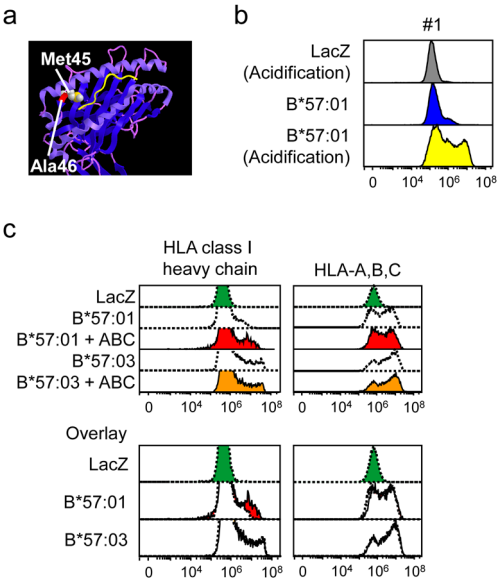

Fig. 7. Identifying Novel Structural Changes in HLA-B*57:01 Induced by Abacavir

(a) The peptide binding groove of HLA-B*57:01 complex (PDB: 3VRI) is displayed. An antigen peptide (yellow) binds to HLA-B*57:01 heavy chain (blue). The molecular structure was drawn by chem3D (Pro 16.0) software. (b) HLA-B*57:01- or lacZ-introduced HeLa cells were treated with 0.1 M glycine–HCl (pH 3.0, acidification). The cells were incubated with phage antibody #1, and then the binding phages were detected with an anti-M13 antibody using flow cytometry. (c) HLA-B*57:01-, HLA-B*57:03-, or lacZ-introduced HeLa cells cultured with abacavir (ABC, 100 μM, solid lines) or without ABC (dotted lines) were incubated with anti-HLA class I heavy chain antibody (HC10) or anti-HLA A,B,C antibody (W6/32) (HLA-A,B,C). Then, the binding antibody was detected using flow cytometry. (Color figure can be accessed in the online version.)

HLA-B*57:01, we used a commercially available anti-HLA class I heavy chain antibody (HC10) that only detects peptide-deficient forms of HLA class I.[27] Consequently, the binding capacity of HC10 to HeLa cells expressing HLA-B*57:01 was enhanced following exposure to abacavir (Fig. 7c). In the case of HLA-B*57:03, the HC10 signal was not enhanced upon exposure to abacavir (Fig. 7c). A series of experiments revealed that a novel unpredicted structural change in HLA-B*57:01 could be detected by phage display technology, and exposure to abacavir increased the expression of peptide-deficient HLA-B*57:01.

DISCUSSION

In this study, we selected phage antibodies with the ability to bind to HLA-B*57:01, and demonstrated that the binding affinity of these antibodies to HLA-B*57:01 was affected by exposure to abacavir. Previous reports have indicated that abacavir can interact directly with the HLA-B*57:01 molecule, resulting in modification of the antigen peptide repertoire on HLA-B*57:01 molecules.[12,13] Although the modified peptide-presenting HLA-B*57:01 is recognized by specific T cells and can induce the hypersensitivity syndrome, the phage antibodies selected in our study recognized peptide-deficient HLA-B*57:01, and its expression was increased following exposure to abacavir (Figs. 7b, c). Since the existence of peptide-deficient HLA-B*57:01 has not been reported, it is unclear why expression was enhanced in response to abacavir exposure. One hypothesis is that intracellular HLA-B*57:01 binds to abacavir and bypasses an HLA quality control system in the endoplasmic reticulum (ER). In general, HLA class I molecules are assembled with antigen peptides in the ER, and peptide-deficient HLA class I molecules tend to be retained in the ER by the quality control system.[28] However, several peptide-deficient HLA class I allotypes are able to bypass this system.[29] For example, HLA-B*57:03 can bypass this system more readily than HLA-B*57:01; only two amino acids at the F pocket region differ between these two HLA allotypes. This suggests that the F pocket structure affects the HLA complex, allowing the quality control system to be bypassed. In addition, abacavir binds to the F pocket of HLA-B*57:01.[12] Collectively, these data suggest that abacavir-bound HLA-B*57:01 may bypass the ER quality control system because of the interaction between the F pocket and abacavir, and be expressed in a peptide-deficient form.

Several reports indicate that peptide-deficient HLA relates to the onset of autoimmune disease.[30–32] For example, a peptide-deficient form of HLA-B27, which is strongly associated with ankylosing spondylitis, is expressed on the cell surface.[32] Here, one hypothesis proposes that ankylosing spondylitis results from immune recognition of the abnormal peptide-deficient HLA-B27 molecule. Therefore, peptide-deficient HLA-B*57:01 may affect the onset of abacavir hypersensitivity syndrome. Another possible effect of peptide-deficient HLA-B*57:01 is supporting the interaction between the altered peptide-presenting HLA-B*57:01 and specific TCR. A recent study showed that peptide-deficient HLA-B*35:01 interacted with various $CD8^+$ T cells *via* CD8-dependent binding, without recognition by TCR.[33] This indicates that peptide-deficient HLA class I nonspecifically promotes the interaction between antigen presenting cells and $CD8^+$ T cells. Considering that abacavir-exposed HLA-B*57:01-positive cells express both peptide-deficient and altered peptide-presenting HLA-B*57:01, peptide-deficient HLA-B*57:01 may interact with various $CD8^+$ T cells, resulting in the altered-peptide presenting HLA-B*57:01 being easily recognized by specific T cells.

Using phage display, we identified unpredicted structural changes in HLA-B*57:01, indicating that this method is useful for evaluating unknown structural changes in HLA complexes. Recently, several approaches have been developed to predict drug-mediated immunogenicity. One such approach is *in silico* docking simulation, which is used to evaluate the binding affinity between compounds and HLA molecules.[11,16,17] This approach can evaluate the risk of structural alterations in HLA complexes in a low cost and timely manner. However, it remains unclear whether static *in silico* docking simulation can reproduce drug-mediated dynamic conformational alterations in HLA complexes. Another approach is the priming of human naïve T-cells to drugs using cells from HLA-typed healthy volunteers.[34] Using this approach, it is possible to determine whether a compound can activate T cells derived from humans possessing specific HLA-allotypes. However, this approach does not determine how drugs induce immunogenicity at the molecular level. Presently, it remains difficult to evaluate structural changes in HLA complexes directly under *in vitro* condition.

In this study, we developed a phage display approach that was able to evaluate abacavir-mediated structural changes on the cell surface of HLA-B*57:01. This approach may enable researchers to detect structural changes in HLA complexes and to evaluate the risk of hypersensitivity, regardless

of the underlying mechanism. In addition, to select specific phage antibodies that recognize drug-mediated changes in the structure of HLA, the use of HLA complexes exposed to individual drugs is not needed at the affinity panning cycles. This is because the selected phage antibodies, which recognize drug-free HLA-B*57:01, could detect structural changes in HLA-B*57:01 induced by abacavir. Therefore, to evaluate the risk of drug-induced hypersensitivity, phage antibodies that recognize drug-free HLA should be selected, and then the interaction between the selected antibodies and the HLA complexes should be evaluated with or without drugs. If the drugs alter the binding affinity, they should induce structural changes in HLA, with potential to induce immune activation. In addition, three out of four selected phage antibody clones can evaluate the structural alteration of HLA-B*57:01 in our experiment. Therefore, to evaluate structural alteration of an HLA allotype, experimenting several phage antibody clones might be essential.

There are currently 26512 HLA and related allotypes included in the IPD-IMGT/HLA Database (http://www.ebi.ac.uk/ipd/imgt/hla/). However, for example, when focusing on HLA-B allotypes in Japanese population, only 25 HLA-B allotypes cover 96% of Japanese allele frequency (The Allele Frequency Net Database, http://www.allelefrequencies.net/). Evaluating structural alteration of major HLA allotypes may provide a hint to reduce an onset of drug-induced hypersensitivity in Japanese population. We believe that the phage display approach may be used for predicting HLA-related drug hypersensitivity reactions in pre-clinical studies.

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Conflict of Interest The authors declare no conflict of interest.

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