Carriage of *HLA-B*5701* and a Haplotypic Hsp70-Hom Variant is Associated with a Class I MHC-Restricted Hypersensitivity Response to Abacavir

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Background - Susceptibility to a clinically significant drug hypersensitivity syndrome associated with abacavir use has a significant genetic component. We have shown that the presence of *HLA-B*5701* strongly predicts abacavir hypersensitivity (ABC HSR), particularly in combination with other allelic markers specific to the 57.1 ancestral haplotype (AH), and identified a potential susceptibility locus within a 300kb region between *MEGT1* and *C4A6* loci in the central MHC. Here we used fine recombinant haplotype mapping to identity the susceptibility loci. **Methods** - 248 consecutive abacavir-exposed individuals were studied, representing full ascertainment of abacavir use in the Western Australian HIV Cohort study. 18 cases of definite ABC HSR (7.3%) and230 tolerant controls were identified, utilising an updated clinical classification that included corroborative epicutaneous skin patch test. Patients were typed for genetic markers using standard molecular techniques. Intracellular measurement of TNF (three colour flow cytometry) and intracellular localisation of Hsp70 and HLA-B57 (confocal microscopy) were undertaken on abacavir exposed *ex vivo* polymorphic blood mononuclear cell (PBMC) cultures. **Results** - Recombinant mapping in patients with allelic markers of the 57.1 AH suggest a susceptibility locus within the Hsp70 gene cluster. *HLA-B*5701* was present in 94.4% of hypersensitive cases and 1.7% of controls (OR 960, pc<0.00001). A haplotypic non-synonymous polymorphism of Hsp70-Hom (HspA1L, M493T) was found in combination with *HLA-B*5701* in 94.4% of hypersensitive cases and 0.4% of controls (OR 3893, pc<0.00001). The Hsp70-Hom M493T allele was present in 22% of controls (OR 60, pc<0.00001), suggesting that the combination of *HLA-B*5701* and Hsp70-Hom M493T conferred susceptibility. Individuals with ABC HSR exhibited a significantly higher proportion of monocytes expressing TNF in response to *ex vivo* abacavir stimulation, which was abrogated, on depletion of $CD8^+$ T cells from whole blood. Increased intracellular expression of Hsp70 and HLA-B57 molecules in abacavir exposed *ex vivo* cultured PBMCs was observed in hypersensitive patients compared with controls. Hsp70 and HLA-B57 molecules co-localised within discrete vesicles.

Conclusions - These data indicate that the presence of *HLA-B*5701* and Hsp70-Hom M493T are predisposing factors in the development of ABC HSR, and implicates them in the generation of a Class I-restricted pathogenic immune response.

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INTRODUCTION

Exposure to abacavir is accompanied by a rare and sometimes lethal hypersensitivity reaction that typically involves multiple organs and rapid and more severe recurrence on rechallenge[1,2,3]. The presence of alleles carried on the 57.1 ancestral haplotype (AH), the *HLA-B*5701* allele in combination with *HLA-DR7,-DQ3*[4] or independently[5] and in association with TNF-238A[5,6] has been shown to predispose patients to this reaction. The putative susceptibility region was mapped to a 300kb region between C4A6 and MEGT1 within the central MHC[1]. Further fine mapping using microsatellite, SNP and recombinant haplotype mapping techniques will help identify the most parsimonious region carrying the gene(s) contributing to abacavir hypersensitivity[7]. Previous studies have indicated that the positive predictive value of *HLA-B*5701* testing (>70%) may be sufficient for use in clinical practice particularly in Caucasian populations[1]. Development of other idiosyncratic drug reactions involves MHC-restricted presentation of a drug or its reactive metabolite to the immune system[8,9]. We therefore examined the association of MHC alleles including HLA-B57 and Hsp70 in *ex vivo* abacavir stimulated cultures of PBMCs.

METHODS

Subjects-Retrospective study: The first 200 participants of the Western Australian HIV Cohort Study prescribed abacavir to 31 December 2001, who were included in our previous study[1], were reclassified based on updated diagnostic criteria. Clinical diagnostic criteria included the occurrence of classical symptoms suggestive of multi-system involvement that were present within 6 weeks of exposure. An appropriate temporal relationship between symptom resolution and cessation of abacavir was also required in these diagnostic criteria. Finally, in cases where the clinical diagnostic criteria were fulfilled but where an alternative explanation for these symptoms was present, epicutaneous patch testing and assessment of the specific immunological response to *ex vivo* abacavir stimulation were undertaken to clarify the role of abacavir. Prospective study: Forty-eight individuals exposed to abacavir since December 2001 were classified by a clinician blinded to the results of genetic analysis utilising the updated diagnostic criteria. However, individuals with *HLA-B*5701 and/or HLA-DRB1*0701/HLA-DQ3* were actively excluded from exposure to abacavir.

Patch test Epicutaneous patch testing was undertaken according to the protocol published by Phillips[10]. Patches containing 0% to 20% abacavir sulphate in white petrolatum were prepared by the Royal Perth Hospital pharmacy and applied on the left side of the thoracic spine, and were then read after 24 and 48 hours of application. A positive result required absence of an allergic response in the control vehicle, and demonstration of typical skin changes consisting of erythema and vesicular rash limited to the patch area. Attempts were also made to patch test all individuals who had been previously assigned as abacavir hypersensitive cases, including those carrying markers of the 57.1 AH (n=14.9 consented to patch testing) as well as those who did not (n=4, 3 consented to patch testing). Within the latter group, the three individuals who had an alternative explanation for their symptoms (concurrent nevirapine therapy) were tested. The remaining individual, who fulfilled diagnostic criteria for abacavir hypersensitivity in the absence of an alternative explanation, had relocated interstate and was therefore unavailable for further testing. Within the group clinically assigned as abacavir hypersensitivity not excluded (n=35) in the previous study (1), three individuals consented to patch testing. Testing was also performed in abacavir tolerant individuals carrying markers of the 57.1 AH (n=12, 4 consented to patch testing), including two who carried the *HLA-B*5701* allele, as well as in two individuals carried the full 57.1 AH (*HLA-B*5701, C4A6, HLA-DRB1*0701,-DQ3*) who had not been exposed to abacavir. Results were negative in all these individuals.

Typing of MHC markers and assignment of alleles specific to the 57.1 AH Typing of MHC markers was carried out using standard genetic assays and sequencing. SNPs within genes located in the *C4A6* to *MEGT1* region were examined from the following websites: (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?). Primers were either designed based on oligonucleotide sequences reported within the databases. Primers and conditions used in the assay are available on request. A set of well-characterised UBIWS EBV transformed homozygous B cell lines representing commonly occurring Caucasian AHs have used to determine alleles carried on the 57.1 AH. Full length sequencing across the Hsp70 gene cluster was carried out in our laboratory using overlapping primer pairs.

Measurement of TNF response to abacavir Intracellular expression of TNF was assessed in cultured peripheral blood mononuclear cells (PBMCs), derived from previously frozen aliquots, by three colour flow cytometry. PBMCs from 25 mL lithium heparin blood were separated by density gradient centrifugation on Ficoll and cryopreserved in aliquots of 2 x 10^6 cells/mL at -80°C in 10% DMSO and 90% heat-inactivated fetal calf serum (FCS)[11]. Thawed PBMCs were washed once and resuspended at 10^6/mL in 10% HIFCS- RPMI. PMA and abacavir sulphate at 4 mg/mL (prepared from Ziagen 300mg commercial grade tablet dissolved in PBS pH 7.4). TNF response was compared in PBMCs cultured in the presence and absence of abacavir. Aliquots of 0.5 x 10^6 cells/mL were cultured with and without abacavir for 3 hrs at 37°C in a CO_2 incubator. Briefly, Brefeldin A (10 µgm/mL, Sigma Chemical Co.) was added after incubation for 1hr with the stimulants. The PBMCs were then labeled with either CD45-RD1, CD14-FITC or IgG1-FITC (isotype control) antibodies (all obtained from Immunotech) and analysed on a Coulter™ EPICS® XL-MCL Flow cytometer using the Coulter XL List Mode Data Analysis program.

CD4 and CD8 cell depletion TNF levels were measured in the absence and presence of abacavir (1 µg/mL, Mozrosek Ltd) in cultured whole blood. One milliliter of whole blood was exposed to abacavir for 24 hrs at 37°C. The blood was centrifuged at 1500rpm for 5min and TNF levels were measured in the supernatant (plasma). Whole blood was also depleted of either $CD4^+$ or $CD8^+$ T cells using the Dynabead kit (Dynal, A.S. Oslo, Norway). TNF expression was compared in supernatant of the depleted blood cultured in the presence and absence of abacavir.

Confocal microscopy Ex vivo abacavir (kind gift from GSK) exposed cultured PBMCs from individuals carrying markers of the 57.1 AH with ABC hypersensitivity (n=10), ABC tolerant (n=5) and abacavir unexposed (n=2) were studied. TNF was measured using three colour flow cytometry and intracellular localisation of $Hsp70^{12}$ (kind gift of Anne Fourie, J&J, San Diego) and HLA-B57 (B17 allo-antisera) was done using differentially labelled fluorescent-tagged secondary antibodies and subsequent visualisation by confocal microscopy. Sub-cellular compartmentalisation of these two molecules was examined using markers specific for endoplasmic reticulum (tapasin) early and late endosomes (Rab4 and Rap1 respectively). Immuno-phenotyping of cells expressing Hsp70 and TNF was done using CD4, CD8 and CD14 (BD Biosciences) markers. A fluorescent microscope equipped with a Bio-Rad MRC 1024 UV confocal laser scanning system, controlled by Lasersharp image acquisition, was used to capture digital images of stained cells. All images for longitudinal samples were acquired during the same day for all experimental conditions, using identical instrument settings that avoided saturation of the brightest pixels. An average of 12 sequential tissue images was collected separately by Kalman, averaging 4 scans per image and using a ×40 oil immersion objective at optical zoom of 1.0 or 2.5. The series of optical sections was collected at 1µm micron increments along z z-axis, and Confocal Assistant software was used to compile 3-dimensional views. Red and green fluorophores, colocalising as yellow to label HLA-B57 and Hsp70, were assessed using Lasersharp 3.1 software and showed a colocalisation coefficient of 95%.

Patient demographics

Table 1.

Variable		Abacavir hypersensitive (n=18)	Abacavir tolerant (n=230)	P value
Sex	Male	15 (83%)	197 (86%)	0.73
	Female	3 (17%)	33 (14.3%)	
Ethnicity	Caucasian	18 (100%)	196 (85%)	0.15
	Non Caucasian	0	34 (15%)	
Mean (SD) age (years)		45 (10.3%)	42.6 (10.3%)	0.35
Mean (SD) CD4 (cells/µL)		446 (299)	435 (284)	0.85
Mean (SD) percentage CD8		51.8 (12.1%)	51.5 (12.1%)	0.92

Table 2

MHC marker	Abacavir hypersensitive (18)	Abacavir tolerant (230)	P-value (corrected)	Odds Ratio	Positive predictive value*	Negative predictive value*
HLA-B*5701	17 (94.4%)	4 (1.7%)	<0.00001	960	78.9%	99.4%
C4A6	14 (77.8%)	7 (3.0%)	<0.00001	111.5	72.2%	98.3%
HLA-DR7, -DQ3	14 (77.8%)	11 (4.8%)	<0.00001	69.7	65.0%	98.3%
Hsp70 Hom M493T	17 (94.4%)	51 (22.2%)	<0.00001	59.7	25.4%	99.3%
HLA-B*5701, C4A6	14 (77.8%)	0	<0.00001	1485	100%	98.4%
HLA-B*5701, -DR7, -DQ3	14 (77.8%)	0	<0.00001	1485	100%	98.4%
HLA-B*5701, Hsp70Hom M493T	17 (94.4%)	1 (0.43%)	<0.00001	3893	93.8%	99.5%

*Positive and negative predictive values were calculated within the retrospective abacavir-exposed cohort (n = 280), to exclude potential confounding caused by the active exclusion of abacavir use among those carrying 57.1 AH markers in the prospective cohort.

Figure 1

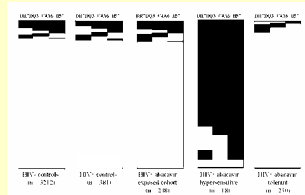

Figure 2

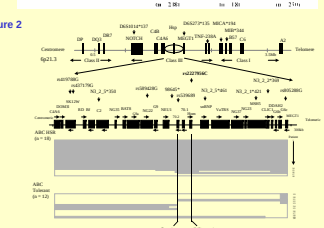

Figure 3

Figure 3. Significant increase in the number of CD14+ cells expressing TNF in abacavir stimulated cultures of PBMCs from patients with ABC HSR.

Figure 4

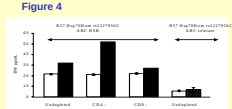

Figure 4 Abrogation of TNF production on depletion of $CD8^+$ T cells. TNF levels in ABC unstimulated (white: HSR, white stippled: non HSR) and stimulated (Black: HSR, black stippled:non HSR)

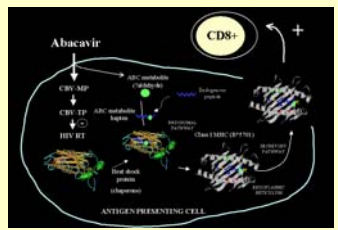

Figure 5 Possible pathogenic model for the generation of a hypersensitive response to abacavir. We hypothesise that abacavir gets metabolised intracellularly sequentially to carbovir mono phosphate (CBV-MP) and tri-phosphate (CBV-TP) to inhibit HIV RT. Hsp70 Hom M493T may facilitate loading of a reactive intermediate (possibly an aldehyde derivative[13]) of ABC haptenated peptide onto HLA-B*5701.

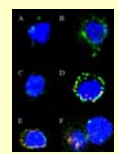

Figure 6 Co-localisation of HLA-B57 and Hsp70 . Partial HLA-B57 expression in PBMCs ABC tolerant patient without (A) and with abacavir (B). Substantial increase in HLA-B57 and Hsp70 expression in ABC unstimulated (C) and stimulated (D) culture of PBMCs after 3 hrs (A,B,C, D) and 24 hrs (E, F)

RESULTS

An updated restrictive definition of abacavir hypersensitivity individuals was used in this study. In a prospective study, 48 abacavir-recipients were HLA typed prior to exposure, All patients with HLA-B*5701, and DR7, -DQ3 were actively excluded. Two out of the 48 patients (4.2%) developed an ABC HSR reaction, both of whom carried the HLA-B*5701 allele, whilst the remaining 46 patients remained asymptomatic on abacavir therapy. Hence, the incidence of abacavir hypersensitivity among *HLA-B*5701* negative individuals in this prospective cohort was 0% (95% confidence interval 0%-0.075%).

The distribution of MHC alleles was significantly different in the abacavir hypersensitive group compared to the abacavir tolerant group (Table 2, Fig.1). With regard to alleles that are specific to the 57.1 AH, *HLA-B*5701* was present in 17/18 cases (94.4%) with abacavir hypersensitivity and 4/230 (1.7%) abacavir tolerant controls (OR=960, P_c<0.0001). *C4A6* was present in 14/18 cases (77.8%) and 7/230 (3.0%) controls (OR=111, P_c<0.0001), and the haplospecific combination of *HLA-DRB1*0701* and *-DQ3* was present in 14/18 cases (77.8%) and 11/230 (4.8%) controls (OR=70, P_c<0.0001). Hence, *HLA-B*5701* provided the strongest univariate association with susceptibility to abacavir hypersensitivity. Combining these markers, the previously described *HLA-B*5701, C4A6, HLA-DRB1*0701, -DQ3* haplotype (1) was present in 14/18 cases (77.8%) and in none of the 230 controls (OR=1485, P_c<0.0001)[13].

Recombinant haplotype analysis to map the putative susceptibility locus/loci was carried out on the restricted patient sample recombinant for the 57.1AH in the MHC region (Fig. 2). Polymorphic markers between *C4A6* and *MEGT1* were identified by comparing the 57.1 AH with a subset of well-characterised EBV-transformed cell lines representative of common Caucasian AHs (data not shown). With reference to abacavir hypersensitive individuals, the boundary of the putative susceptibility region included the Hsp70 region of the 57.1AH. The translated sequences of Hsp70.1 and Hsp70.2 were similar in both abacavir hypersensitive individuals (cases 15-17) and controls with recombinant 57.1 haplotypes (cases 19-22), and were therefore excluded as candidate susceptibility loci. We observed that the Hsp70 Hom M493T allele, carried on the 57.1 AH, was detected in 94.4% of the hypersensitive group compared with 22.2% of tolerant controls (17/18 vs 51/230, P_c<0.00001, OR=59.7, P_c<0.00001). However, in combination with *HLA-B*5701*, the Hsp70-Hom M493T allele was strongly associated with abacavir hypersensitivity (17/18 vs 1/230, P_c<0.00001, OR=3893, P_c<0.00001) (Table 2).

The proportion of TNF-positive cells was higher in patients with abacavir hypersensitivity (n=8) than abacavir tolerant controls (n=9) with a median 13.4-fold increase, (median 13.4, IQR=13.0) compared with a median 8.9-fold decrease in proportion of TNF positive cells in tolerant controls (median –8.9, IQR=24.1, P<0.008 Mann Whitney test) (Fig.3). Extracellular levels of TNF were higher in abacavir stimulated whole blood cultures of the ABC HSR individual compared with the tolerant control (Fig.4). In addition, TNF levels were attenuated in the abacavir-stimulated blood of an abacavir hypersensitive patient when $CD8^+$ T cells were depleted, compared with undepleted or $CD4^+$-depleted cultures (Fig.4), suggesting the involvement of MHC class I molecules and $CD8^+$ T cells in the development of this immune reaction.

A parsimonious model that suggests Hsp70-Hom M493T assisted cross-presentation of abacavir and or its hapten by HLA-B*5701 to the immune system has been presented (Fig 5)[13].

Co-localisation of HLA-B57 and Hsp70 was observed in discrete, punctate vesicles in peri-cell membrane and peri-nuclear region of a patient with ABC HSR but not in a tolerant control (Fig 6) studied using confocal microscopy CD14+ cells expressed (Fig 7) Hsp70 molecules. HLA-B57 and Hsp70 appear to co-localise within late endosomes (Fig 8) early endosomes (Fig 9) and the ER (Fig 10). A model describing the expression and subsequent compartmentalisation has been suggested (Fig 11).

CONCLUSIONS

1. **The putative HSR region includes a 14kb Hsp70.2 to Hsp70.1 gene interval.**
2. **The association of HLA-B*5701 and Hsp70Hom M493T is highly predictive of abacavir hypersensitivity.**
3. **Co-localisation of HLA-B57 and Hsp70 occur in early, late endosomes and endoplasmic reticulum of $CD14^+$ cells of ABC HSR individuals.**

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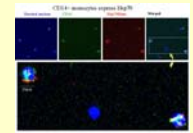

Figure 7 Expression of Hsp70 in CD14+ cells after stimulation Nuclei stained with Hoechst dye in an ABC hypersensitive pa

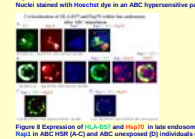

Figure 8 Expression of HLA-B57 and Hsp70 in late endosom Rap1 in ABC HSR (A-C) and ABC unexposed (D) individuals AH after stimulation with abacavir

Figure 9 Marginal co-localisation of HLA-B57 and Hsp70 in stained with Rab4 after ABC stimulation in an ABC HSR (A) individual. Note absence of HLA-B57 in the ABC tolerant indi

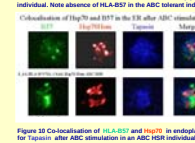

Figure 10 Co-localisation of HLA-B57 and Hsp70 in endoplas for Tapasin after ABC stimulation in an ABC HSR individual

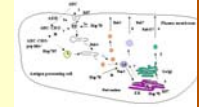

Figure 11 Schematic model describing possible intracellular