SEVERE BULLOUS HYPERSENSITIVITY REACTIONS AFTER EXPOSURE TO CARBAMAZEPINE IN A HAN-CHINESE CHILD WITH A POSITIVE HLA-B*1502 AND NEGATIVE IN VITRO TOXICITY ASSAYS: EVIDENCE FOR DIFFERENT PATHOPHYSIOLOGICAL MECHANISMS

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ABSTRACT

Background
Drug hypersensitivity syndrome (DHS) can present in several clinical forms ranging from simple maculopapular skin rash to severe bullous reactions and multi-system dysfunction. Genetic analysis of DHS patients has revealed a striking association between carbamazepine (CBZ)-induced severe bullous reactions, such as Steven-Johnson Syndrome, and toxic epidermal necrolysis in individuals from Southeast Asia who carry a specific HLA allele (HLA-B*1502). This ethnic-specific relationship with a disease phenotype has raised the question of the commonality of the pathogenesis mechanisms of these diseases. The aim of this study was to investigate the genetic and metabolic bases of DHS development to help predict patient susceptibility.

Method
A case of carbamazepine-induced Steven-Johnson Syndrome reaction in a HLA-B*1502 positive child of Han Chinese origin, a carbamazepine-induced DHS case in a Caucasian patient and 3 healthy controls were investigated. We performed two types of in vitro toxicity assay, the lymphocyte toxicity assay (LTA) and the novel in vitro platelet toxicity assay (iPTA) on cells taken from the Chinese child 3 and 9 months after recovery from the reaction and from two healthy volunteers. We also tested the Caucasian patient, who developed CBZ-induced DHS, 3 months after the reaction.

Results
Both LTA and iPTA tests were negative 3 and 9 months after the reaction on samples from the Chinese child whereas the tests were positive in the Caucasian patient.

Conclusion
These results strongly suggest more than one mechanistic pathway for different CBZ-induced hypersensitivity reactions in patients with different ethnic backgrounds.

Key Words: Adverse drug reactions; drug hypersensitivity syndrome; in vitro diagnosis; drug-induced Stevens-Johnson Syndrome; in vitro toxicity assay

Drug hypersensitivity syndrome (DHS) or drug rash with eosinophilia and systemic symptoms (DRESS) is a life-threatening type of adverse drug reaction (ADR). It is unpredictable, unrelated to the drug’s direct pharmacological action and does not have a clear dose-effect relationship. These features
put DHS among type-B (bizarre) ADRs as opposed to Type-A (augmented) reactions, which are predictable from the pharmacological action of the drug, and are dose-dependent.\(^1\) DHS is defined as a constellation of symptoms that may include fever, skin rash and internal organ involvement following drug exposure.\(^2\) The true incidence of DHS is unknown; however, some authors have reported a rate as high as 13.5% of all ADRs.\(^3,4\) The lack of a clear clinical definition for this disorder and the absence of any safe, validated diagnostic test have limited the ability to confirm this type of ADR, and may have contributed to the significant morbidity and mortality related to delayed diagnosis.

The clinical manifestations of DHS can be quite diverse, ranging from mild self-resolved maculopapular eruptions to severe life-threatening cutaneous reactions involving multi-system dysfunction.\(^5\) The reactions may also take the form of a severe bullous skin eruption with systemic involvement (e.g., Stevens-Johnson syndrome, SJS and toxic epidermal necrolysis, TEN) with a mortality rate of up to 40%.\(^6\) The DHS spectrum has also been classified according to the type of skin reaction: bullous cutaneous ADRs (cADRs), which include SJS; and TEN and non-bullous cADRs, which include other types of reactions that comprise DHS. The diagnostic criteria of DHS have been a subject of lengthy debate, notably as to whether the severe forms of the disorder are variants of the same syndrome or completely different pathological entities.\(^7,8\) The nomenclature of DHS is far from consensus; however, for the purpose of this study we have used ‘non-bullous CBZ-DHS’ to indicate carbamazepine-induced hypersensitivity syndrome that is not SJS or TEN. The latter are denoted as CBZ-SJS/TEN.

The lymphocyte toxicity assay (LTA) is an \textit{in vitro} diagnostic test that has been used for decades to investigate patient susceptibility to DHS.\(^9\) The test is based on the hypothetical framework that susceptible patients have diminished ability to detoxify reactive electrophilic metabolites of the culprit drugs (or more recently reactive electrophilic by-products formed during the metabolism of the drug such as the lipid peroxidation product, 4-hydroxynonenal); and thus, form them at amounts that can cause DHS (the reactive metabolite hypothesis), presumably by the development of misdirected immune response. The latter hypothesis has been applied to clinical cases for the diagnosis of DHS.\(^10,11\)

Several lines of evidence exist supporting the genetic basis of patient susceptibility to DHS. Familial occurrence of DHS has been documented with cells isolated from relatives of patients that are also susceptible to \textit{in vitro} toxicity.\(^9,12\) Other evidence comes from the discovery of an association between the existence of certain HLA alleles and patients’ susceptibility to DHS induced by drugs such as the anti HIV reverse-transcriptase inhibitor, abacavir and the antiepileptic, carbamazepine (CBZ).\(^13,14\) These findings also strengthened the proposed immunological etiology of DHS as genetic loci within the major histocompatibility complex (MHC) region have been suggested to be involved. One of the strongest associations was found between the susceptibility to CBZ-induced SJS/TEN (CBZ-SJS/TEN) in Han Chinese patients and the occurrence of the HLA-B*1502 allele.\(^13,15\) This genetic variation has 100% sensitivity and 97% specificity for prediction of severe bullous reactions due to CBZ exposure in Han-Chinese populations. This discovery has prompted the US-FDA to issue a recommendation to test any patient with Asian ancestry for the HLA-B*1502 allele before initiating CBZ therapy.\(^16\) It appears, however, that having the HLA-B*1502 allele is predictive of only severe bullous reactions in the Southeast Asian population to CBZ.\(^17\) Of note, other studies have not found any association between this specific genetic marker and the disease in other ethnic groups.\(^18-20\)

To the best of our knowledge, the present study is the first to attempt to unravel the complexity of DHS pathogenesis using both genetic and biochemical evidence.

**MATERIALS AND METHODS**

**Case Report**

An 11 year-old boy with epilepsy of Han-Chinese origin presented to our Emergency Department with SJS after receiving CBZ treatment for 2 weeks. He had previously been treated with phenobarbital (PHB) for more than a year. The ADR started with fever and rash that rapidly progressed to significant cutaneous and corneal involvement. Dermatological
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examination confirmed mucosal ulceration followed by maculopapular rash on the trunk, face and arms progressing to confluent macules covering more than 50% of the body with extensive bullae and erythematous vesicles on peripheries, and epidermolysis of 10% of body surface area, requiring skin debridement by a plastic surgeon. Ophthalmological examination revealed bilateral epithelial defects and blurred vision. Blood haemoglobin content was 126 g/L, but dropped to 61 g/L on day 5 requiring transfusion (2 units of blood). C-reactive protein (CRP) content was 84.9 mg/L. There was also mild elevation of liver enzymes in the plasma, (ALT, 325 U/L; Amylase, 389 U/L; AST, 375 U/L; LDH, 1941 U/L) and transient hyperglycemia. Serology showed negative IgM for CMV and Mycoplasma pneumonia. The boy was treated with IVIG 1g/kg/day for 3 days, corticosteroid eye drops, and surgical debridement of bullae. He recovered well and was discharged from hospital. The Research Ethics Boards of the University of Western Ontario and the Hospital for Sick Children approved this study.

HLA Typing
HLA-A, B, C and DR low resolution typing was performed using polymerase chain reaction-sequence-specific oligonucleotide (PCR-SSO) method and HLA-B*15 and Cw*08 high resolution typing was performed using polymerase chain reaction-sequence specific primer (PCR-SSP) method (Toronto Regional Histocompatibility Laboratory, Toronto, ON, Canada).

In vitro Toxicity Testing
The lymphocyte toxicity assay (LTA) was performed as described previously. Briefly, 100 µl of a peripheral blood monocyte suspension (PBMCs, lymphocytes), at a density of 1x10^6 cell/ml, were placed in each well of 96-flat-bottom multiwell plates. Cells were incubated with different concentrations of CBZ for 2 hrs in a humidified atmosphere at 37°C and 5% CO₂ partial pressure. Microsomal protein (0.25 mg/ml) was added followed by an NADPH generating system (NADP, 0.6 mM; glucose-6-phosphate, 2.4 mM; glucose-6-phosphate dehydrogenase, 2 U/ml). Plates were then spun at 500 g for 15 min and media was replaced with fresh RPMI 1640 media containing 10% FBS and penicillin/streptomycin cocktail. Cells were allowed to recover for 18 hrs in a humidified atmosphere at 37°C and 5% CO₂ partial pressure. At this point, plates were spun down and contents resuspended in 100 µl HEPES buffer. An aliquot (25µl) of a 5 mg/ml aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated at 37°C for 4 hrs. Reactions were stopped by adding 100 µl stop solution (50% N, N-dimethylformamide, DMF; sodium dodecyl sulphate, SDS, 20%) and allowed to set overnight at room temperature, protected from light. The absorbance was measured at 590 nm by a Molecular Device Spectrophotometer (Beckman, Palo Alto, CA, USA). A standard curve was generated by seeding the cells at 0, 25, 50, 75 and 100% of cell concentration in the corresponding buffer in quadruplicate. The data were analyzed using the Softmax™ Molecular Device Group Analytical software version 2.35 and statistical graphs were created using Microsoft Excel™ 2007 software.

The in vitro platelet toxicity assay (iPTA) was performed in a similar procedure as above except, calcium-free Locke’s solution was used as the medium in the experiments. Platelets were incubated at a density of 7.5x10^8 cells/ml and plates were centrifuged at 900 x g, in each step to pellet platelets.

Statistical Analysis of Data
Data were analyzed using Microsoft Excel™ 2007 software and differences between cells from the patient and those from controls were determined by Student’s t-test with Bonferroni correction for multiple comparisons. Values are expressed as percentage of control (vehicle only) and presented as mean ± standard error (SE).

RESULTS
We performed the in vitro toxicity assays using cells from healthy volunteers and the patient 3 months and 9 months after his recovery from the reaction. The patient’s cells (PBMCs, LTA and platelets, iPTA) did not show any significant increase in cell death upon incubation with up to 250μM CBZ in the presence of fortified rat liver
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Microsomes (RLM) compared to healthy controls (Table 1). We performed an identical in vitro toxicity assay to cells of a Caucasian patient who had typical non-bullous DHS to CBZ (non-bullous CBZ-DHS). Cells from the non-bullous CBZ-DHS patient showed a significant increase in cell death (~20%) upon exposure to CBZ and its metabolites compared to cells from the healthy volunteer (p<0.05, n=12) (Table 1). Genotyping for HLA-A, B, C and DR and for HLA-B*15 and Cw*08 revealed that the CBZ-SJS Chinese patient carries the HLA-B*1502 allele.

### TABLE 1 Results of in vitro toxicity testing of CBZ-SJS patient, CBZ-DHS patient and healthy controls

<table>
<thead>
<tr>
<th>Time of the in vitro toxicity assay</th>
<th>Subject</th>
<th>LTA</th>
<th>iPTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>CBZ-SJS Patient</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>9 months</td>
<td>Healthy control</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>3 months</td>
<td>CBZ-SJS patient</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td></td>
<td>Healthy control</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td></td>
<td>CBZ-DHS patient</td>
<td>POS</td>
<td>POS</td>
</tr>
<tr>
<td></td>
<td>Healthy control</td>
<td>NEG</td>
<td>NEG</td>
</tr>
</tbody>
</table>

a: Time elapsed between the reaction and the test. b: To determine test result we used 20% increase in cell death as a cut-off value, see Method section for details. LTA- lymphocyte toxicity assay; iPTA- in vitro platelet toxicity assay; CBZ- carbamazepine; SJS- Stevens Johnson syndrome; DHS- drug hypersensitivity syndrome.

**DISCUSSION**

Aromatic anticonvulsants (AACs) are one of the drug classes most commonly associated with DHS.\(^21\) They include clinically important drugs such as Phenytoin (DPH), Carbamazepine (CBZ), Phenobarbital (PHB) and Lamotrigine (LMG). CBZ is the drug of choice as first line therapy for certain types of epileptic seizure, including partial seizures and tonic-clonic seizures. CBZ is also used to treat chronic pain and certain psychiatric disorders. Carbamazepine-induced DHS is characterized by variable clinical presentations and different degrees of severity. The condition can present as maculopapular skin rash that may be accompanied with fever and other systemic symptoms in virtually any organ. The disease can also develop as a severe bullous reaction that involves skin, mucosal membranes and ocular tissue (SJS and TEN).

Carbamazepine and its oxidative metabolites can activate T-cells from DHS patients in vitro\(^22,23\) and both CBZ and its major metabolite, CBZ-epoxide, yield a positive patch test in CBZ-hypersensitive patients.\(^24,25\) Carbamazepine-specific CD4\(^+\), CD8\(^+\) and CD4\(^+\)/CD8\(^+\) T-cell clones have been generated in vitro from blood samples of CBZ-hypersensitive patients.\(^26\) In light of the currently available data, it may be naive to define CBZ-induced hypersensitivity reactions as a constellation of symptoms ranging from simple maculopapular skin rash with fever to severe bullous skin manifestation with multisystem dysfunction; as this definition does not address the
distinct histopathological and immunological features of different forms of CBZ-induced hypersensitivity reactions. Additionally, genetics may play a major role in determining the susceptibility of patients to CBZ-induced hypersensitivity reactions, because of their familial and ethnic occurrence, as certain types of AACs-induced hypersensitivity reactions were found to run in families.12,20,27

The search for genetic markers for ADRs has been underway for a long time and was accelerated by the recent improvement in quality and decreased cost of gene analysis methods. Because it was known that metabolite bioactivation plays an essential role in the development of some hypersensitivity reactions, the first candidates were genes that control the main metabolic enzymes (e.g., cytochrome P450 monooxygenases (CYP) and epoxide hydrolase (EH)). However, studies have failed to identify any association between DHS and polymorphism in genes encoding these drug metabolizing enzymes.9,15,28 Subsequently, attention was focused towards genes involved in immune response, such as the HLA gene, which encodes the major histocompatibility complex (MHC), a major player in the antigen presentation process.29 A number of HLA alleles were found to be associated with hypersensitivity reactions to different drugs; however, the strongest association was found between the HLA-B*1502 allele and the development of CBZ-SJS/TEN in Southeast Asian populations, with an odds ratio of 2504.13 This allele was also found in high frequency in these populations, which may explain the higher prevalence of SJS/TEN cases due to CBZ use among Southeast Asians.17 This finding suggests a functional role of the HLA-B*1502 allele in CBZ-SJS/TEN pathogenesis, a hypothesis that is not yet proven.27

The cases presented here provide further biochemical and genetic insights into the distinct pathogenesis of non-bullous CBZ-DHS and CBZ-SJS/TEN. A clinically confirmed typical case of non-bullous CBZ-DHS Caucasian patient. Our biochemical approach using two in vitro testing systems revealed unexpected results (Table 1). We believe that our data are consistent with distinct pathophysiological pathways within subsets of CBZ-DHS. Specifically, in this case the immunological pathway is active whereas the toxic metabolite pathway is not.

Several working hypotheses have been proposed to explain the pathophysiological mechanisms underlying DHS. The first to be introduced was the hapten hypothesis (HH), which assumed that a small drug molecule can be recognized by the immune system only after forming an adduct with endogenous peptide.32 The reactive metabolite hypothesis attributes DHS to imbalance in bioactivation and detoxication of the drug resulting in larger quantities of toxic metabolites in the body.10 The danger hypothesis considers signals released by stressed and dying cells (e.g., cytokines, HSP, NO, ROS) as a requirement to fully activate an immune response.33 Finally, the pharmacological interaction with immune receptor hypothesis (p-i Hypothesis) has proposed another scenario to activate the immune system by a small molecule. It postulates that drug molecules (parent drug or metabolites) can directly bind non-covalently to T-cell receptors (TCRs) and activate T-cells independent of gene processing and presentation.34

Undoubtedly, activation of the drug to an active metabolite is a prerequisite to initiate a cascade of events leading to development of DHS. As depicted in Figure 1, activation of the parent drug to an electrophilic reactive metabolite is likely to represent the first step in a cascade of events leading to the ADR. Several lines of evidence strongly suggest that metabolic activation is the first step in DHS.35 Cells (peripheral blood monocytes, PBMCs and platelets) from drug hypersensitive patients are more susceptible to the in vitro toxicity of the toxic drug metabolites than are cells from healthy control individuals.11,36,37

Enhanced in vivo concentrations of the toxic metabolites fit well with the suggested DHS mechanism. First, high level of cytotoxic reactive metabolites in either the metabolizing cells (e.g., hepatocytes or skin cells) or other tissues can cause cell necrosis and death providing 'danger signals' to prime antigen presenting cells (APCs) and T-cells to be activated. These signals can be in the form of cytokines, HSP, NO or ROS.
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released from necrotic or apoptotic cells. Second, these ‘dying’ cells can also release haptenated proteins and peptides which can be processed by the APCs and presented as antigens. Finally, the metabolites can interact directly with the T-cells receptors (TCRs) and form a bridge with the MHC (class I and II) on APCs causing T-cell activation and expansion of T-cell clones (the p-i hypothesis). It must be pointed out that these various hypotheses are not mutually exclusive. Thus, the formation of ROS can result in the production of 2-hydroxynonenal, an endogenous electrophile that could mimic a drug in terms of covalent reaction with proteins, and enhance the immunological response and the severity of an ADR.

FIG. 1

Figure 1: The reactive metabolite, hapten, danger and p-i hypotheses and their suggested involvement in DHS. When a lipophilic drug molecule enters a biological system it will, in most cases, be readily metabolized to either chemically reactive or non-reactive metabolites. The reactive metabolites are in turn converted to less toxic or non-toxic products. An imbalance in these processes can result in the enhanced concentration of reactive toxic metabolites in vivo that can cause either necrotic or apoptotic cell death, releasing the ‘danger signals’ and haptenated self-peptides that can be processed by specialized antigen presenting cells (APCs) and presented to specific T-cell clones. These T-cell clones expand upon activation and mediate the immune response (see text for more details). Certain alleles that were found to associate with a high risk of CBZ-DHS are presented in boxes along the pathway. CBZ: carbamazepine, APCs: antigen presenting cells, MHC, major histocompatibility complex.
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According to the classical theory of antigen presentation, activated APCs carrying the antigen will migrate to a local lymph node and present the antigen in the context of the MHC (classes I and II) to naive or memory T-cells which will then expand and initiate the immune response. Antigens formed in the cytosol of APCs are presented on MHC I to CD8+ cytotoxic T-cells; whereas, extracellular antigens are presented on MHC II to CD4+ helper T-cells. Each of these types of immune response has its own characteristic pathway and cytokine profile and can result in distinct clinical manifestations. Consequently, the subsequent events along the pathway are determined by the expansion of specific T-cell clones that will propagate the immune response and determine the clinical signature of the reaction.

CD8+ T-cells are known to produce cytokines such as IFN-γ and TNF-α, which increase the surface expression of MHC, and their cytotoxic effects are thought to be mediated by Fas death receptor through increasing the expression of its ligand FasL or by a perforin/granzyme B dependent pathway. These mediators are highly expressed in skin from SJS/TEN patients. On the other hand, CD4+ T-cells produce IL-4 and IL-5 leading to eosinophil recruitment and features characteristic of non-bullous DHS. However, it is noteworthy that most of the details of DHS signalling pathways are unknown at the present time, in part because of the lack of validated animal models for these types of reactions. It is unlikely that CBZ works only as a classic hapten-forming drug (e.g., penicillin’s) because penicillin’s can induce all types of hypersensitivity reactions in the ‘Coombs and Gell’ classification (types I-IV) whereas CBZ induces only type IV reactions. Furthermore, no endogenous immunologically-relevant protein target has been identified to be haptenated by CBZ metabolites.

The recent finding of genetic predisposition to CBZ-SJS/TEN (but not non-bullous CBZ-DHS) in individuals from Southeast Asian suggests a difference in the pathophysiology of these two variations of ADRs caused by CBZ. This concept is supported by our current study in which a Han-Chinese descendent with CBZ-SJS and positive HLA-B*1502 allele was negative to both LTA and iPTA in vitro toxicity assays. We earlier reported that LTA has a sensitivity of 85-100% in well documented CBZ-DHS cases. Such a finding strongly suggests that the severe bullous reactions caused by CBZ (CBZ-SJS/TEN) may have distinct pathophysiology from non-bullous CBZ-DHS, which is not detected by these in vitro toxicity assays.

It is apparent that hypersensitivity reactions (bullose and non-bullous) have different pathophysiological mechanisms. Individuals prone to form enhanced concentrations of toxic metabolites of drugs that cause ADRs may also carry genes that predispose to immunotoxicity from metabolite-protein or by-product-protein adducts. While these variables might co-exist in individuals with a high frequency of HLA-B*1502 allele, such as is the case for Southeast Asians, development of CBZ-SJS/TEN in patients who carry the HLA-B*1502 allele does not seem to depend on reaching a threshold of reactive metabolites.

Another feature that differentiates non-bullous reactions from SJS/TEN is the involvement of viral reactivation, particularly human herpes virus 6 (HHV-6) which has been observed in most non-bullous DHS cases but is not observed in SJS/TEN cases. This has led some investigators to establish HHV-6 reactivation as one of the diagnostic criteria of DHS.

Overall, these observations suggest that the process of activating APCs and antigen presentation may not be a major contributor in CBZ-SJS/TEN development but, rather, that interaction among the parent drug (or metabolite(s)), TCR and MHC (Probably class I, HLA-B*1502) are the main components that initiate the immune response that manifests in CBZ-SJS/TEN. In line with the p-i hypothesis this scenario is quite possible and may explain why certain HLA alleles are associated with specific immune responses. Such an effect would be independent of drug bioactivation and hapten formation and processing, which could explain why the in vitro toxicity assays were negative in the Han-Chinese child case we presented here.
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