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T-Cells from HLA-B*57:01+ Human Subjects Are Activated with Abacavir through Two Independent Pathways and Induce Cell Death by Multiple Mechanisms

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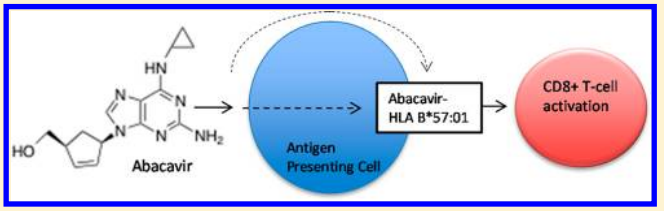
ABSTRACT: Susceptibility to abacavir hypersensitivity has been attributed to possession of the specific human leukocyte antigen allele HLA-B*57:01. HLA-B*57:01-restricted activation of CD8+ T-cells provides a link between the genetic association and the iatrogenic disease. The objectives of this study were to characterize the functionality of drug-responsive CD8+ T-cell clones generated from HLA-B*57:01+ drug-naive subjects and to explore the relationship between abacavir accumulation in antigen presenting cells and the T-cell response. Seventy-four CD8+ clones expressing different Vβ receptors were shown to proliferate and kill target cells via different mechanisms when exposed to abacavir. Certain clones were activated with abacavir in the absence of antigen presenting cells. Analysis of the remaining clones revealed two pathways of drug-dependent T-cell activation. Overnight incubation of antigen presenting cells with abacavir, followed by repeated washing to remove soluble drug, activated approximately 50% of the clones, and the response was blocked by glutaraldehyde fixation. In contrast, a 1 h antigen presenting cell pulse did not activate any of the clones. Accumulation of abacavir in antigen presenting cells was rapid (less than 1 h), and the intracellular concentrations were maintained for 16 h. However, intracellular abacavir was not detectable by mass spectrometry after pulsing. These data suggest that T-cells can be activated by abacavir through a direct interaction with surface and intracellular major histocompatibility complex (MHC) molecules. With the former, abacavir seemingly participates in the MHC T-cell receptor binding interaction. In contrast, the latter pathway likely involves MHC binding peptides displayed as a consequence of abacavir exposure, but not abacavir itself.



INTRODUCTION

Abacavir, {(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl} methanol, is an effective nucleoside reverse transcriptase inhibitor used for the treatment of HIV. Unfortunately, the therapeutic application of abacavir has been restricted due to the appearance of serious delayed-type hypersensitivity reactions in 4−8% of patients commencing therapy,[1] though cases of clinically confirmed reactions (i.e., positive patch test with abacavir) are considerably lower.[2] Expression of the HLA allele B*57:01 is strongly associated with susceptibility to abacavir hypersensitivity,[2,3] and a recent change in drug labeling, highlighting the need for HLA-B*57:01 testing prior to abacavir exposure, has effectively reduced the incidence of hypersensitivity.[4,5]

Abacavir stimulates the specific release of proinflammatory cytokines, including interferon-γ[6] and TNF-α,[7] from peripheral blood mononuclear cells (PBMC) of hypersensitive patients *ex vivo*. Biopsies from inflamed skin of hypersensitive patients show a marked infiltration of CD8+ T-cells, suggesting that tissue damage develops as a result of cytotoxic T-cell activity.[8] Innovative studies by Chessman et al. defined the role of HLA-B*57:01 in the disease pathogenesis. Abacavir-specific T-cell responses were detected *in vitro* using lymphocytes from healthy volunteers expressing HLA-B*57:01 but not other closely related HLA alleles.[9] The fine specificity of the abacavir−major histocompatibility complex (MHC) interaction was mapped to the B*57:01 antigen-binding F pocket where a serine residue at position 116 was important for drug antigen presentation. Indeed, an interaction between abacavir and cell surface and endogenous HLA-B*57:01 has recently been postulated,[10,11] with the latter having an effect on the display of HLA-restricted peptide epitopes.[10,12,13] Most notably, these authors suggest that activation of T-cells occurs in the absence of drug metabolism and hapten protein binding.

The aims of this study were to utilize abacavir-responsive CD8+ T-cells cloned from volunteers expressing HLA-B*57:01 to explore the profile of effector molecules released by activated T-cells and the relationship between drug accumulation in antigen presenting cells and the T-cell response.

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Table 1. Volunteer Characteristics and Generation of Abacavir-Specific T-Cell Clones

								abacavir clones	
volunteer	HLA type			age	sex	LTT[a]	clones tested	CD4	CD8
1	A*03:01	B*14:02	C*06:02	26	F	−	276	0	67
	A*24:02	B*57:01	C*08:02						
	DRB1*13:02	DQB1*03:03	DQA1*01:02						
	DRB1*07:01	DQB1*06:09	DQA1*02:01						
2	A*01:01	B*49:01	C*06:02	38	M	−	208	0	5
	A*01:01	B*57:01	C*07:01						
	DRB1*16:01	DQB1*03:03	DQA1*01:02						
	DRB1*07:01	DQB1*05:02	DQA1*02:01						
3	A*01:01	B*08:01	C*06:02	27	M	−	314	0	2
	A*02:01	B*57:01	C*07:01						
	DRB1*03:01	DQB1*02:01	DQA1*05:01						
	DRB1*03:01	DQB1*02:01	DQA1*05:01						
4	A*03:01	B*07:02	C*07:01	26	M	−	96	0	0
	A*31:01	B*08:01	C*07:02						
	DRB1*03:01	DQB1*02:01	DQA1*03:01						
	DRB1*04:04	DQB1*03:02	DQA1*05:01						
5	A*02:01	B*15:01	C*03:04	24	F	−	72	0	0
	A*11:01	B*51:01	C*15:02						
	DRB1*01:01	DQB1*03:02	DQA1*01:01						
	DRB1*04:01	DQB1*05:01	DQA1*03:01						
6	A*02:01	B*14:02	C*07:01	31	M	−	24	0	0
	A*03:01	B*18:03	C*08:02						
	DRB1*13:02	DQB1*02:01	DQA1*01:02						
	DRB1*07:01	DQB1*06:04	DQA1*02:01						

[a]A lymphocyte transformation test (LTT) was performed in order to rule out any prior sensitization to the drug. A stimulation index of >2 is considered a positive result.

EXPERIMENTAL PROCEDURES

Chemicals. All reagents were obtained from Sigma-Aldrich (Dorset, United Kingdom), unless otherwise stated. Abacavir was a kind gift from GlaxoSmithKline.

Expression of HLA Molecules and PBMC Proliferation. Ethical approval for the study was obtained from the local research ethics committee, and consent was obtained from all volunteers prior to blood donation. Four hundred healthy volunteers were recruited from North West England. A total of 100 mL of blood was collected for both DNA and PBMC isolation. Further donations were requested to replenish cell stocks when required. PBMCs were stored at −150 °C and rapidly thawed when required. Confidentiality was maintained throughout the study.

Genomic DNA was extracted from whole blood using Chemagic magnetic separation (Chemagen, Baesweiler, Germany) and high-resolution sequence-based HLA typing was performed by the Histogenetics laboratory (Histogenetics, New York, USA) at the following loci: HLA-A, -B, -C, -DRB1, and -DQB1. Results of the HLA typing have been reported previously.[14]

Three HLA-B*5701 positive and negative volunteers were randomly selected for experiments described from this point forward. Proliferation of PBMCs (1.5×10^5/well; total volume 200 μL) against abacavir (1−100 μM) and tetanus toxoid (1 μg/mL; positive control) was initially measured using the lymphocyte transformation test.[15]

Generation of Autologous EBV-Transformed B-Cell Lines. Epstein−Barr virus (EBV) transformed B-cell lines were created from PBMCs by transformation with supernatant from the virus-producing cell line B9.58. Lines were maintained in medium comprising RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Paisley, U.K.), 25 mM HEPES, 1000 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine, and used as a source of autologous antigen presenting cell.

Cell Lines and T-Cell Cloning. PBMCs (1×10^6; 0.5 mL) were cultured with abacavir (50 μM) in complete medium comprising RPMI 1640 supplemented with 10% human AB serum (Innovative Research, Class A), 25 mM HEPES, 1000 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine, and 25 μg/mL transferrin. Cultures were supplemented with rhIL-2 (Peprotech, U.K.) on days 6 and 9. On day 14, CD4+ and CD8+ cells were separated using MultiSort kits (Miltenyi Biotec, Surrey, UK). Separated cells were then cloned by serial dilution using established methods.[16]

Abacavir specificity was assessed by the addition of autologous irradiated EBV-transformed B-cells (1×10^4/well) and abacavir (50 μM) to T-cell clones (5×10^4/well; 200 μL). [^{3}H]-Thymidine (0.5 μCi) was added after 48 h, and proliferation was measured by scintillation counting. Clones with a stimulation index of greater than 2 were expanded by repetitive mitogen stimulation in IL-2 containing medium for further analysis. CD and Vβ phenotyping was performed with a panel of over 30 antibodies using a BD FACSCanto II flow cytometer. The effect of drug concentration on the T-cell response was measured by incubating clones and irradiated EBV-transformed B-cells with abacavir (1, 5, 10, 50, or 100 μM) and measuring proliferation and secretory molecules (IL-5, IL-13, IFN-γ, FAS ligand, perforin, granzyme B) by [^{3}H]-thymidine incorporation and ELISpot, respectively. Supernatants from abacavir-stimulated cultures and control wells were collected and stored for global analysis of cytokine secretion using a Millipore multiplex assay kit (Th1/Th2 panel including IL-9; Millipore, Hertfordshire, U.K.) on a Bio-Plex Suspension Array System (model Luminex100). For the ELISpot assays, plates were developed after 48 h according to the manufacturer's instructions.

Mechanism of Antigen Presentation to Abacavir-Responsive T-Cells. A stepwise approach was adopted to study pathways of abacavir presentation to T-cell clones. First, autologous EBV-transformed B-cells were omitted from the proliferation and IFN-γ ELIspot assays described above. Second, clones were stimulated with autologous and allogeneic EBV-transformed B-cells expressing different HLA-B (5702, 5801, 7, 8, 18, 27, 35, 44, 45, 65, and 62) allotypes. Third, autologous EBV-transformed B-cells, some of which were subjected to aldehyde fixation to prevent intracellular processes, were pulsed for 16 h with abacavir (50 μM). Following repeated washing,

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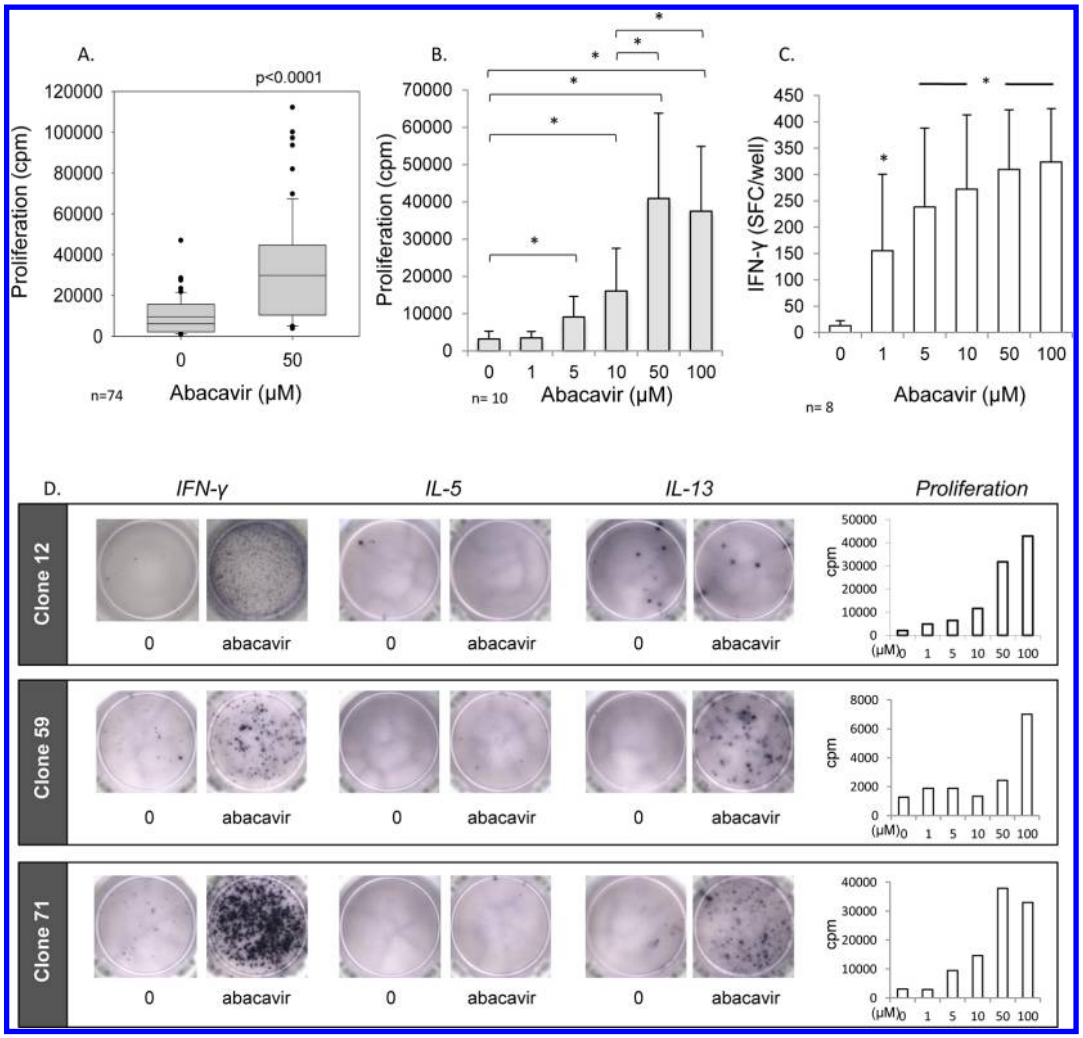

Figure 1. Proliferation and cytokine secretion from abacavir-stimulated CD8+ clones. (A) Proliferation of 74 abacavir-responsive clones from three HLA-B*5701 positive volunteers stimulated with EBV-transformed B-cells in the presence or absence of abacavir (50 μM). (B, C) Concentration-dependent proliferation (n = 10 clones) and interferon-γ secretion (n = 8 clones) from T-cell clones stimulated with abacavir (*p < 0.05; one-way ANOVA). Data shows mean ± SD. For the individual clones, each abacavir concentration was analyzed in duplicate. (D) Representative IFN-γ, Il-5, and IL-13 ELISpot data for three CD8+ clones cultured with and without abacavir (100 μM) for 48 h. For comparison, the abacavir-specific concentration-dependent proliferative response is also shown. Data shows the mean of replicate cultures. Coefficient of variation less than 20%.

the pulsed antigen presenting cells were irradiated and incubated with T-cell clones. Intracellular abacavir concentrations prior to and after washing were measured by liquid chromatography coupled to mass spectrometry. Deuterated abacavir (D_2 labeled on the methanol side chain; Emma Yang, University of Liverpool; 200 nM; 50 μL) was used as internal standard. Acetonitrile (10 volumes) was added, and precipitated proteins were removed by centrifugation at 4 °C. Samples were then dried and reconstituted in mobile phase. Abacavir concentrations were analyzed using a 4000 QTRAP mass spectrometer (AB Sciex, Warrington, U.K.) coupled to an Ultimate 3000 liquid chromatography system (Dionex, Warrington, U.K.). Chromatographic separation was performed on an Eclipse XBD C18 (2.1 mm × 150 mm, 5 μm) column (Agilent, Cheshire, U.K.) at 30 °C. Mobile phase A consisted of 10 mM ammonium acetate, pH 3.8, while mobile phase B consisted of acetonitrile. The compounds were eluted from the chromatographic column using a linear gradient (5−20% over 20 min) with a flow rate of 0.2 mL/min. The mass spectrometer was operated in positive polarity. Nitrogen (20 psi) was used as both the nebulizing and drying gas. The source was operated at 500 °C, and electrospray voltage was set to 5 kV. Analyses were performed in MRM scanning mode. Data was processed using Analyst v1.5 software. Lower limit of quantitation (LLOQ) for abacavir was 0.3 nM. Quantification was carried using the ratio of analyte to internal standard peak areas.

Characterization of the Relationship between Abacavir Distribution in Antigen Presenting Cells and the Activation of CD8+ T-Cell Clones. The kinetics of abacavir uptake into antigen presenting cells was measured to explore the consequences of drug distribution on the T-cell response. EBV-transformed B cells (2×10^7; 5 mL) were incubated with 10−50 μM abacavir for 1, 4, and 16 h. At each time point, cells were harvested and divided into two fractions. Fraction 1 was subjected to repeated washing, prior to irradiation and incubation with T-cell clones in the absence of soluble drug. Fraction 2 was pelleted by centrifugation, suspended in 5 mL water, sonicated (4 × 10 s), and processed for mass spectrometric analysis. In order to determine the effect of glutaraldehyde on cellular uptake of abacavir, the experiment was repeated using fixed cells.

Statistical Analysis. The Mann−Whitney test was used to compare T-cell responses in drug-treated vs control wells.

RESULTS

Expression of HLA Molecules and PBMC Proliferation and Cytokine Secretion. The demographics and HLA alleles detected in our HLA-typed volunteer cohort are described in detail elsewhere.[14] HLA-B*57:01 was present in 26 individuals

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Table 2. Cytokine Secretion from Abacavir Stimulated T-Cell Clones

ID	proliferation (SI)	IL-2[a]	IL-4	IL-5	IL-9	IL-10 (pg/mL)	IL-12	IL-13	IFN-γ	TNF-α
12	22.8	16 ± 27	*b*	*b*	*b*	*b*	*b*	43 ± 12	86 ± 38	212 ± 110
13	7.2	*b*	*b*	*b*	*b*	*b*	*b*	20 ± 53	2 ± 1	22 ± 17
19	39.4	72 ± 100	*b*	*b*	*b*	*b*	*b*	71 ± 8	75 ± 2	188 ± 115
37	5.8	*b*	*b*	8 ± 13	*b*	3.2	*b*	4008 ± 502	3 ± 1	11 ± 3
59	13.3	*b*	*b*	8 ± 7	*b*	*b*	*b*	2857 ± 288	*b*	37 ± 3
71	12.3	72 ± 11	*b*	*b*	*b*	*b*	*b*	1454 ± 349	286 ± 21	560 ± 20
105	8.6	46 ± 2	*b*	36 ± 13	*b*	1.5	*b*	7089 ± 420	108 ± 26	351 ± 95
116	20.6	*b*	*b*	*b*	*b*	*b*	*b*	336 ± 221	*b*	20 ± 9
160	10.8	3500 ± 2600	*b*	182 ± 57	*b*	*b*	*b*	>10000	8002 ± 720	>10,000
168	40.6	928 ± 310	*b*	412 ± 287	*b*	*b*	*b*	>10000	1649 ± 68	2994 ± 450

[a]Supernatant was collected from clone cultures with abacavir (1, 5, 10, 50, or 100 μM) for 48 h. Abacavir concentrations associated with a maximum proliferative response (50 or 100 μM) were used to analyze cytokine secretion. Data shows mean ± SEM of duplicate cultures with cytokine levels in drug-free wells subtracted (Cytokine levels in control [no abacavir] wells: less than 5 pg/mL IL-4, IL-9, IL-10, IL-12, IFN-γ, or TNF-α; less than 20 pg/mL; less than 50 pg/mL IL-2, IL-5, IL-13). [b]Not detectable.

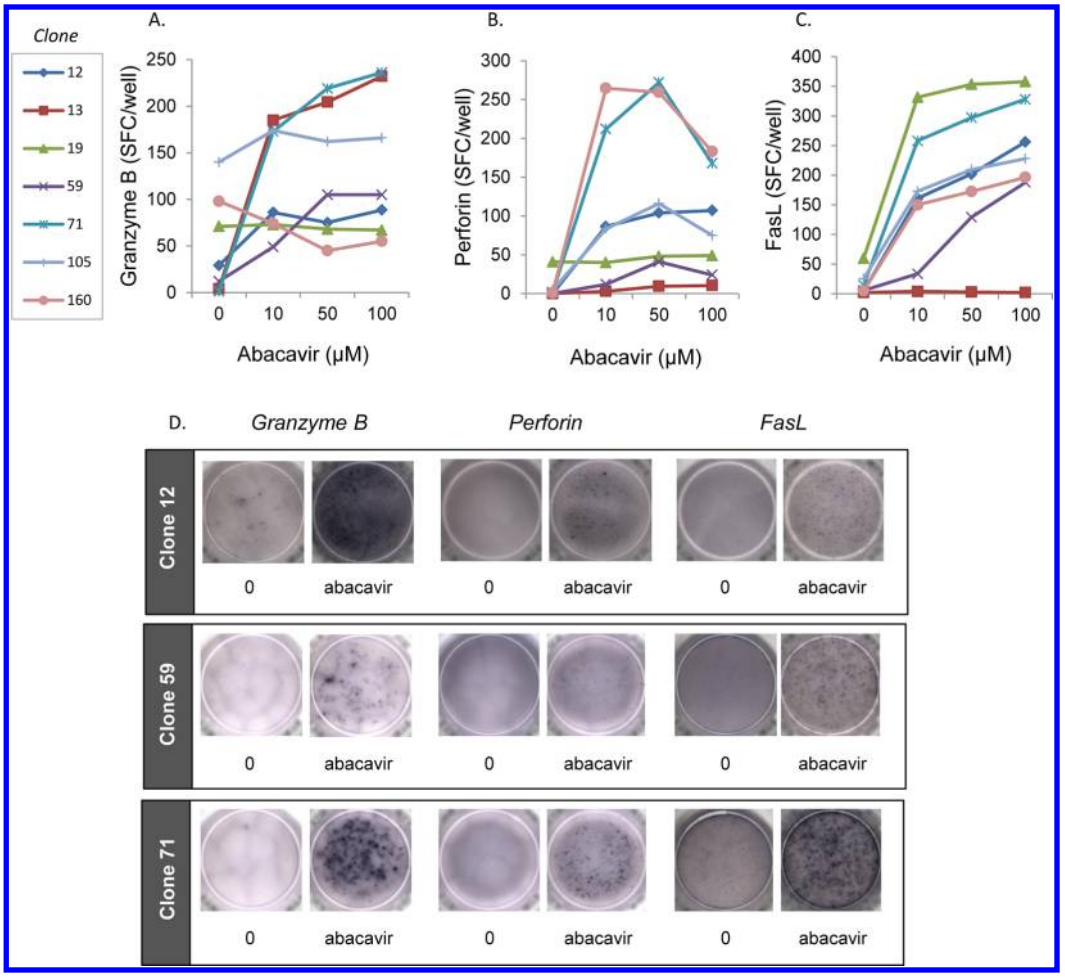

Figure 2. Abacavir-specific cytotoxic activity of CD8+ clones. Concentration-dependent secretion of the effector molecules (A) granzyme B, (B) perforin, and (C) FasL from eight abacavir-stimulated CD8+ clones. Data show the mean of replicate cultures. The coefficient of variation was consistently less than 20%. (D) Representative ELIspot data for three clones. Clones were cultured with and without abacavir (100 μM) for 48 h prior to the analysis of effector molecules.

(1 homozygote, 25 heterozygotes). For the purpose of these mechanistic studies, PBMCs from three volunteers expressing HLA-B*57:01 and three volunteers expressing other HLA-B alleles were used (Table 1). Proliferative responses and IFN-γ secretion were not detected when PBMCs were directly stimulated with abacavir. In contrast, tetanus toxoid-specific lymphocyte responses were readily detectable (results not shown).

Generation and Characterization of Abacavir-Responsive T-Cell Clones. Seventy-four abacavir-responsive CD8+ clones were generated from the three volunteers expressing HLA-B*57:01 (Table 1; Figure 1A, 0, 9556 ± 9131 cpm; abacavir [50 μM], 33409 ± 26548 cpm; $P < 0.0001$). In

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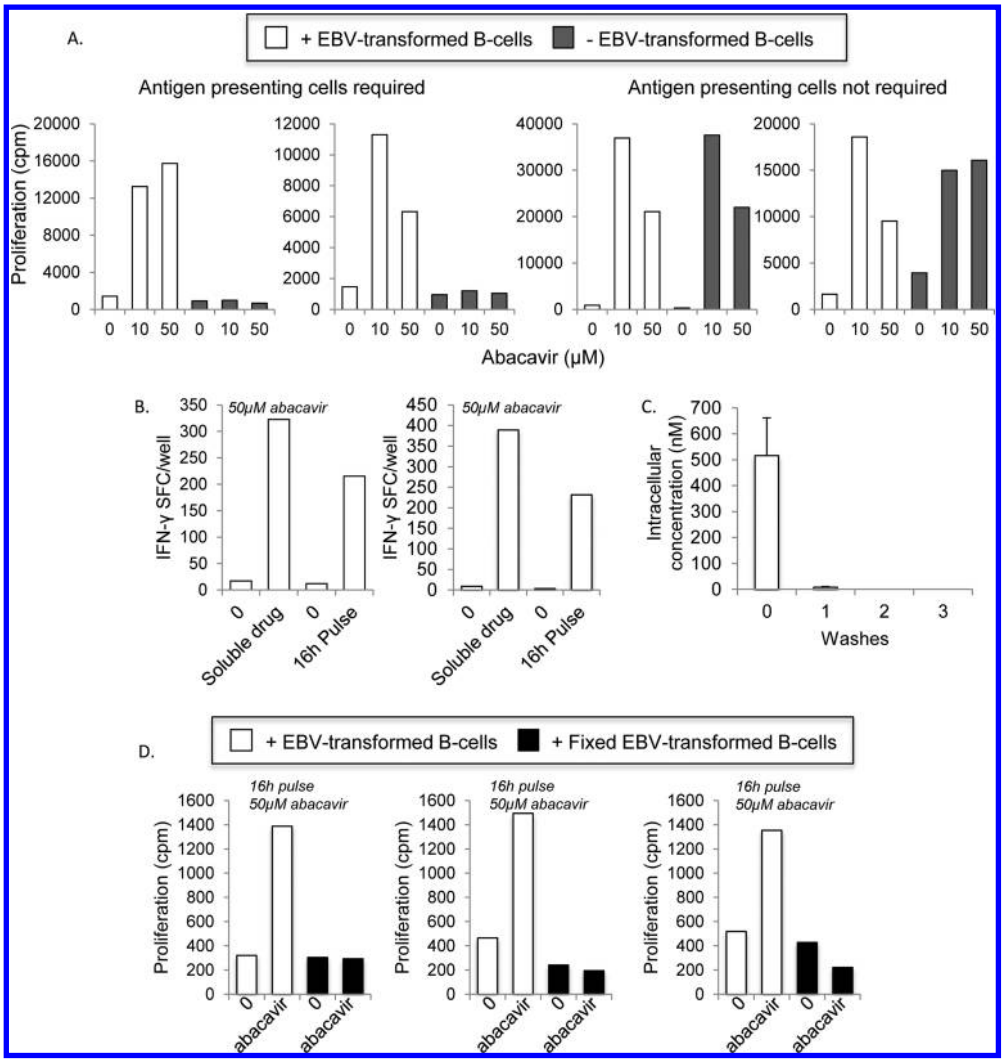

Figure 3. Abacavir presentation to CD8+ clones. (A) Activation of clones with abacavir in the presence or absence of EBV-transformed B-cells. [^{3}H]thymidine was added for the final 16 h of the experiment to measure the abacavir-specific proliferative response. Representative clones are shown; each data point depicts the mean of replicate cultures. Coefficient of variation was consistently less than 20%. All subsequent experiments to study mechanisms used the clones that needed antigen presenting cells to stimulate an abacavir-specific response. (B) Activation of clones with EBV-transformed B-cells pulsed with abacavir for 16 h. IFN-γ ELIspot was used to measure the abacavir-specific T-cell response. Similar data was obtained using [^{3}H]thymidine (results not shown). Each data point shows the mean of replicate cultures. The coefficient of variation was consistently less than 20%. (C) Intracellular abacavir concentration after 16 h before and after washing with drug-free medium. (D) Activation of clones with glutaraldehyde-fixed or unfixed abacavir pulsed EBV-transformed B-cells. [^{3}H]thymidine was added for the final 16 h of the experiment to measure the abacavir-specific proliferative response. Representative clones are shown; each data point depicts the mean of replicate cultures. Coefficient of variation was consistently less than 20%.

contrast, when over 400 CD4+ clones were incubated with EBV-transformed B-cells and abacavir, specific reactivity was not detected. One hundred ninety-two CD4+ or CD8+ clones were generated from the three HLA-B*57:01 negative volunteers, and specific activity with abacavir tested. Abacavir-specific responses were not observed.

The T-cell receptor Vβ repertoire on abacavir-responsive CD8+ clones from HLA-B*57:01+ volunteers was heterogeneous (results not shown). Proliferation was observed with abacavir concentrations ranging from 5 to 100 μM (Figure 1B). As described elsewhere,[9] the T-cell response with abacavir was restricted by the HLA-B*57:01 allotype. Clones were stimulated with abacavir when the drug was presented by transformed B-cells expressing HLA-B*57:01 but not other common HLA-B alleles (data not shown).

The abacavir-specific T-cell proliferative response was associated with high levels of IFN-γ, IL-13, and TNF-α secretion (Table 2 [luminex analysis]; Figure 1C [IFN-γ ELISpot]; Figure 1D [IFN-γ, IL-5, and IL-13 ELISpot showing representative clones]) and the killing of [^{51}Cr]-loaded target cells (results not shown). In contrast, cytokines IL-4, IL-5, IL-9, and IL-10 were secreted at lower levels and only by a limited number of clones. T-cell activation was associated with the release of cytolytic molecules including perforin, granzyme B, and FasL (Figure 2A–C). Clones were broadly divided into three categories: (1) clones secreting effector molecules and displaying increased FAS ligand expression (e.g., clones 12, 59, and 71); (2) clones primarily secreting effector molecules (e.g., clone 13); and (3) clones primarily displaying increased FAS ligand expression (e.g., clone 19).

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Direct and Processing-Dependent Activation of CD8+ T-Cell Clones. Abacavir stimulated 45% of the clones to proliferate and secrete IFN-γ directly, in the absence of irradiated EBV-transformed B-cells. The remaining clones were activated only in the presence of EBV-transformed B-cells (Figure 3A); accordingly, these clones were used to probe mechanisms of drug antigen presentation. At least two different mechanisms were observed. EBV-transformed B-cells pulsed with abacavir for 16 h, prior to three washes with drug-free medium, stimulated 10 out of 19 clones to proliferate and secrete IFN-γ (Figure 3B). Intracellular abacavir was not detectable by mass spectrometry after two washes (Figure 3C). To determine whether the response of clones against abacavir-pulsed antigen presenting cells was dependent on intracellular processing, transformed B-cells were fixed with glutaraldehyde prior to abacavir exposure. Clones were activated with soluble abacavir using fixed and unfixed antigen presenting cells. However, the T-cell response directed against abacavir pulsed antigen presenting cells was blocked by fixation (Figure 3D).

Abacavir Accumulates Rapidly in Antigen Presenting Cells, But an Overnight Incubation Is Needed To Activate CD8+ T-Cell Clones. Exposure of abacavir to antigen presenting cells for a short duration (1 h) did not activate the clones (Figure 4A). Thus, experiments were designed to compare the cellular accumulation of abacavir to the drug-specific activation of T-cell clones. Intracellular concentrations of abacavir were measured in glutaraldehyde-fixed and unfixed EBV-transformed B-cells after 1, 4, and 16 h (Figure 4B). Abacavir was found to enter fixed and unfixed cells rapidly (within 1 h), and the intracellular concentration was maintained throughout the course of the 16 h incubation.

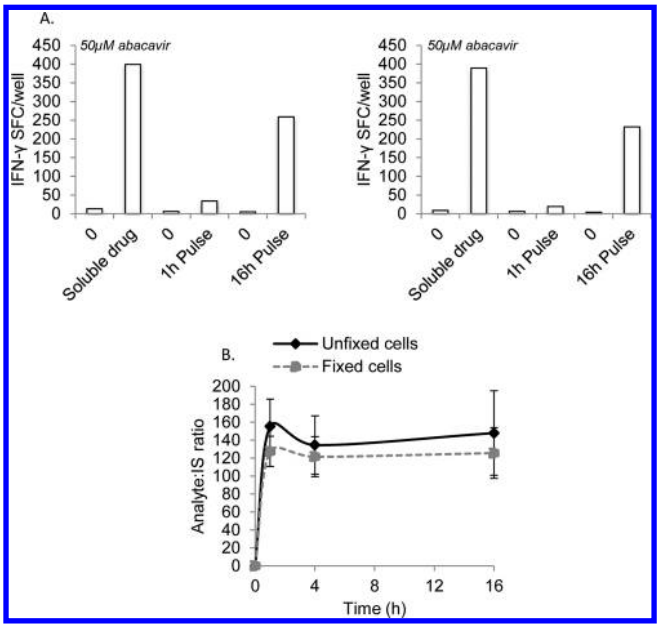

Figure 4. Abacavir accumulation in antigen presenting cells and the activation of CD8+ clones. (A) Activation of clones with soluble abacavir and EBV-transformed B-cells pulsed with abacavir for 1 or 16 h. IFN-γ ELIspot was used to measure the abacavir-specific T-cell response. Similar data was obtained using [^{3}H]thymidine (results not shown). Representative clones are shown; each data point depicts the mean of replicate cultures. Coefficient of variation was consistently less than 20%. (B) Accumulation of abacavir into glutaraldehyde-fixed and unfixed EBV-transformed B-cells. Intracellular abacavir concentrations were measured by mass spectrometry. Each data point shows the mean ± SEM of replicate experiments.

DISCUSSION

Immunological drug reactions are frequently referred to as unpredictable with no clear relationship discernible between dose and the development of clinical signs. However, the detection of associations between specific HLA alleles and hypersensitivity to a diverse group of drugs (e.g., abacavir, carbamazepine, allopurinol, nevirapine, ximelagatran, flucloxacillin, lumiracoxib)[3,17−22] has changed the way in which immunological drug reactions are defined; reactions are no longer entirely unpredictable. For abacavir and, more recently, the anticonvulsant carbamazepine in patients of South East Asian origin, genetic screening for expression of the HLA alleles HLA-B*57:01 and B*15:02, respectively, has been shown to decrease the incidence of reactions.[2,23] Innovative studies of Chessman et al.[9] describe the immunogenetic basis for the development of HLA-restricted abacavir hypersensitivity. The authors established that it was possible to detect abacavir-specific CD8+ T-cells *in vitro* using PBMCs from drug naive volunteers expressing HLA-B*57:01. A number of groups have now shown that (1) the T-cell response was entirely restricted by the HLA-B*57:01 allotype and (2) the activation of T-cells can occur independently of drug metabolism.[10−13]

In our study, abacavir-responsive CD8+ clones, with a clear Th1 cytokine secretion profile, were generated from volunteers expressing HLA-B*57:01 following a 14-day abacavir-driven T-cell enrichment step. Varying numbers of clones were generated from each individual, which may relate to technical issues; in particular, the extensive handling needed to isolate and culture CD8+ clones.[15] However, because 45% of individuals that carry the HLA-B*57:01 allele do not develop hypersensitivity when exposed to abacavir, it is also possible that the frequency of drug-responsive clones is one factor that determines susceptibility.[2] CD4+ clones were generated in large numbers, but abacavir-specific responses were not detected. This is consistent with the class I HLA association. Following drug-induced T-cell receptor triggering, target cell killing might result from increased surface expression of FAS ligand or release of soluble mediators such as granzymes, granulysin, and perforin. FAS ligand activates the apoptotic cascade through ligation of FAS receptors expressed on target cells. Perforin disrupts cell membranes, while granzymes and granulysin induce apoptosis, at least in part, through direct activation of caspases (granzymes, caspase 3 and 9; granulysin, caspase 7).[24,25] The different death pathways activated by T-cells prompted the study of effector molecules released from individual abacavir-responsive clones that are capable of activating the apoptotic cascade. Our findings demonstrate that clones release perforin, granzyme B, increase surface expression of FasL and kill target cells following activation with abacavir. Furthermore, it was possible to group the clones into three categories: (1) clones secreting effector molecules and displaying increased FAS ligand expression; (2) clones secreting effector molecules; (3) clones displaying increased FAS ligand expression. These data support a recent report describing distinct granzyme-containing and FAS ligand-associated secretory lysosomes in human T-cells[26] and suggest that lysosomes are differentially expressed in clones or differentially activated in response to T-cell receptor signaling. New insights in cell biology have revealed that other modes of T-cell mediated killing exist, such as programmed necrosis and

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autophagic death.[27] Although beyond the scope of the current investigation, it would be interesting to study whether IFN-γ secretion from abacavir clones triggers autophagy-related necrotic death as has been described in a concanavalin A hepatitis model.[28]

Considerable advances have been made in our understanding of the abacavir HLA-B*57:01 interaction and the nature drug-specific T-cell response. Our data must therefore be discussed in this context. Adam et al.[11] demonstrated that CD8+ T-cell clones are activated rapidly with abacavir bound directly to MHC molecules expressed on the cell surface and argue that reactivity patterns are dependent on HLA-B*57:01 surface densities. However, modification of surface MHC molecules is only part of the story. A number of groups have independently proposed an additional pathway for the abacavir-specific activation of T-cells.[10,12,13] Abacavir modification of specific residues located within the binding groove of HLA-B*57:01 was shown to induce the loading of novel self-peptides that trigger "abacavir-specific" T-cell responses. Key aspects of this work include (1) the elucidation of a crystal structure of a self-peptide bound to HLA-B*57:01 in the presence of abacavir and (2) demonstration that T-cell recognition of self-peptides is dependent on intracellular class I processing.

Importantly, neither of these proposed pathways, when evaluated in isolation, fully explains our findings relating the activation of CD8+ clones to the cellular disposition of abacavir. Herein, we report that (1) abacavir is taken up rapidly by antigen presenting cells and intracellular drug concentrations remain constant for 16 h, (2) antigen presenting cells pulsed with abacavir for 16 h, but *not* 1 h stimulate approximately 50% of the drug-responsive clones, (3) intracellular abacavir is not detectable after washing antigen presenting cells with drug-free medium, and (4) activation of clones with abacavir-pulsed antigen presenting cells (not soluble abacavir) is blocked by glutaraldehyde fixation, which inhibits metabolic processes but not the cellular accumulation of abacavir. Collectively, these data indicate that T-cells can be activated by abacavir through a direct interaction with surface and intracellular MHC molecules. With the former, abacavir seemingly participates in the MHC T-cell receptor binding interaction. With the latter, the altered repertoire of HLA-restricted peptides displayed as a consequence of abacavir MHC binding for 16 h are likely to be the primary antigens that activate T-cells.

We have recently used mass spectrometry to detect low levels of isomeric abacavir carboxylic acids in subcellular fractions of antigen presenting cells incubated with abacavir (unpublished data). In contrast to abacavir oxidative metabolism in liver preparations,[29] carboxylic acid formation in antigen presenting cells was not blocked by inhibitors of alcohol dehydrogenase. With no information on the enzyme(s) that catalyze abacavir metabolism in antigen presenting cells or synthetic HLA-B*57:01 binding peptides modified irreversibly with the abacavir hapten, it is difficult to study whether drug metabolites additionally activate abacavir-responsive T-cells.

To conclude, we have shown that the activation of CD8+ T-cells with abacavir triggers different cytotoxicity pathways. Furthermore, our data reveals a complex relationship between drug disposition in antigen presenting cells and the T-cell response.

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Notes

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ABBREVIATIONS USED

EBV, Epstein−Barr virus; PBMCs, peripheral blood mononuclear cells; SI, stimulation index

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