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Predicting Drug Hypersensitivity Reactions: Mechanistic and Clinical Implications

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Predicting Drug Hypersensitivity Reactions: Mechanistic and Clinical Implications

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Abstract

Adverse drug reactions (ADRs) are responsible for a high number of morbidities and mortalities worldwide and estimated to be the fourth most important cause of death in the US and Canada after heart diseases, cancer and stroke. ADRs are either type A (~80%) which are predictable, related to the drug pharmacology and dose-dependent or type B (~20%), which are unpredictable, unrelated to the drug pharmacology and have no clear dose-dependency. Drug hypersensitivity reactions (DHRs) represent the majority of type-B ADRs, which are rare but potentially fatal and unpredictable. The latter aspect makes DHRs very difficult to diagnose and necessitate the development of a reliable and safe *in vitro* diagnostic test to aid prediction and confirm diagnosis. The currently used tests are not well characterized and their predictive value is unknown. The aim of this work was to evaluate the clinical value of the currently used diagnostic tests for DHRs; to develop a simple, reliable and safe test; and to explore the pathophysiology of DHRs using different approaches for further understanding of the DHRs pathophysiology which will allow us to develop new means for prevention prediction and diagnosis.

Methodology used involved performing systematic literature reviews, population survey on previously tested patients, patient recruitment and laboratory techniques that include preparation and testing of liver microsomes from human and animal origin, using hematopoietic cell lines and primary cultures of different blood cell types as a surrogate model to explore DHRs pathphysiology and test patient susceptibility for DHRs.

Systematic review of available literature revealed that the currently used diagnostic tools for DHRs lack any characterization or standardization and much more

work is needed to further characterize and improve these tools. We developed a novel laboratory approach for diagnosis of DHRs that proved to be less cumbersome and potentially more reliable than other currently used tests. Using different biochemical and genetic methods, we introduced novel concepts that explain some aspects of the pathophysiology of DHRs.

The main achievement in this research was the development of a novel diagnostic test for DHRs, the *in vitro* platelet toxicity assay (*i*PTA), which has a great potential as a clinical tool due to its simple procedure and good reproducibility. We hope that these features will allow its wider clinical use as oppose to other currently used tests. In addition, expanding our understanding of the molecular pathophysiology of DHRs using recent technical advances in genetic analysis and laboratory techniques will have a great impact on the management of these cases.

Keywords

Adverse drug reaction, drug hypersensitivity, in vitro diagnosis, in vitro platelet toxicity assay, the lymphocyte toxicity assay.

Co-Authorship Statement

Chapter 2: Patch testing for the diagnosis of anticonvulsant hypersensitivity syndrome: a systematic review.
Ms Knowles, Dr Shear and Dr Bend critically revised the manuscript. Drs Koren and Rieder provided guidance during the systematic review design and preparation of the manuscript.

Chapter 3: In vitro testing for the diagnosis of anticonvulsant hypersensitivity syndrome: A Systematic Review.
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Chapter 4: Predictive value of the lymphocyte toxicity assay in the diagnosis of drug hypersensitivity syndrome.
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Chapter 5. The In vitro Platelet Toxicity Assay (iPTA): a Novel Approach for Assessment of Drug Hypersensitivity Syndrome.
Drs Koren and Rieder provided guidance during the experimental work design, analysis of data and preparation of the manuscript.

Chapter 6. Severe bullous hypersensitivity reactions after exposure to carbamazepine in a Han Chinese child with a positive HLA-B*1502 and a negative lymphocyte toxicity assay: Evidence for different pathophysiological mechanisms.
Drs Garcia-Bournissen and Finkelstein identified patients and provided blood samples from patients and controls. Dr Bend critically revised the manuscript. Drs Koren and Rieder provided guidance during the study design, analysis of data and preparation of the manuscript.

Chapter 7. Challenges and Future Directions - In Vitro Testing for Hypersensitivity-Mediated Adverse Drug Reactions.
Drs Koren, Bend and Rieder provided guidance during writing of the review and critically revised the manuscript.

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"*The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher* ***inspires****"*.

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List of Abbreviations

5-HT	5-Hydroxytryptophan
ACDs	Anticonvulsant drugs
ADR	Adverse drug reaction
AGEP	Acute generalized exanthematous pustulosis
AHS	Anticonvulsant hypersensitivity syndrome
AST	Aspatate transaminase
BE	Boullus exanthema
cADR	Cutaneious adverse drug reaction
CBZ	Carbamazepine
CFSE	Carboxy fluorescein succinimidyl ester
CMV	cytomegalovirus
CRP	C-reactive protein
CYP	Cytochrome P450
DHS	Drug hypersensitivity syndrome
DIDMOHS	Drug-induced delayed multiorgan hypersensitivity syndrome
DIHS	Drug-induced hypersensitivity syndrome
DPH	Phenytoin
DRESS	Drug rash with eosinophilia and systemic symptoms
EH	Epoxide hyrdolase
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema multifomie
FDA	Food and drug agency
GST	Glutathion S-transferase
HEPES	2-[4-(2-hydroxy ethyl) piperazin-1-yl] ethanesulfonic acid
HLA	Human leukocyte antigen
iPTA	in vitro Platelet toxicity assay
IRs	Ideosyncratic reactions
LDH	Lactate dehydrogenase
LMG	Lamotrigine
LPT	lymphocyte proliferation test
LST	lymphocyte stimulation test
LTA	lymphocyte toxicity assay
LTT	lymphocyte transformation test
ME	maculopapular exanthema
MeSh	Medical subheading
MPO	Myeloperoxidase
MTT	4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolim
NADPH	Nicotinamide adenosine dinucleotide phosphate
NPV	Negative predictive value
PBL	Peripheral blood leukocyte
PBMCs	Peripheral blood monocytes
PBS	Phosphate buffered saline
PCR-SSO	Polymerase chain reaction-sequence specific oligonucleotide

PCR-SSR	Polymerase chain reaction-sequence specific primer
PHA	Phytohemoagglutinin
PHB	Phenobarbitone
P-I	Pharmacological interaction with the immune system
PPV	Positive predictive value
PT	Patch test
RLM	Rat liver microsomes
SJS	Stevens-Johnson syndrome
SMX	Sulfamethoxazole
SMX-HA	Sulfamethoxazole-hydroxide amine
TEN	Toxic epidermal necrolysis
TT	Tetanus toxoid
WHO	World Heath Organization

Preface

This '*integrated articles*' thesis is based on 6 papers I have published over the last 3 years, all focusing on examination of the predictive value of *in vitro* tests for drug hypersensitivity reactions (DHRs). These idiosyncratic reactions are often very severe and may result in serious morbidity and mortality. Hence, it is critical to create and validate laboratory methods that can identify vulnerable patient before serious reactions occur.

After presentation of my hypotheses and objectives, I present two systematic critical reviews of the currently available *in vivo* and *in vitro* methods used for diagnosis and prediction of DHRs. This is followed by description and validation of a new laboratory method, the *in vitro* platelet toxicity assay (iPTA), developed by me during the tenure of my PhD.

The thesis is concluded by an overall discussion of the state of the art of all aspects of *in vitro* testing for DHRs.

Thank you for your participation and interest in my work.

Chapter 1: Objectives and hypotheses.

1.1. Objectives:

1) To systematically review the literature on patch testing for diagnosis of hypersensitivity reactions (DHRs).

2) To systematically review the literature on *in vitro* testing for diagnosis of DHRs.

3) To characterize the predictive value of the lymphocyte toxicity assay (LTA) for the diagnosis of DHRs.

4) To develop and validate an *in vitro* platelet toxicity assay (*i*PTA) for drug hypersensitivity syndrome.

5) To distinguish between different mechanisms of carbamazepine (CBZ)-induced hypersensitivity syndrome using novel tests and biological markers.

1.2. Hypotheses:

1) The available patch tests are not sufficiently standardized and their sensitivity and specificity are not adequately determined.

2) The available *in vitro* tests for hypersensitivity syndrome are not standardized and reproducible to be used clinically.

3) The predictive value of the LTA for aromatic anticonvulsants and sulfonamides-induced DHRs allows it is clinical use.

4) The *i*PTA is more sensitive and predictive than the older LTA.

5) *In vitro* testing using iPTA and LTA and the use of genetic markers such as the HLA allow identifying two separate mechanisms of CBZ-induced DHRs.

Chapter 2: Patch testing for the diagnosis of anticonvulsant hypersensitivity syndrome: a systematic review.

This chapter has been published previously:

Elzagallaai AA, Knowles SR, Rieder MJ, Bend JR, Shear NH, Koren G. Patch testing for the diagnosis of anticonvulsant hypersensitivity syndrome: a systematic review. Drug Saf. 2009;32(5):391-408.

2.1. Introduction

Adverse drug reactions (ADRs) have been defined as undesirable effects associated with the therapeutic use of drugs.[1] An ADR is defined by the WHO as noxious and unintended response to a drug that occurs at a dose normally used in man for prophylaxis, diagnosis or therapy.[2] ADRs represent a major health problem world-wide with high rates of morbidity and mortality.[3-6] Lazarou and colleagues[4] have estimated in a meta-analysis that ADRs were responsible for nearly 100 000 deaths in the US in 1994. Despite the fact that this study has been criticized,[7] it does lend credence to the seriousness of this problem. Indeed, the authors of this study have estimated that ADRs are ranked between the fourth and sixth leading cause of death, after heart disease, cancer, stroke, pulmonary disease and accident, in the US and Canada. It has also been demonstrated that drug-related injuries occur in at least 7% of hospitalized patients,[4] although accurate estimation of such cases is difficult due to under-reporting.[8] In addition, ADRs also represent a serious economic burden on the health care system.[9]

ADRs have been classified into the following two types: type-A reactions which are usually predictable, dose-dependent and related to the pharmacological action of the drug; and type-B reactions, which are unpredictable, have a delayed onset and cannot be explained by the pharmacological action of the drug.[10] Type-B reactions are typically dose-independent; however, dose-dependence of these type of drug reactions can exist at higher dose ranges than conventional pharmacological dose-response relationships.[11] Type-B ADRs or idiosyncratic reactions (IRs) comprise various types of reactions such as immune-mediated (allergic, immunological reactions), which include drug hypersensitivity reactions or drug hypersensitivity syndrome (DHS), and non-immune-

mediated (sometimes called metabolic idiosyncrasy).[12] Gell and Coombs[13] classified immune-mediated reactions into four types: type I reactions (immunoglobulin E-mediated); type II reactions (through cytotoxic mechanisms); type III reactions (immune complex-mediated); and type IV reactions, which involve activation of T cells and are known as "delayed hypersensitivity". Type IV reactions have recently been subdivided according to the heterogeneity of T-cell function into Types IVa, IVb, IVc and IVd.[14, 15] Although an elegant and mechanism-based classification system, many serious and probable immune-mediated ADRs do not fit into these established categories.[16] DHS is thought to belong to type IV, T-cell mediated delayed reactions.[17]

Drug hypersensitivity syndrome is a rare but potentially lethal host-dependent ADR that occurs in susceptible patients upon exposure to specific agents. It has been estimated that IRs, of which DHS represents a major component (around 10%), constitute from 3% to 25% of all ADRs.[18] Because of its unpredictable nature and potential severe morbidity and mortality, DHS is a major problem for patients, clinicians, drug regulators and the pharmaceutical industry and often deprives patients of effective therapy.

The nomenclature of this type of drug hypersensitivity reaction has long been a topic of debate.[19, 20] Dilantin hypersensitivity syndrome, sulfone syndrome, dapsone hypersensitivity syndrome, allopurinol hypersensitivity syndrome, drug-induced delayed multiorgan hypersensitivity syndrome (DIDMOHS), anticonvulsant hypersensitivity syndrome (AHS), drug rash (reaction) with eosinophilia and systemic symptoms (DRESS) and drug-induced hypersensitivity syndrome (DIHS) have all been suggested as names and acronyms for this disorder.[20, 21] Although no consensus has emerged thus far, the last three are the most widely used terms. However, for the purpose of this review, it

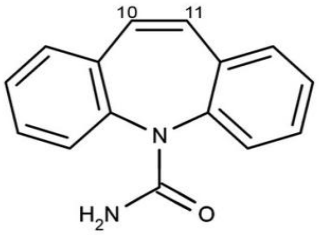
was felt that AHS is the most relevant term because only reactions related to aromatic anticonvulsant drugs (ACDs) were reviewed.

The objective of this systematic review was to critically review all the relevant publications related to the use of the patch test in the diagnosis of AHS. We also aimed at discussing the technical aspects of this *in vivo* test that contribute to its performance.

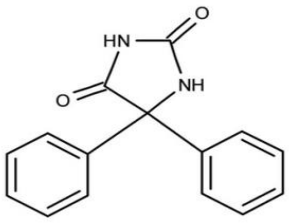
2.1.1. Anticonvulsant Hypersensitivity Syndrome (AHS)

Aromatic ACDs such as phenytoin, carbamazepine and phenobarbitol as well as some newer agents, including lamotrigine, oxcarbazepine, felbamate and zonisamide (figure 1), have been implicated in eliciting a whole repertoire of hypersensitivity reactions ranging from simple maculopapular skin eruptions to a severe life-threatening disorder. Upon exposure to an implicated drug, a constellation of symptoms develop including fever, skin eruption and internal organ dysfunction.[22-33] Implicated drugs include aromatic anticonvulsants (carbamazepine, phenytoin, phenobarbital, lamotrigine), sulfonamide antibacterials, dapsone, minocycline, terbinafine, azathioprine and allopurinol.[34] Although AHS is typically defined by the triad of symptoms (i.e. fever, skin rash and internal organ involvement), it is quite difficult to associate a typical clinical picture to this syndrome as AHS can manifest as a wide range of clinical symptoms. Affected patients may develop fever, a skin eruption (from a mild skin rash to severe eruptions such as Stevens-Johnson syndrome and toxic epidermal necrolysis), and internal organ involvement (either asymptomatic or symptomatic).[1, 28, 35] The multivisceral involvement of this illness may include blood dyscrasias (e.g. eosinophilia, thrombocytopenia), hepatitis, nephritis, myocarditis, thyroiditis, interstitial pneumonitis

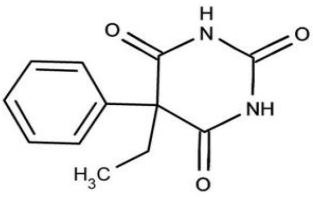
Fig. 1. Chemical structure of aromatic anticonvulsant drugs.



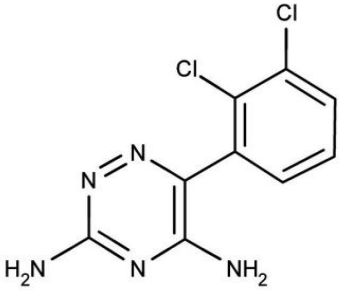
Carbamazepine



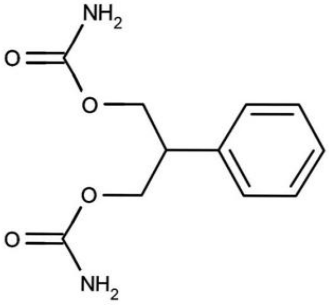
Phenytoin



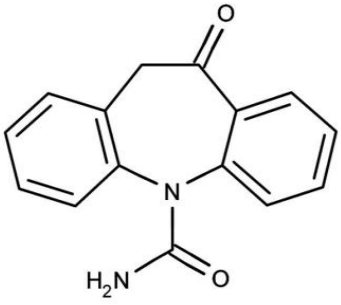
Phenobarbital



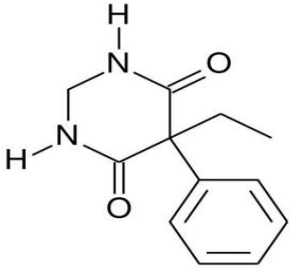
Lamotrigine



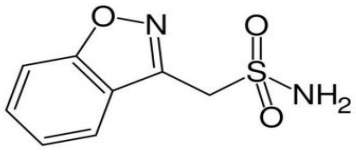
Felbamate



Oxcarbamazepine



Primidone



Zonisamide

and encephalitis. Other clinical features of AHS are facial oedema, tonsillitis, pharyngitis, mouth and lip ulcers, enlargement of liver and spleen, myopathy and disseminated intravascular coagulation.[35-39] It has been estimated that the incidence of AHS lies between 1 in 1000 to 1 in 10 000 among patients chronically treated with phenytoin and carbamazepine.[40] However, these incidences are believed to be inaccurate as a result of under-reporting.[41]

The exact molecular mechanisms involved in AHS are not well understood. In fact, it is thought that multiple mechanisms are involved, sometimes simultaneously, to produce a single event.[39, 42] Discussing detailed molecular mechanisms underlying AHS is beyond the scope of this review; nonetheless, some recent comprehensive reviews on this subject are available.[39, 43, 44] In general, AHS is believed to be immune-mediated in all cases,[17, 45] and the generation of reactive electrophilic drug metabolites that react selectively and non-enzymatically at nucleophilic sites on multiple proteins to form immunogenic drug metabolite-protein adducts is proposed to be the initial mechanistic step in the cascade of cell-based reactions that results in the clinical symptoms.[33, 46-48] At least a few of the proteins that are covalently modified by metabolites of drugs causing AHS are likely to be involved in eliciting the immune response that characterizes these hypersensitivity reactions.[39, 46, 49]

2.1.2. Diagnosis of AHS

A validated, gold standard *in vitro* test for diagnosis or prediction of AHS is not yet available. In fact, the value of all currently used *in vivo* and *in vitro* tests is widely controversial and their sensitivities, specificities and variability are yet to be

determined.[50-54] Currently, the diagnosis of AHS is based on clinical expertise that is comprised of: (i) a thorough clinical history, including detailed medication history; (ii) a comprehensive physical examination; and (iii) available laboratory data. Misdiagnosis of AHS is very common because the syndrome resembles other conditions such as infections, collagen vascular disorders and haematological/oncological conditions.[24, 39] An *in vivo* systemic rechallenge (drug provocation testing or controlled re-exposure) is considered to be the gold standard in AHS diagnosis,[55] although ethically this is highly contentious, as a rechallenge with the implicated drug may result in severe morbidity or even death. Presently, there are at least three tests available for diagnosis of AHS, namely the patch test, the lymphocyte transformation test (LTT) and the lymphocyte toxicity assay (LTA).[50, 55-58] The use of the patch test for the diagnosis of AHS is reviewed here.

2.2. Research Methodology

We performed the systematic literature search using the databases PubMed, EMBASE and MEDLINE from their commencement to the 4th week of August 2008 (figure 2).

2.2.1. Search strategies

The first search (Search strategy I) was carried out using key words "anticonvulsant" and "antiepileptic" in their singular, plural and truncated forms. These terms were also mapped to their medical subject headings (MeSH) terms. We also searched for individual aromatic ACDs including 'carbamazepine', 'phenytoin', 'phenobarbital', 'oxcarbazepine', 'primidone', 'lamotrigine', 'felbamate' and 'zonisamide' both as key words and as MeSH terms when available and the option 'explode' was used. The obtained results were combined using 'OR'.

In addition, a second search (search strategy II) was carried out using the key words ‘skin test’, ‘patch test’ and ‘epicutaneous test’ in their singular, plural and truncated forms. These terms were also mapped to their MeSH terms when available and the option ‘explode’ was used.

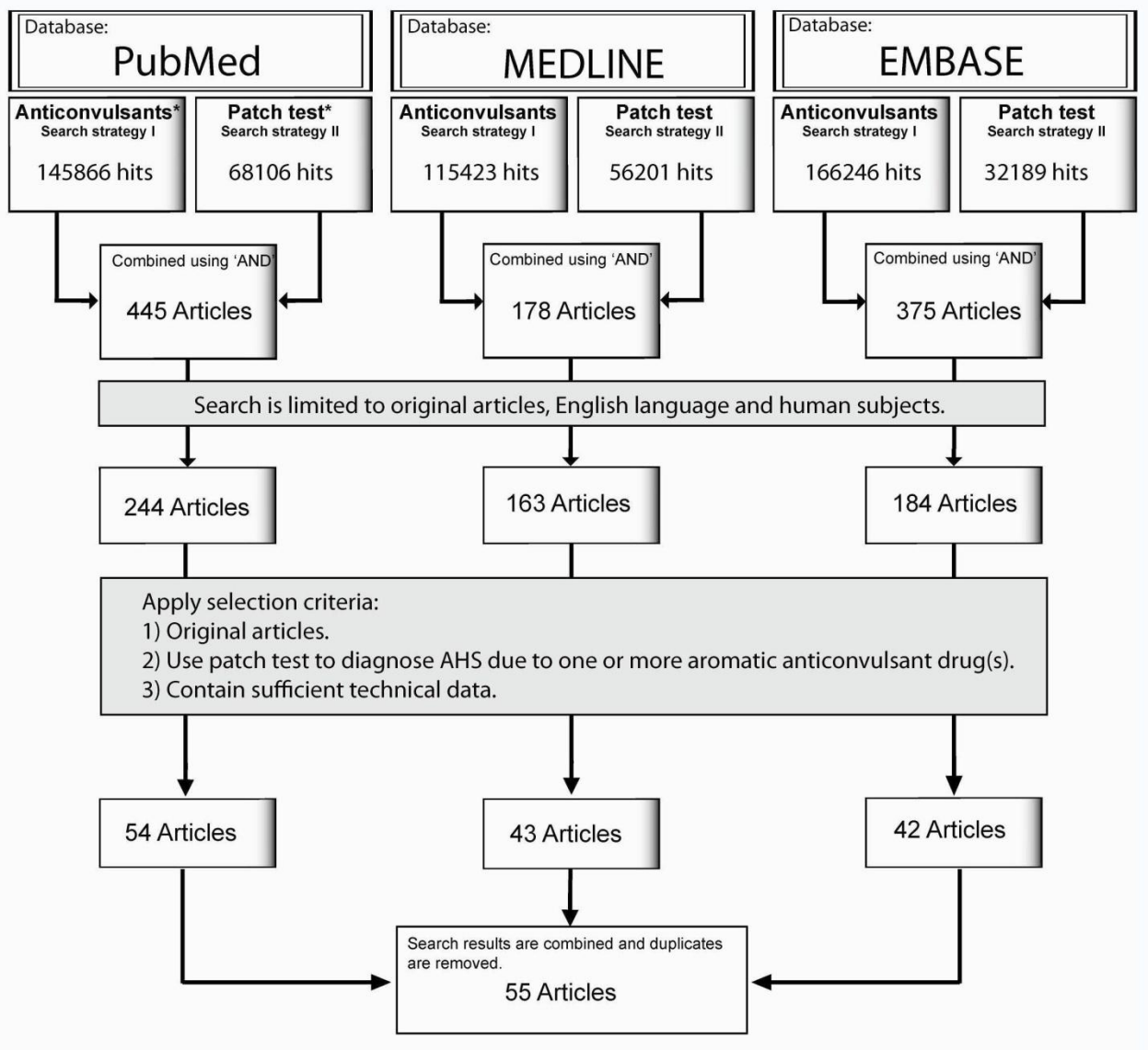
The results of the first and second searches were then combined using ‘AND’. The search results were then limited to original articles that were published in English language and performed on human subjects. At this point, we retrieved 244 articles from PubMed, 163 articles from MEDLINE and 184 articles from EMBASE. These publications were then manually reviewed and the following selection criteria were applied: (i) original articles; (ii) used patch testing for the purpose of diagnosis of suspected AHS as a result of one or more aromatic ACD(s); and (iii) contained sufficient technical data.

Applying our selection criteria, 54 articles from PubMed, 43 articles from MEDLINE and 42 from EMBASE were found to meet our selection criteria. The search results from the three databases were then combined and duplicates were removed. The final number of included articles from the three databases was 55.

2.3. Patch Tests in the Diagnosis of AHS

Patch testing utilizes the concept that a localized, confined, immune-mediated reaction to the agent of concern can be reproduced by introducing the agent through the skin. Briefly, the patch test is performed by applying the drug to the tested (ground commercially available tablets, liquid forms or pure drug powder) on the skin (usually the upper back) using different devices that give standard surface area exporure. One widely

Fig. 2. Flow chart of literature search and retrieval process. 1, Search strategies ‘anticonvulsants’ and ‘patch test’ include all relevant medical subject headings and key words as described in the Research Methodology section; AHS = anticonvulsant hypersensitivity syndrome.


Database:
PubMed
Database:
MEDLINE
Database:
EMBASE
Anticonvulsants*
Search strategy I
145866 hits
Patch test*
Search strategy II
68106 hits
Anticonvulsants
Search strategy I
115423 hits
Patch test
Search strategy II
56201 hits
Anticonvulsants
Search strategy I
166246 hits
Patch test
Search strategy II
32189 hits
Combined using 'AND'
445 Articles
Combined using 'AND'
178 Articles
Combined using 'AND'
375 Articles
Search is limited to original articles, English language and human subjects.
244 Articles
163 Articles
184 Articles
Apply selection criteria:
1) Original articles.
2) Use patch test to diagnose AHS due to one or more aromatic anticonvulsant drug(s).
3) Contain sufficient technical data.
54 Articles
43 Articles
42 Articles
Search results are combined and duplicates are removed.
55 Articles

used device is the Finn chamber. The drug is diluted in suitable media (usually petrolatum, water or ethanol) and the media alone is used as control. The test is then read for appearance of local reaction after different time periods (20 minutes to 4 days).[59]

This concept has been proven and extensively used for contact irritants and systemically administered drugs such as the β-lactam antibacterials.[60, 61] Presently, the positive predictive value (PPV) and the negative predicative value (NPV) of the patch test in the diagnosis of AHS remain to be determined and its real value is still unknown.[50] The percentage of concordance between clinically suspected hypersensitivity reactions and positivity of patch testing varies considerably because of lack of test standardization.[42, 45, 62]

2.4. Determinants Affecting Patch Test Results

2.4.1. Epicutaneous Penetration

An important determinant of patch test success is the ability of the tested agent (drug) to cross the skin (epicutaneous penetration) and come into contact with the processing cells of the immune system (presumably dendritic cells).[63] This property depends largely on the physicochemical characteristics of the drug to be tested, its concentration/formulation and the vehicle in which the drug is dispersed or solubilized. The physicochemical characteristics of the drug determine its polarity and lipid-solubility, thus affecting the ability of the drug to cross the skin barrier and reach the target cells.[64] In this regard, either the drug itself or its reactive metabolite can be used, although many reactive metabolites are not available commercially because of their instability, and purity of the reactive metabolites tested in this manner is an issue. In

addition, reactive metabolites may not be able to cross the epithelial barrier as they tend to be less lipophilic and, in some cases, the reactive metabolite is unknown. Unfortunately, it is not yet possible to comment on the benefit of using metabolites of ACDs in patch testing because of the paucity of literature on this subject.[53, 62] There are also cases where opposing results have been obtained when patch testing a drug and its main metabolite in the same patient.[62, 65] Surveying the literature, it seems that the ACDs that are most commonly involved in eliciting AHS are carbamazepine and, to some degree phenytoin. This may be because of frequency of use of carbamazepine and phenytoin as opposed to prevalence of AHS, therefore, it is not surprising to find many more investigators interested in studying the toxicity of these drugs compared with other ACDs. Another possible reason for choosing to work with carbamazepine is because it is easier to work with in regard to the frequency of positive results in highly imputable cases.

Once absorbed, carbamazepine is initially metabolized in the liver (or skin) via cytochrome P450 (CYP) 3A4 and CYP2C8, into at least 33 different metabolites.[66-69] One of the main metabolites that is also known to have pharmacological activity is carbamazepine 10,11 epoxide, which is stable and available commercially for research purposes. Lee et al.[62] patch tested both carbamazepine and carbamazepine 10, 11 epoxide on 13 patients who had exhibited a skin reaction to carbamazepine manifested as a maculopapular cutaneous eruption. Seven of the 13 patients gave positive patch tests with the parent drug but negative results with the metabolite; two reacted only to the metabolite and 1 patient tested positive to both agents. In the same study, all 39 control subjects who were taking antiepileptic drugs including carbamazepine gave negative

patch test results to both carbamazepine and carbamazepine 10,11 epoxide. Although the PPV of the patch test for carbamazepine in this study was good (61.5%), the low percentage of positive tests when using carbamazepine 10,11 epoxide (23.0%) is difficult to explain. The authors interpreted these results to be due to either the low concentration of carbamazepine 10,11 epoxide used or to efficient metabolism of carbamazepine 10,11 epoxide, for example by epoxide hydrolase, in some of the patients. The latter explanation is more likely.

The use of a reactive metabolite in patch testing has always been hindered by lack of knowledge of the role of each metabolite of a drug in eliciting hypersensitivity reactions and response to the exact testing procedure as well as lack of availability of most of the suspected metabolites due to their chemical instability. Duhra and Foulds[66] patch tested carbamazepine and oxcarbazepine as well as some of their metabolites (but not carbamazepine 10,11 epoxide) in a patient with suspected carbamazepine hypersensitivity. Only carbamazepine gave positive patch test results and they suggested that the double-bond between positions 10 and 11 of the azepine ring (figure 1) is critical for skin reactivity. No other study is available in the published literature using patch testing with metabolites of ACDs.

2.4.2. *Type of Drug Tested*

It has been shown that the predictive value of a patch test depends largely on the type of drug implicated in the ADR.[59] Galindo et al.[70] have investigated 23 different types of ADR, including generalized rash, fever, arthralgia, lymphadenopathy, palpable purpura, facial erythema, angio-oedema and erythema multiforme, developed to ACDs in 15 patients using patch testing. They found the patch test to be most useful for ADRs

involving carbamazepine (PPV 75%) and phenytoin (PPV 60%), whereas the rate for a positive test was very low (25%) with phenobarbital and lamotrigine. The good PPV observed with carbamazepine does not seem to be affected by the vehicle used, i.e. whether it is liquid (water or ethanol) or semisolid (petrolatum).[30, 71] One explanation for the good PPV of patch testing with carbamazepine could be its high lipophilicity, which may facilitate its percutaneous penetration and intracellular movement during patch testing. Indeed, carbamazepine has very good lipophilic properties and a log K_{oct} value of 2.7, which is near the optimum value of 2.5 for transdermal permeation, although other parameters can be enhanced through some modifications to the chemical structure of the compound.[72]

2.4.3. Concentration of Tested Drug

The ideal drug concentration in patch testing of anticonvulsants is critical in obtaining positive results in affected patients without inducing non-specific local irritation, which may be falsely interpreted as positive results.[60] The concentration selected should give negative results in control subjects.[73] Because the exposed surface area of the skin is standard (e.g. using Finn chambers) the amount of drug used is always expressed as concentration (weight by volume). In published data, the drug concentration used with ACDs ranged from 0.0001% to 100% pure substance, but the most commonly used concentrations were between 1% and 10%. However, 0.1% has been the lowest reported concentration at which a positive patch test to carbamazepine was observed.[74-76]

It has been recommended to use pure drug, whenever available, in order to avoid false-positive results due to hidden additives in the drug formulations[77],degradation

products or impurities. In all cases, certain guidelines for the preparation of commercially available drug formulae for patch testing have been suggested.[59, 73]

2.4.4. Vehicle

Petrolatum has been a preferred medium for patch testing of skin sensitizers because it gives good occlusion and prevents drug degradation as a result of hydrolysis.[78] Its use has yielded satisfactory results with patch testing of ACDs,[27, 30, 62, 64, 74-76, 79-86] although, other liquid solvents, such as water,[87] saline,[79] ethanol,[88] methanol,[89] acetone[90] and propylene glycol,[91] have also been used. Nonetheless, it appears that using different vehicles does not alter the results,[71] although some liquid vehicles evaporate during the test, possibly affecting the concentration at which the drug is introduced.

In addition, applying control patches of the vehicle at the same time as the drug is critical because some patients may be sensitive to the vehicle itself especially if it is not of high purity.[92] The state of the drug in aqueous vehicle or in a semisolid medium, such as petrolatum, are different since the compound may dissolve in the liquid vehicle but be dispersed as undissolved crystals in the semisolid medium. Thus, we might expect to have better delivery of the drug using the liquid vehicle rather than petrolatum. In fact, using *in vitro* mounted human skin and chromate preparations as a model, Gammelgaard et al.[93] demonstrated a better skin permeation of the chemical (potassium dichromate) with aqueous vehicle. It is also interesting to note that paracetamol (acetaminophen) gave a positive patch test when using an aqueous vehicle and negative patch test when petrolatum was used as the vehicle.[27]

2.4.5. Timing

Another factor that seems to be critical to the final result of patch testing is the timing of the test in regard to the beginning of the hypersensitivity reaction. Some authors[94] have recommended performing the patch test within 6 months following the reaction to avoid false-negative results because it is not known how long drug reactivity lasts. However, others have recorded positive patch tests 6 months to 2 years after the reaction.[76, 80, 84, 87, 95, 96] In fact, positive patch test results have been obtained in patients tested 12 years after the adverse reaction to drugs such as sulfamethoxazole.[97] This may not be surprising as drug-specific T cells can be detected for decades following an adverse reaction.[45] It is not known if this phenomenon of long-lasting drug reactivity is drug-dependent, although the frequency of drug-specific T cells is apparently drug-dependent.[97]

On the other hand, Jones and coworkers[80] have reported false-negative patch test results to carbamazepine when the test was performed during or right after the hypersensitivity episode. In contrast, others[30] have warned about false-positive patch test results if the test is performed during the increased reactivity period of the hypersensitivity reaction and recommend waiting for at least 2 months after the subsidence of the reaction before performing the test. However, positive patch test results have been obtained when the test was performed during or right after recovery from the reaction.[27, 71, 79, 85, 90, 98-100]

In reviewing different studies, it seems obvious that performing patch tests during the acute phase of the reaction appears to yield low rates of positive results,[88, 100-102] and the optimal timing for the test in this regard appears to be between 2 and 6 months after the reaction. No mechanistic explanation is available as to why the reaction is not

detectable early on; however, some have speculated that transient immune depression during the reaction produces this refractory period.[102] Others propose that transient selective recruitment of antigen-specific lymphocytes into target organs may lead to the low number of such cells in the peripheral blood, and thus low reactivity. [84] However, in the case reported by Okuyama et al.,[102] other factors may have contributed to the negative results of the patch tests for carbamazepine hypersensitivity during and immediately after the reaction, including topical and oral co-administration of steroids during the illness. This observation is supported by the appearance of slightly positive LTTs during the early stages of the reaction.

2.4.6. Clinical Picture

The clinical picture of the AHS seems to correlate with patch test results, in that patients with certain types of clinical manifestations seem to react differently to the test. This is because the clinical manifestations reflect the underlying and integrated immunological mechanisms of the 'reactions', which probably differ in one or more aspects from patient to patient and from one drug to another in individuals.[101, 103] Some of these underlying reactions are unlikely to be recognized by patch testing, or may not involve the immunological mechanisms that the patch test was designed to detect. For instance, when the patch test was used on patients who developed different types of cutaneous ADRs, such as exanthemas, fixed drug eruptions or urticaria, more positive results were observed with exanthema patients than in patients with other types of cutaneous ADRs.[50]

Similarly, Alanko[71] studied 18 patients with different forms of cutaneous reactions to carbamazepine. Of these, 15 were confirmed by oral rechallenge. Patients

with maculopapular exanthematous eruptions, exfoliative erythrodermas or erythema multiforme were found to give positive patch test results in about 70% of those tested, whereas those with other types of skin reactions including fixed drug eruptions, urticaria and other types of exanthema all had negative patch test results. However, Alanko et al.[104] could demonstrate positive patch test results in patients with fixed drug eruption only if the test were performed on the site of old lesion and not on unaffected skin. Similarly, Galindo and coworkers[70] have also suggested a correlation between the histological features of the hypersensitivity reaction and the predictability of testing such as patch tests. Puig et al.[75] reported that the clinical type of ADR plays a critical role in the sensitivity of the patch test, which appears to be maximal for maculopapular or morbilliform reactions.

Of particular importance, delayed hypersensitivity reactions may take more than one cutaneous form, even in the same patient.[45, 105] Cutaneous manifestations of reactions to ACDs come in many different forms,[28] some of which could be of pseudo-allergic nature,[106, 107] i.e. they may not be mediated by the usual immune mechanisms. Those reactions, although they mimic true allergic reactions, are unlikely to be detectable by the patch test.[52] This may explain the low rate of positive patch test results on AHS patients reported by some investigators.[50, 53, 81, 86, 100]

2.4.7. Other Factors

Other factors that may affect the outcome of the patch test in general are age, sex and ethnic origin of the patient. Many parameters of skin function, such as thickness, pH, blood flow and content of lipid, water and protein, are known to change during ageing.[108-111] These changes can affect the ability of the applied drug to penetrate the

skin and elicit its effects. With contact allergy, contradictory literature reports have appeared regarding the effect of age, sex and ethnic origin on results of the patch test.[109, 112, 113] However, these factors have not been evaluated directly in patch testing of ACDs, and further comprehensive work is essential if the contributions of these factors to the variability in patch test results are to be completely understood (table I).

2.5. Discussion

Our systematic review reveals that there is a deficiency in large-scale studies determining the usefulness of patch testing in the diagnosis of AHS. Lammintausta and Kortekangas-Savolainen[50] performed a retrospective study analysing the result of skin tests including patch testing performed on 947 patients with suspected cutaneous ADRs during a 13-year period, of whom 56 patients had been exposed to ACDs. Tested patients had developed a wide range of cutaneous symptoms including exanthema, urticaria, angio-oedema, fixed drug eruption, vasculitis, purpura and erythema multiforme. Unfortunately, the percentage of positive tests among these patients was lower than 20% and no oral rechallenge was performed to validate the predictive value of the patch test in such cases.

In another study to investigate the suitability of the patch test or the LTT to detect carbamazepine allergy, Troost and colleagues[88] tested a number of patients using both techniques. Correlation between positive results of both tests was rather low ($r = 0.39$, $p = 0.0022$). Among a total of 59 patients displaying adverse effects to carbamazepine, 23 had positive LTTs and only 8 of the 23 LTT-positive patients had a positive patch test (35%).

Among the published studies, the PPV of the patch testing seems to depend on the type of antiepileptic drug under investigation, with the highest values obtained with carbamazepine and the lowest with phenobarbital. These values range from 20% to 80%; however, it is difficult to draw a firm conclusion because in most of the cases it is only the medical history of the patients, which provides any evidence of the drug involved. Oral rechallenge would help confirm the identity of the suspect drug, but because of the possible severity of the reaction, a systemic rechallenge is rarely performed.

The PPV of the patch test in the diagnosis of AHS appears to be higher than its NPV. This trend is expected because there are two types of determinants in achieving a positive patch test: (i) the technical and toxicokinetic characteristics of the agent prior to its introduction to the immune cells; and (ii) the readiness of the immune system to recognize this agent and elicit its distinct reaction. Both of these types of factors appear to contribute to the success of the drug in eliciting a positive patch test. In fact, some investigators believe it is quite "astonishing" that the patch test can give a positive reaction at all.[52] This doubt is especially relevant for drugs in which the mechanism of hypersensitivity is believed to involve long and complex pathways. Positive patch test results in AHS can be indicative of patient sensitivity to the drug (high PPV) but negative ones are not conclusive (low NPV) as false-negative results have been described.[136]

The patch test is capable only of detecting a rather strong inflammatory reaction and this capability depends on how many inflammatory components are involved in the hypersensitivity reaction.[52] Therefore, weak or intermediate immune responses are unlikely to be detected by patch testing. Recent advances in genetic research have allowed the discovery of associations between genetic polymorphisms in certain genes

Table I. Summary of data: use of patch testing to investigate anticonvulsant hypersensitivity syndrome

Type of study	No. of pts	No. of controls	Drug[a]	Conc. [w/v] (% unless otherwise indicated)	Vehicle	Time[b]	Frequency of positive result (%)	Reference
Case report	1	10	Phenytoin	1, 5	Sal	6 mo	1/1	
	1	10	Carbamazepine	0.1–20	Sal	6 mo	0/1	
	1	10	Phenobarbital (phenobarbitone)	10–20	Sal	6 mo	0/1	[114]
Case series	1	0	Carbamazepine	0.1–10	NA	4 wK	1/1	[115]
Case series	1	0	Phenytoin	1	Wat	DUR	1/1	
	4	0	Carbamazepine	5	Petr	DUR	3/4 (75)	[27]
Case series	8	34	Carbamazepine	5–20	Petr	1–120 mo	6/8 (75)	
	1	34	Phenobarbital	5–20	Petr	1–120 mo	1/1	
	1	34	Oxcarbazepine	5–20%	Petr	1–120 mo	1/1	
	1	34	Valproic acid	15–60	Petr	1–120 mo	1/1	[116]
Case report	1	0	Phenytoin	1, 10	Petr/wat	3 mo	1/1[c]	
	1	0	Carbamazepine	1, 10	Petr/wat	3 mo	1/1[c]	[30]
Case report	1	0	Carbamazepine	NA	NA	NA	1/1	[117]
Retr. cohort	37	5	Carbamazepine	1–30 μg/mL	Petr/sal/eth	2 mo–20 y	7/37 (18.9)	

Type of study	No. of pts	No. of controls	Drug[a]	Conc. [w/v] (% unless otherwise indicated)	Vehicle	Time[b]	Frequency of positive result (%)	Reference
	6	5	Phenytoin	1–30 μg/mL	Petr/sal/ eth	2 mo–20 y	2/6 (33.3)	
	8	5	Oxcarbazepine	1–30 μg/mL	Petr/sal/ eth	2 mo–20 y	1/8 (12.5)	
	5	5	Lamotrigine	1–30 μg/mL	Petr/sal/ eth	2 mo–20 y	0/5 (0%)	[50]
Case report	1	3	Phenytoin	50 mg/mL	NA	2 mo	1/1	[118]
Case report	1	10	Carbamazepine	10	Petr	NA	1/1	[83]
Case series	1	10	Phenytoin	12.5	PBS	NA	1/1	
	1	10	Carbamazepine	20	PBS	NA	1/1	
	1	10	Oxcarbazepine	12.5	PBS	NA	1/1	[119]
Case series	10	40	Phenytoin	10	Petr/eth	NA	3/10 (30)	[81]
Case report	1	3	Valproic acid	Pure	Pure	3 mo	1/1	[120]
Case report	13	39	Carbamazepine	10	Petr	NA	7/13 (53.8)	
	13	39	Carbamazepine 10, 11 epoxide	1 μg/mL	Eth	NA	3/13 (23)	[62]
Case series	8	20	Carbamazepine	5	Wat	>2 mo	5/8 (62.5)	
	5	20	Phenytoin	5	Petr	>2 mo	3/5 (60)	

Type of study	No. of pts	No. of controls	Drug[a]	Conc. [w/v] (% unless otherwise indicated)	Vehicle	Time[b]	Frequency of positive result (%)	Reference
	4	20	Phenobarbital	5	Petr	>2 mo	1/4 (25)	[70]
Case report	1	5	Lamotrigine	10	Petr	DUR	1/1	[98]
Case report	1	0	Carbamazepine	NA	NA	1 wk	0/1	[121]
Cohort study	1	20	Carbamazepine	400 μg/mL	PBS	6–8 wk	0/1	[122]
Case report	1	0	Carbamazepine	0.1, 1, 2	Petr	1–2 wk	1/1	[123]
Case report	1	0	Lamotrigine	50	Petr	2 d	1/1	[124]
Case report	1	0	Carbamazepine	5	Petr/sal	Aft. Rec.	1/1	[87]
Case report	1	0	Valproic acid	20	Wat	9 mo	1/1	[125]
Case report	1	15	Carbamazepine	1, 5	Petr	5 mo	1/1	[121]
Case series	2	0	Carbamazepine	1, 5	Petr	NA	1/2	[125]
Case report	1	20	Phenytoin	1–20	Petr/wat	2 mo	1/1	[126]
Case series	20	0	Carbamazepine, phenytoin, phenobarbital	10	NA	NA	12/20 (60)	[34]
Case series	4	5	Carbamazepine	1, 10	Petr	>1 mo	4/4 (100)	[86]
Case report	1	0	Carbamazepine	0.1–10	Petr/wat	3 mo	0/1	[127]
Case series	11	20	Carbamazepine, Phenobarbital	1	Petr	3–8 wk	5/11(45.5)	[86]

Type of study	No. of pts	No. of controls	Drug[a]	Conc. [w/v] (% unless otherwise indicated)	Vehicle	Time[b]	Frequency of positive result (%)	Reference
Case report	1	0	Carbamazepine, phenytoin, oxcarbazepine	10	Eth	2 mo	1/1	[99]
Case series	61	11	Carbamazepine	10	Eth	DUR	12/61 (20)	
	59	11	Oxcarbazepine	10	Eth	DUR	8/59 (14)	[88]
Case series	7	40	Carbamazepine	1, 5, 10	Petr	>1 mo	6/7 (85.7)	[75]
Case report	1	10	Phenytoin	1	Petr	NA	1/1	
	1	10	Carbamazepine	1	Petr	NA	1/1	
	1	10	Phenobarbital	5	Petr	NA	0/1	[128]
Case report	1	5	Carbamazepine	2	Petr	Right after	0/1	
	1	5	Carbamazepine	1	Petr	3 mo	1/1	[102]
Case series	4	12	Carbamazepine	0.1–100	Petr/ace	0, 1.5,4 and 6 y	4/4 (100)	[129]
Case report	1	0	Carbamazepine	0.1–10	Per	4 mo	0/1[d]	[130]
Case report	1	5	Carbamazepine	1 and 5	Meth	NA	1/1	[89]
Case series	5	20	Carbamazepine	1	Petr	3 mo–5 y	4/5 (80%)	[80]
Case series	3	0	Carbamazepine	10	Petr/eth/DMSO	NA	3/3 (100)	[131]

Type of study	No. of pts	No. of controls	Drug[a]	Conc. [w/v] (% unless otherwise indicated)	Vehicle	Time[b]	Frequency of positive result (%)	Reference
Case report	1	9	Carbamazepine	10, 20,40	YSP	3 y	1/1	[96]
Case series	18	20	Carbamazepine	3, 10	Petr/wat/eth	DUR	9/18 (50)	[71]
Case report	1	0	Carbamazepine	100	Pure	Right after	0/1	[66]
Case report	1	20	Carbamazepine	0.1, 1	Petr	4 wk	1/1	
	1	0	Carbamazepine	10	Petr	4 wk	0/1	[132]
Case report	1	0	Carbamazepine	0.001–5	Petr	NA	1/1[c]	[74]
Case series	25	10	Carbamazepine, Oxcarbazepine	NA	NA	NA	6/25 (24)	[53]
Case series	6	0	Carbamazepine	0.3–20	Petr/sal	NA	4/6 (67)	
	2	0	Phenytoin	0.3–20	Petr/sal	NA	1/2 (50)	
	10	0	Phenobarbital	0.3–20	Petr/sal	NA	4/10 (40)	
	5	0	Valproic acid	0.3–20	Petr/sal	NA	4/5 (80)	[64]
Case report	1	0	Carbamazepine	1, 10, 100	Ace/petr	DUR	1/1	[90]
Case report	1	0	Carbamazepine	Cr.Tab	Petr/wat	DUR	1/1[c]	[85]
Case report	1	0	Carbamazepine	0.0001–0.1	Petr	6 mo	1/1[c]	[76]
Case series	10	80	Carbamazepine	1, 5, 10	Petr	NA	3/10 (30)	[133]
Case report	1	0	Carbamazepine	1–10	Petr	DUR	0/1	[100]

Type of study	No. of pts	No. of controls	Drug[a]	Conc. [w/v] (% unless otherwise indicated)	Vehicle	Time[b]	Frequency of positive result (%)	Reference
Case series	3	0	Carbamazepine	1–10	Petr	4–7 mo	3/3 (100)	[84]
Case report	1	0	Carbamazepine	Pure, 1	Petr/ace	3 mo	1/1	[134]
Case report	1	0	Phenobarbital	20	Pr. gly	Right after	1/1	[91]
Case series	7	18	Carbamazepine	10, 20, 40	Petr	14 wk–7 y	6/7 (85.7)	[95]
Case report	1	4	Phenytoin	1, 5, 10	NA	5 mo	0/1	
	1	4	Carbamazepine	1, 5	NA	5 mo	1/1	[135]

* = concentration in μg/ml.
** = time elapsed between the reaction and the test.
*** = Frequency of positive results (Percentage).
§: positivity depends on concentration and/or vehicle used.
¥: The suspected drug causing the reaction as suggested by at least the medical history of the patient(s).
¶: Pt with 10% in petr. was slightly positive at 3 days.
Abbreviations: AC = anticonvulsant, Ace = acetone, AHS = anticonvulsant hypersensitivity syndrome, CBZ = Carbemezepine, CBZ-E = carbamazepine epoxde, Cr.Tab = crushed tablet, CT = number of control subjects, DPH = Phenytoin, DRG = drug, DUR = during, Eth = ethanol, LMT = Lamotrigine, M = month, Meth = methanol, Petr = petrolatum, PHB = Phenobarbital, Pr. gly = propylene glycol, PT = number of patients, Retr. = retrospective, Sal = saline, VA = valproic acid, Wat = water, Wk = week, Y = year, YSP = yellow soft paraffin.

(e.g. *HLA-B* and *heat shock protein 70*) and the risk of specific types of drug hypersensitivity reactions.[137-143] However, no genetic marker has yet been identified that has sufficient predictive value to be used as a screening tool for AHS predisposition in the general population.[138] A recent alert has been issued by the US FDA recommending screening all patients with Asian ethnicity for the *HLA-B*1502* allele before prescribing carbamazepine because of the proven genetic association between this allele and a high risk of developing severe forms of hypersensitivity reactions (SJS/TEN).[144] However, the Asian population consists of multiple ethnic groups that vary considerably in terms of genetic composition, including the frequency of the *HLA-B*1502* allele. Furthermore, no link was found between this type of mutation and other non-bullous forms of carbamazepine-induced hypersensitivity reactions, making genetic screening useless in predicting patient susceptibility to these reactions.[145] However, it is of interest that different polymorphic alleles were found to associate with specific forms of hypersensitivity reaction (maculopapular eruption, multiple organ syndrome, SJS, TEN), implying varying pathological mechanisms for each reaction. This may partially explain differences in patch test performance in patients developing different clinical manifestations of AHS.

2.5. Conclusion

Patch testing is one of the tools that can be used to diagnose or predict AHS. It is apparent that patch testing can detect only a small portion of the immunological reactions that underlie AHS, therefore, other diagnostic methods, such as systemic rechallenge, LTA and/or LTT, should be utilized to make testing more reliable. However, the benefit of testing appears to be maximal with certain drugs (i.e. carbamazepine and phenytoin)

and for specific clinical manifestations (strong reactions). It should be performed 2–6 months after recovery from the date of the ADR for best results, with adequate vehicle control. In addition, the test procedure must be standardized in order to evaluate its performance in the diagnosis of drug-induced hypersensitivity reactions.

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Chapter 3: *In vitro* testing for the diagnosis of anticonvulsant hypersensitivity syndrome: A Systematic Review

This chapter has been published previously:

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3.1. Introduction

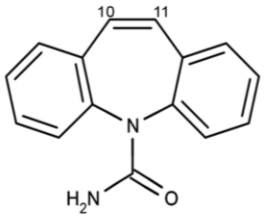
Anticonvulsant hypersensitivity syndrome (AHS), also known as drug hypersensitivity syndrome (DHS) or drug rash with eosinophilia and systemic symptoms (DRESS), is a type B "Bizarre" adverse drug reaction (ADR) that develops in susceptible patient following exposure to certain drugs including aromatic anticonvulsants(figure 1).[1, 2] Although lacking a defined clinical picture, AHS is typically associated with development of skin rash, fever and internal organ dysfunction that may include blood discrasias, hepatitis, nephritis, myocarditis, thyroditis and interstitial pneumonitis and encephalitis.[3] The pathophysiological mechanisms underlying AHS are not well understood; however it is believed to be immune mediated in general and involve generation of nucleophilic reactive metabolites that react covalently with macromolecules to form immunogenic adducts able to activate the immune system. [4, 5] The accurate incidence of AHS is unknown due to underreporting but it has been estimated to be from 1 in 1000 to 1 in 10,000 in patient newly exposed to aromatic anticonvulsants. [6] While the disorder is rare,it is potentially fatal and represents a clinical dilemma to treating doctors. Diagnosis of AHS is challenging as a reliable and safe diagnostic test is not available to confirm causality or identify the culprit drug. A number of in vivo and invitro tests have been devised and used to aid the diagnosis of AHS.[7, 8] These include skin tests (patch test, prick test, intradermal test), the lymphocyte transformation test (LTT) and the lymphocyte toxicity assay (LTA).[9] The use of patch test in the diagnosis of AHS has been reviewed recently.[10] This systematic review is an attempt to evaluate the utility of in vitro tests used for the purpose of diagnosis of the T-cell-mediated type IV delayed

AHS reactions. Other tests used for other types of allergic reactions (e.g. IgE measurement, radioallergosorbent test, basophil activation test) are not reviewed here.

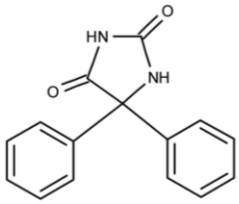
In vitro diagnostic tests have the advantage over *in vivo* tests (patch test and rechallenge) of bearing no potential harm to patients. A number of *in vitro* diagnostic tests have been used to aid the diagnosis of delayed-type drug hypersensitivity reactions[7, 8, 11-13]; however, their true value is yet to be defined. Among these tests are those that utilize peripheral blood monocytes (PBMCs) as target cells, including the LTT and the LTA. Unfortunately, these techniques require expensive equipment and sophisticated laboratories as well as specialized experience with biochemical and molecular methods so only a few centres are sufficiently equipped to perform them. Hence, these methods, although successfully employed as research tools, have not been successfully translated to diagnostic tests.[14, 15] The specific aims of the current systematic review are 4-folds: (i) to evaluate the use of LTT and LTA in the diagnosis of AHS; (ii) to describe the advantages and limitations of these tests; (iii) to discuss different technical aspects of both tests with the scope of possible improvement; and (iv) to identify potential future work to increase the diagnostic value of these tests. The overall objective of this review is to identify gaps that must be closed to allow these tests to become validated, mainstream diagnostic tools.

Leukocytes are present in peripheral blood at densities of 5-7 X 10^3 cells/mm^3; 20 to 50% of these cells are lymphocytes whereas 2 to 10% are monocytes. Lymphocytes are favoured as a model for investigation of immune-mediated diseases because of their unique characteristics, which include that (i) they are easily obtained at adequate density; (ii) they play a key role in the immune system by orchestrating different elements of the

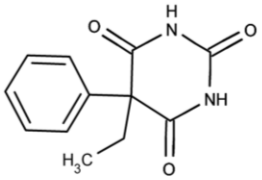
Fig. 1. Chemical structures of aromatic anticonvulsant drugs.



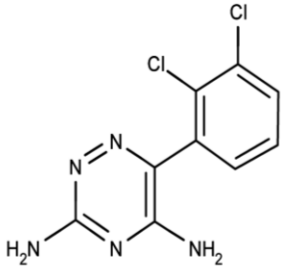
Carbamazepine



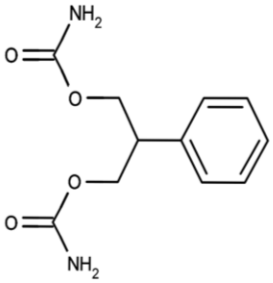
Phenytoin



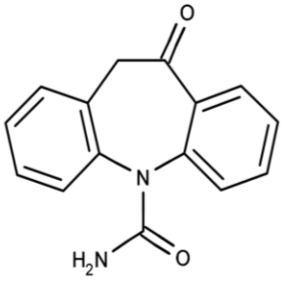
Phenobarbiral



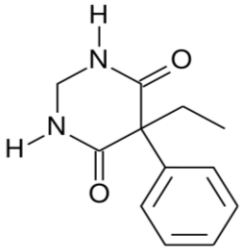
Lamotrigine



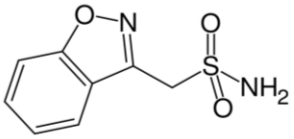
Felbamate



Oxcarbamazepine



Primidone



Zonisamide

immune response and thus representing the state of the immune system in the specific patient; (iii) they are metabolically active and expressing most of the enzymes that are required for drug detoxication; and (4) individual genetically-based defects in the expression or activity of these detoxication enzymes are phenotypically expressed in lymphocytes.[1]

3.1.1. Isolation of peripheral blood mononuclear cells (PBMCs)

Several methods have been used to isolate lymphocytes from heparinised whole blood including the gelatine method, passage through glass wool or beads as well as magnetic separation after cellular ingestion of carbonyl iron [16], However, the most successful and currently most used method is that developed by Böyem[17]. This method involves centrifugation of diluted blood samples through a gradient of Ficoll®, a synthetic high molecular weight polymer of sucrose that is highly branched and has low intrinsic viscosity. This method permits recovery of 60 ± 20% of lymphocytes from original blood samples with cell viability greater than 90%. This technique allowed the use of isolated peripheral blood monocytes (PMBCs) in tests such as the LTT and LTA.

3.1.2. The lymphocyte transformation test (LTT)

The *in vitro* lymphocyte transformation phenomenon was first described during the late 1950s. In short, human peripheral leukocytes differentiate in short-term primary cultures, forming plasts. This effect was later attributed to the presence of a constituent, phytohemoagglutinin (PHA), of plant extract from red kidney beans (*Phaseolus vulgaris*), used to isolate blood peripheral leukocytes.[18] PHA causes erythrocytes to aggregate and sediment allowing leukocytes to separate from whole blood preparations.[19, 20] In a later report, Nowell[21] demonstrated that PHA also initiates

mitotic activity (transformation) in cultured human leukocytes. To confirm the assumption that the effect of PHA on isolated peripheral blood leukocytes (PBL) has an immunological basis, Pearmain *et al.*[22] exposed PBLs isolated from both tuberculin-sensitive and non-sensitive patients to tuberculin *in vitro*. Only PBLs from tuberculin-sensitive patients showed mitotic activity whereas cells from patients not previously exposed to the antigen showed no mitosis.

One of the first reports of using this test for diagnosis of drug allergy was by Holland and Mauer who evaluated the effect of diphenylhydantoin (phenytoin; DPH) on cultured lymphocytes isolated from patients sensitive to the drug and non-sensitive (control) subjects. In these experiments, PHA, used as a positive control showed non-specific stimulation of all cells sampled whereas DPH stimulated only the cells from DPH-sensitive patients.[23] With peripheral lymphocytes isolated from a sulphadiazine-sensitive patient, this effect was found to be concentration-dependent.[24] In 1966, Vischer[25] replaced the lengthy visual counting of mitotic figures from fixed slides with a faster and less subjective method by measuring radiolabelled thymidine incorporation into cellular DNA as a reflection of the rate of cell division. During the late 1960s and early 1970s a great deal of work was done by Schellekens and colleagues to optimize the *in vitro* lymphocyte transformation technique.[26-30]

The terms Lymphocyte Transformation Test (LTT), Lymphocyte Stimulation Test (LST) and Lymphocyte Proliferation Test (LPT) are interchangeably used to describe this technique. The procedure includes incubation of PMBCs isolated from drug-hypersensitive patients with the incriminated agent at non-toxic concentrations and observation of any increase in the rate of cell proliferation measured by [^{3}H]thymidine

incorporation (figure 2). The increase in cell proliferation is expressed as a ratio between proliferation of cells incubated with and without the drug (control). This ratio is defined as the stimulation index (SI) and it is calculated as follows:

$$SI = \frac{\text{Value of } [^3H]\text{thymidine uptake, count per minute (cpm) in the presence of the drug}}{\text{Value of } [^3H]\text{thymidine uptake (cpm) in the absence of the drug (vehicle}}$$

Cell cultures from drug-exposed and unexposed non-sensitive individuals are also used to confirm the specificity of a potential drug effect. The final result of the test depends on several factors such as the value of background cell proliferation and the type of the drug, however, an SI of > 3 is always considered indicative of a positive reaction.[31] Other endpoints for measurement of T-cell activation such as elevation of released cytokines (using an enzyme-linked immunoabsorbent assay, ELISA) have been proposed and could be a more sensitive method for detection of T-cell activation than measurement of the rate of cell proliferation.[31, 32] A recent technique based on staining of intracellular proteins with carboxyfluorescein succinimidyl ester (CFSE) has been used successfully to measure T-cell proliferation *in vitro*.[33, 34] This fluorescent dye is used to non-specifically label intracellular proteins. In cell proliferation, the intensity of the fluorescent signal is progressively decreased as the stained proteins are divided during mitosis. An increase in number of low fluorescent cells indicates cell proliferation that can be measured by flow cytometry.[35, 36]

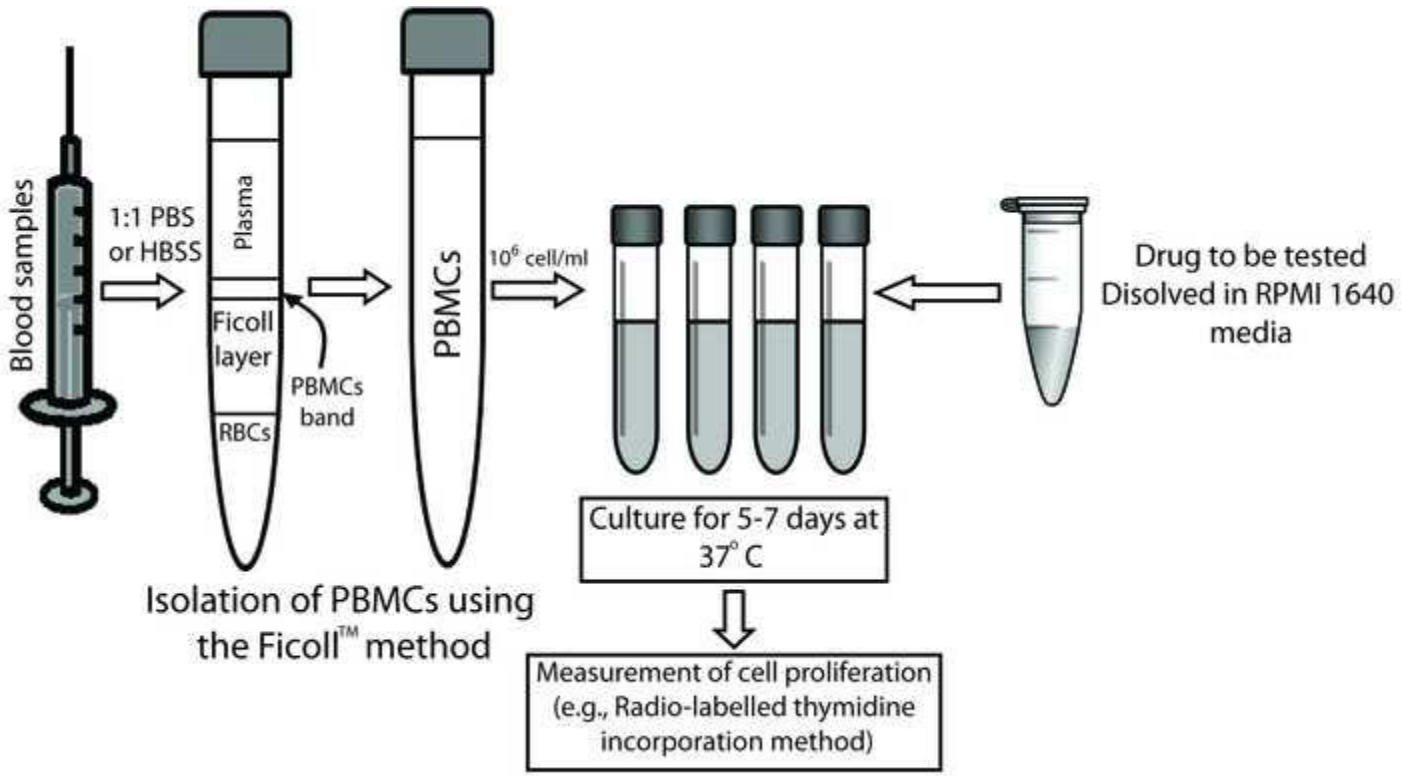
The LTT has been used by some investigators for diagnosis of potential drug allergy cases for more than 20 years.[37] However, its value in diagnosis and prediction of AHS remains controversial.

3.1.3. The lymphocyte toxicity assay (LTA)

Introduced by Spielberg and colleagues[1, 38-41] in the 1980s, the LTA is an *in vitro* test which utilizes isolated PBMCs to investigate the pathogenesis of idiosyncratic drug reactions. The test is based on the hypothesis that idiosyncratic reactions develop as a result of imbalance between generation of toxic reactive metabolites (metabolic activation or toxication) and detoxication capacity which leads to accumulation of toxic metabolites (the "reactive metabolite" hypothesis).[4, 42-44] In this test, lymphocytes are used not as immunogenic cells but rather as easy to obtain surrogate target cells.[41] The procedure for the test entails incubation of PBMCs isolated from the patient with the culprit drug in the presence of phenobarbital-induced mouse, rat or rabbit liver microsomal 9,000 x g supernatant fraction (S9), as a source of cytochrome P450 (CYP) monooxygenase activity).

CYP activity in the rodent (or sometimes human) liver preparation is hypothesized to oxidize drug to its active (cytotoxic) metabolite(s). Lymphocytes contain enzymes that are required for drug detoxication including epoxide hydrolases (EHs) and glutathione S-transferases (GST) and any genetic defect in the function of these enzymes is phenotypically expressed in these cells.[1] The % of cell death is then determined using different methods for assessing cell death (e.g., trypan blue exclusion or with a tetrazolium dye, for example by the MTT method). Cell death is assumed to reflect the vulnerability of the cells to the toxic effects of the drug which is hypothesized to indicate the susceptibility of the patient to develop hypersensitivity reactions to the parent drug and its reactive metabolite(s), presumably via differences in detoxication capacity and immune processing.

Fig.2. Steps of the lymphocyte transformation test (LTT). HBSS = Hanks balanced salt solution; PBMCs = peripheral blood monocytes; PBS = phoaphate buffered saline; RBCs = red blood cells; RPMI = Roswell Park Memorial Institute medium.


Blood samples
1:1 PBS
or HBSS
Plasma
Ficoll
layer
PBMCs
band
RBCs
PBMCs
10^6 cell/ml
Drug to be tested
Disolved in RPMI 1640
media
Culture for 5-7 days at
37° C
Isolation of PBMCs using
the Ficoll™ method
Measurement of cell proliferation
(e.g., Radio-labelled thymidine
incorporation method)

Aromatic anticonvulsants are excellent examples of metabolically activated cytotoxicants, metabolized, primarily by hepatic CYP isozymes into reactive electrophilic arene oxide metabolites.[5] These unstable and highly reactive intermediate metabolites are readily detoxified by EH and/or GST enzymes usually to non-electrophilic products (dihydrodiols and S-glutathione conjugates, respectively)[45, 46]

Although the same cell model (isolated PBMCs) is used in both types of assay, LTT and LTA are completely different approaches to the diagnosis of AHS. Whereas the former detects the *in vivo* immunological generation of drug-specific T-lymphocytes used as a sign of hypersensitivity, the latter detects genetic defects that lead to accumulation of toxic metabolites which are assumed to be a major factor in the etiology of drug hypersensitivity in addition to possible differences in cell death. Because the two tests use the same cell model and have similar nomenclature, it is not uncommon for individuals to confuse the LTT for LTA or vice versa[47] or to use different nomenclature to describe these tests.[2, 48, 49]

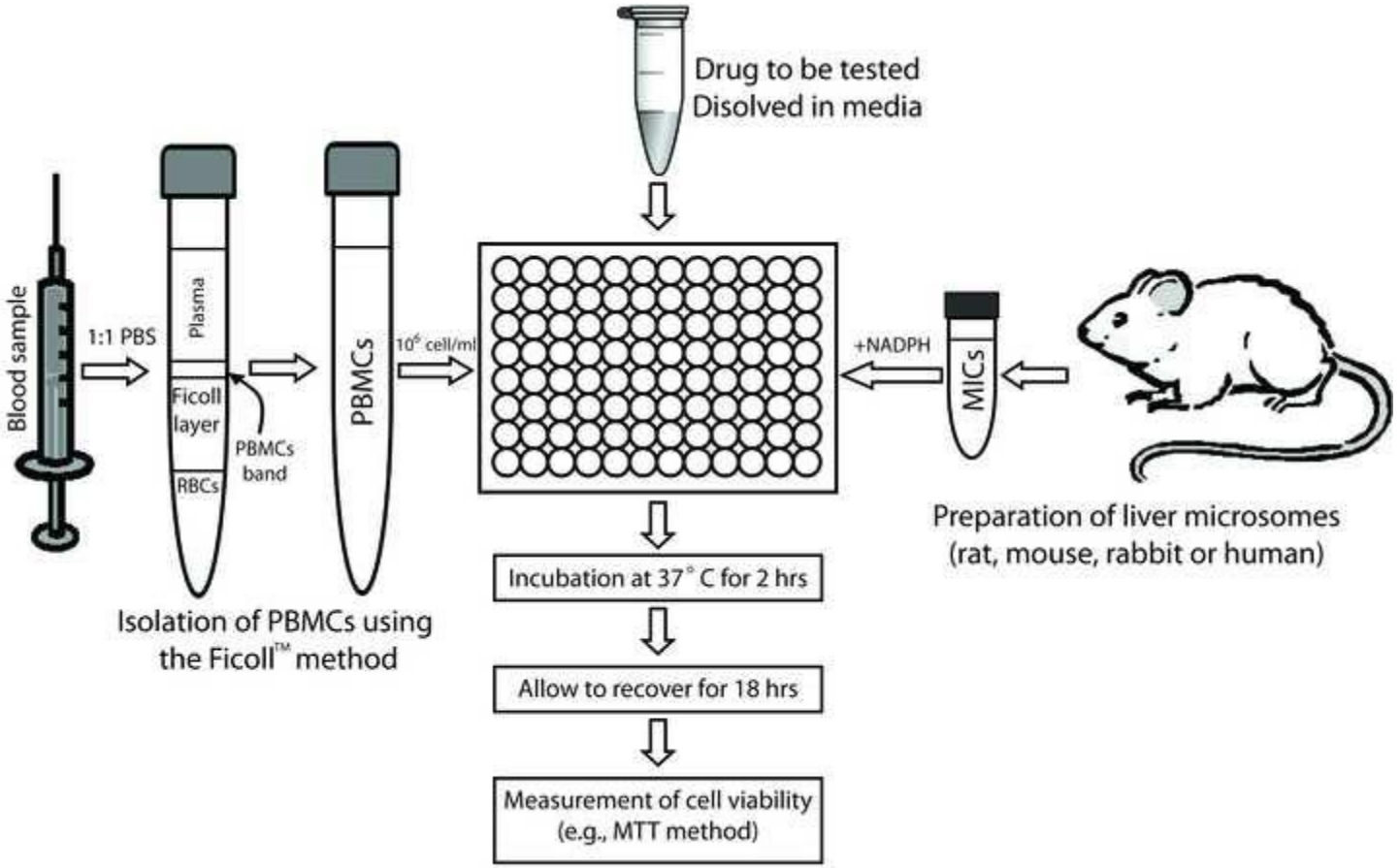
3.2. Research Methodology

In order to evaluate the clinical usefulness of these tests in diagnosis of AHS, we performed a systematic literature search using three major biomedical citation databases, PubMed, EMBASE and MEDLINE without any restriction on date from their commencement to the fourth week of May 2009

3.2.1. Search strategies

Search strategy I: the search was carried out using the key words “anticonvulsant” and “antiepileptic” in their singular, plural and truncated forms. These terms were also

Fig. 3. Steps of the lymphocyte stoxicity assay (LTA). MICs = microsomes; MTT = 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoluim; NADPH = nicotinamide adenosine dinucleotide phosphate; PBMCs = peripheral blood monocytes; PBS = phosphate buffered saline; RBCs = red blood cells.


Drug to be tested
Disolved in media
Blood sample
1:1 PBS
Plasma
Ficoll layer
PBMCs band
RBCs
PBMCs
10^6 cell/ml
+NADPH
MICs
Isolation of PBMCs using the Ficoll™ method
Preparation of liver microsomes (rat, mouse, rabbit or human)
Incubation at 37° C for 2 hrs
Allow to recover for 18 hrs
Measurement of cell viability (e.g., MTT method)

mapped to their medical subject headings (MeSH) terms. We also searched for individual aromatic anticonvulsant drugs including ‘carbamazepine’, ‘phenytoin’, ‘phenobarbital’, ‘oxcarbazepine’, ‘primidone’, ‘lamotrigine’, ‘felbamate’ and ‘zonisamide’ both as key words and as MeSH terms when available and the option ‘explode’ was used. The obtained results were combined using ‘or’.

Search strategy II: In parallel, we used as key words ‘lymphocyte toxicity assay’, ‘LTA’, ‘lymphocyte toxicity test’, ‘in vitro lymphocyte toxicity assay’, ‘in vitro lymphocyte toxicity test’, ‘lymphocyte transformation test’, ‘lymphocyte stimulation test’, ‘lymphocyte proliferation test’, ‘LTT’, ‘LST’, ‘LPT’, ‘drug-induced lymphocyte stimulation test’ and ‘DLST’. These terms were also mapped to their MeSH terms when available and the option ‘explode’ was used.

We then combined the results of both searches (search strategy I and search strategy II) using ‘and’ (figure 4).

Retrieved publications were manually reviewed and the following selection criteria were applied: (i) original articles are written in English; (ii) study is performed on human subjects; (iii) LTA or LTT was used to diagnose AHS due to one or more aromatic anticonvulsant drug(s); and (iv) contains sufficient technical data for scientific evaluation.

Thirty-one articles from PubMed, 22 articles from MEDLINE and 28 from EMBASE were found that met our selection criteria. The search results from the three databases were then combined and duplicates were removed. The final number of included articles from the three databases was 48 articles. Thirty six articles used LTT

and 12 used LTA for the diagnosis of AHS (figure 4). Although single case reports were included in the review, none of these reports were used to calculate any of the tests epidemiological characteristics.

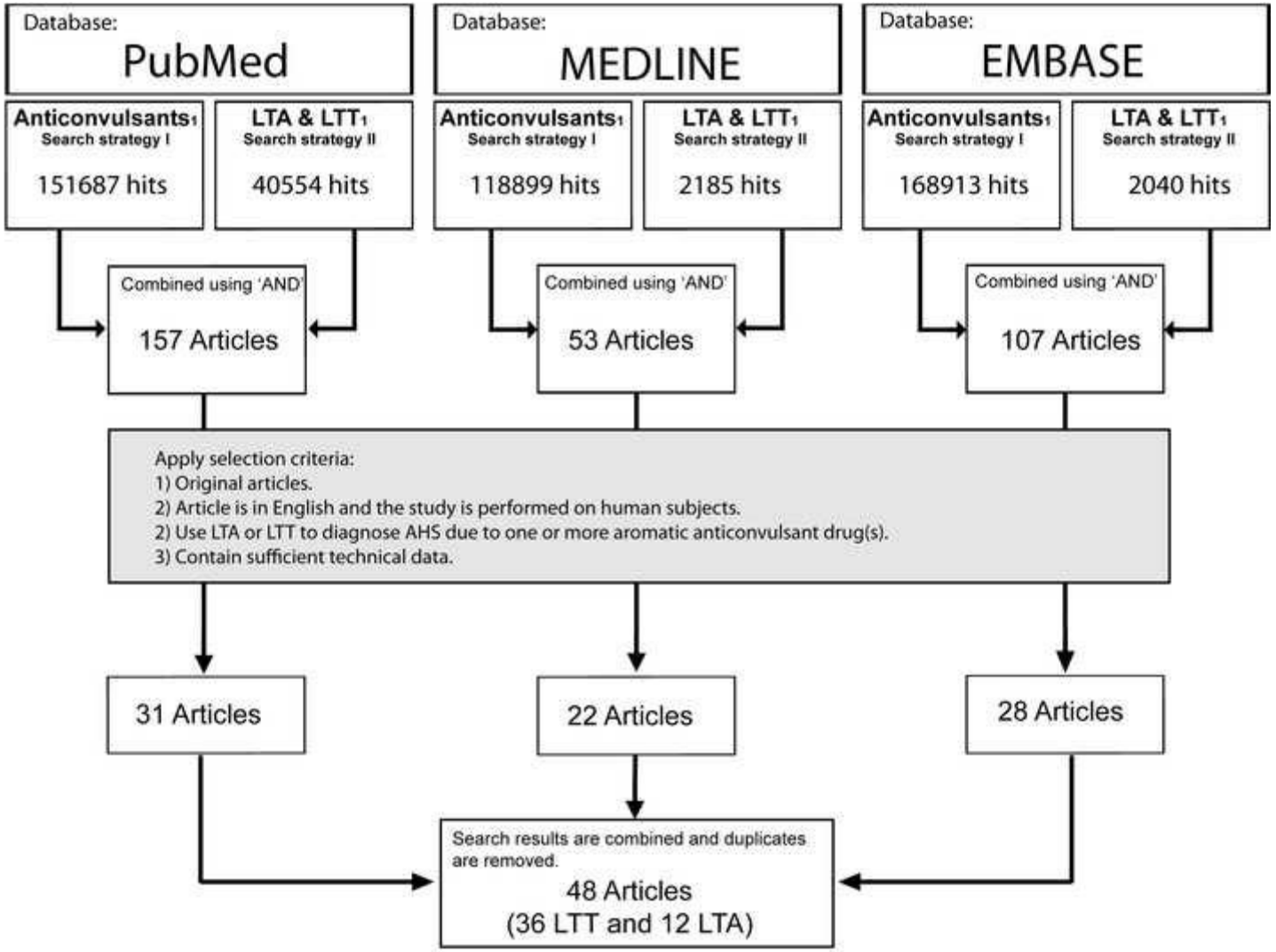
3.3. Results

3.3.1. The LTT in the Diagnosis of Anticonvulsant Hypersensitivity Syndrome (AHS)

The use of the LTT in diagnosis of hypersensitivity to ACDs dated back to the early 1960s, but its use was almost always confined to experienced technicians in well-equipped research centres, primarily for the purpose of investigating the mechanism of T-cell-mediated reactions rather than diagnosis of drug allergy[14, 15, 50-53] In addition, due to its low laboratory-to-laboratory reproducibility[31, 54] and hard-to-interpret results,[31] this test cannot be described as user friendly and requires a great deal of experience for interpretation of results. For this reason the test has not been translated into widespread clinical use. In fact, only a few research groups worldwide use this technique routinely.[31]

Table (I) summarizes data from original publications where LTT has been used to investigate hypersensitivity reactions to anticonvulsants. Troost *et al.*[55] directly addressed the issue of LTT usefulness in AHS diagnosis They collected data from 65 patients who displayed a wide range of adverse effects of carbamazepine (CBZ). They compared the performance of the patch test (PT) with the LTT and found that the LTT had a better positive predictive value than the PT (40% and 20%, respectively). However, the inclusion criteria for the AHS cases were not well described and medical history was the only evidence that incriminated the drug. Furthermore, the low positive predictive value of the PT for CBZ (20%) in this study may indicate that some of the cases included

Fig. 4. Flow chart of literature search and retrieving process. 1 Search strategies ‘anticonvulsants’ and ‘LTA and LTT’ include all relevant medical subject heading and key words as described in section 2.1. AHS = anticonvulsany hypersensitivity syndrome. LTA = lymphocyte toxicity assay, LTT = lymphocyte transformation test.


Database:
PubMed
Anticonvulsants1
Search strategy I
151687 hits
LTA & LTT1
Search strategy II
40554 hits
Database:
MEDLINE
Anticonvulsants1
Search strategy I
118899 hits
LTA & LTT1
Search strategy II
2185 hits
Database:
EMBASE
Anticonvulsants1
Search strategy I
168913 hits
LTA & LTT1
Search strategy II
2040 hits
Combined using 'AND'
157 Articles
Combined using 'AND'
53 Articles
Combined using 'AND'
107 Articles
Apply selection criteria:
1) Original articles.
2) Article is in English and the study is performed on human subjects.
2) Use LTA or LTT to diagnose AHS due to one or more aromatic anticonvulsant drug(s).
3) Contain sufficient technical data.
31 Articles
22 Articles
28 Articles
Search results are combined and duplicates are removed.
48 Articles
(36 LTT and 12 LTA)

were not typical CBZ-induced hypersensitivity reactions. This is supported by the fact that only 23% of the included patients displayed systemic manifestations and that 92% of them had only some form of mild skin reaction as an adverse event.

In an attempt to determine the sensitivity and specificity of the LTT in diagnosis of allergy to different drugs,[37] files of 923 patients with possible hypersensitivity reactions to drugs were studied. These patients were classified based on their medical history, follow-up and provocation tests into four groups where drug allergies were "definite," "probable," "less probable" or "negative". One hundred cases were considered to have a very high probability of drug allergy, of which 78 had a positive LTT. Only 3 of these 100 cases were attributed to ACDs (2 to CBZ and 1 to DPH). The 2 CBZ cases exhibited positive LTT tests whereas for the DPH case the LTT was negative. Although the chemistry of the drug in question appears to play a major role in determining the usefulness of the LTT, the overall specificity and sensitivity of this test in this study were found to be in the range of 85% and 76%, respectively. It is not known whether or not these numbers can be applied to ACDs. However, due to the fact that many different factors are involved in determining the final result of the LTT test as discussed below, one cannot generalize these figures to include all types of drugs taken under various conditions.

Numerous factors have been found to affect the predictive value of the LTT in the diagnosis of drug hypersensitivity reactions. These factors include: the timing of the test in relation to the beginning of the reaction; the type of clinical manifestations caused by the drug; the nature of the suspected drug; and the test procedure itself.

3.3.1.1. Timing of the test.

Performing the LTT during the adverse drug reaction may result in the high incidence of false negative test results due to the high rate of spontaneous T-cell proliferation that does not respond to any additional *in vitro* stimulation (refractory period).[50, 55, 56] Houwerzijl and coworkers[57] demonstrated the relationship between the timing of the test and its outcome. Sequentially testing a number of patients with hypersensitivity reactions to CBZ, they showed that this refractory period may extend up to 11 weeks from the time of the adverse reaction. This effect may be due to impaired T-cell function as the predominant T-cell mitogen, PHA could not stimulate T-cells during this period[57] In contrast,[58] Zakrzewska and Ivanyi obtained positive results when performing LTT on 6 of 9 patients within 2 weeks of the beginning of the adverse reaction. These authors attributed their results to the mild form of the adverse reactions (skin rash only) in their patients. In such cases, a shorter or no refractory period is expected due to the moderate degree of activation of T-cells.

Although drug-specific T-cells have been isolated from patients decades after the time of the reaction,[15] a positive LTT is not guaranteed if the test is performed later than 3-4 years after the reaction. [31]After remission from severe hypersensitivity drug reactions the frequencies of circulating drug-specific T-cells was estimated at 1:250 to 1:5000 (from 1:2000 to 1:10,000 for ACDs) and this rate does not appear to be affected by time. These frequencies were higher than frequencies of T-cells that recognize a full antigen such as tetanus toxoid (TT).[15] It is well documented that circulating drug-specific T-cells may last for years or even decades[15, 31, 59] after the insult but the length of this period may vary considerably for reasons not yet understood.

In a recent study designed to investigate the effects of timing and type of drug involved in adverse reactions on the utility of the LTT for diagnosis of drug hypersensitivity, Kano and colleagues[60] followed 12 patients suffering from different types of drug-induced hypersensitivity reactions (i.e., macula-papular eruption, MP; Steven- Jonson syndrome, SJS; and AHS or DRESS). Six patients developed AHS as a result of taking aromatic ring-containing anticonvulsants (DPH, CBZ and phenobarbital; PHB). Only one of 6 patients showed a positive LTT when the tests were performed within one week of the onset of the reaction. However, all 6 patients gave positive LTT results when tested at a later time (ranging from 5 weeks to 1 year). Interestingly, patients with MP and SJS showed an opposite pattern where the stimulation indices (SI) levels decreased with time. In another study of one case of CBZ-induced hypersensitivity that involved pulmonary symptoms, fever, generalized maculopapular erythmatous skin eruptions and eosinophilia, the LTT performed during the course of the reaction was positive (SI = 2.2). However, when the test was repeated 3 months after recovery, a negative result was obtained.[61]

In contrast, Wu *et al.*[14] did not find any association between timing of the test and the strength of the response in a cohort of cases of hypersensitivity to CBZ. Specifically testing a patient < one month after the reaction resulted in a strongly positive LTT (SI up to 69.4). In the study, similar results were obtained when testing patients at 84 and 180 months after the event, similar to Beeler's results[15] at 4 and 19 months after the reaction. Houwerzijl *et al.*[57] showed that maximum SI values are obtained if the test is performed 10 to 20 weeks after the beginning of the reaction, after which the SI values start to decline over time.

It appears that during the course of the adverse reaction drug-specific T-lymphocytes go through three different stages:

1. A highly reactive stage (very strong response) that shows spontaneous proliferation during which the cells do not respond to any additional *in vitro* stimulation.[31, 60] This stage appears to last for the first 1-4 weeks after the adverse event depending on the strength of the initial reaction.[57] Stronger reactions usually result in longer "refractory periods" than weaker ones.
2. An apparently long stage where drug-specific T-cells can be detected in peripheral blood and are responsive to an *in vitro* stimulation (strong response). This stage starts at the remission of the reaction and may last for years and sometimes for decades.[31]
3. A final stage, where no drug-specific T-cells can be detected in peripheral blood (weak response). This does not mean that the patient is desensitized to the drug or is able to tolerate it. In fact, a severe reaction may develop again once the patient is exposed to the culprit agent.[15, 31]

The so-called refractory period has been attributed to two mechanisms: 1) the circulating peripheral T-lymphocytes are at their maximum activation and do not respond to any further stimulation in the presence of the drug; and 2) drug-specific T-lymphocytes are selectively recruited to the affected target organs leading to a deficiency of these cells in peripheral blood.[55, 57, 64] It has also been observed that PBMCs isolated from patients during the hypersensitivity episode are characterized by a high proliferation rate, presumably related to their recent exposure to the culprit

drug (*in vivo*). These activated T-cells cannot be further stimulated by exposure to the drug or PHA *in vitro*.

3.3.1.2. Clinical manifestation of the reactions.

Hypersensitivity reactions to drugs can manifest in a wide range of distinct clinical symptoms including morbilliforme or bullous exanthema, urticaria, as well as involvement of other internal organs and including fever, blood cell dyscrasia hepatitis, nephritis and interstitial lung disease.[67, 83] Severe forms of AHS include erythema multiform (EM), SJS and toxic epidermal necrolysis (TEN).[84] Some of these reactions cannot be listed under any known classification of immune-mediated diseases. T-cell-mediated drug allergy can take different forms and utilize a variety of mechanisms including activation of different clones of T-cells and secretion of different types of mediators.[85] In some cases such as in patients with drug-induced SJS, the proliferative response of the drug-specific T-cells was found to be low despite the high level of secreted cytokines.[15] This can affect the ability of T-cell proliferation tests such as the LTT to detect low levels of circulating drug-specific T-cells, however, in this particular context measuring cytokines secretion as a readout system for T-cell activation can be more sensitive than the conventional LTT.[32]

Neukomm *et al.* [67] did not find any correlation between the strength of the LTT result (SI) and the type of the adverse drug reaction, but showed positive LTT results for a wide variety of reactions including IgE-mediated reactions. It is quite unexpected that an *in vitro* test such as the LTT can give positive results in all cases where drug-induced

Table I. Summary of data from original work that used the lymphocyte transformation test (LTT) to investigate hypersensitivity reactions to aromativ anticonvulsants.

Type of study	No. of patients	No. of controls	Drug[a]	Conc. (% unless otherwise indicated)	Time[b]	Read-out	Frequency of positive result (%)	Year/ Reference
Case series	2	5	carbamazepine	1, 10, 100	2 M, 14 M	CD69 ^{3}H-T	2/2	
	1	5	Phenytoin	1, 10, 100	2 M, 14 M	CD69 ^{3}H-T	1/1	**2008 [62]**
Case series	4	4	carbamazepine	1/50-1/100	< 1mo > 1 mo	^{3}H-T	2/4 4/4	
	1	4	Phenytoin	1/50-1/100	< 1 mo > 1 mo	^{3}H-T	0/1 1/1	
	1	4	Phenobarbital	1/50-1/100	< 1 mo > 1 mo	^{3}H-T	0/1 1/1	**2007[60]**
Case series	7	11	Phenytoin	50ug/ml	1 mo -4Y	CFSE IFN-γ	7/7 (100%) 5/7 (71.4%)	**2007[63]**
Case report	1	0	Phenytoin	200 µg/ml	2 Wk	^{3}H-T	0/1	
						CFSE	1/1	**2006[34]**
Case series	8	32[c]	carbamazepine	10-200 µg/ml	1-120 mo	^{3}H-T	2/8 (25%)	
	1	32	Oxcabazepine	10-200 µg/ml	1-120 mo	^{3}H-T	1/1	
	1	32	Phenobarbital	10-200 µg/ml	1-120 mo	^{3}H-T	1/1	**2006[64]**
Case series	12	0	carbamazepine	10-100	1-229 mo	^{3}H-T	11/12 (92%)	
	1	0	Oxcabazepine	10-100	23 mo	^{3}H-T	1/1	**2006[14]**
Case series	1	1	Phenytoin	10, 50 µg/ml	19 mo	^{3}H-T CFSE	1/1 1/1	
	1	1	carbamazepine	10 µg/ml	4 mo	^{3}H-T	1/1	**2006[15]**

Type of study	No. of patients	No. of controls	Drug[a]	Conc. (% unless otherwise indicated)	Time[b]	Read-out	Frequency of positive result (%)	Year/ Reference
						CFSE	1/1	
Case series	1	20	Phenytoin	20%	> 6 Wk Aft. Rec	^{3}H-T	1/1	
	1	20	carbamazepine	12.5%	> 6 Wk Aft. Rec	^{3}H-T	1/1	**2005[65]**
Case report	1	0	carbamazepine	NA	29D	NA	0/1	**2003[66]**
Cohort Study	4	8	Lamotrigine	1-100 µg/ml	5-10Y	^{3}H-T	3/4 (75%)	**2003[51]**
Cohort Study	5	8	carbamazepine	1-100 µg/ml	1-72 mo	^{3}H-T	5/5 (100%)	**2003[53]**
Cohort study	2	2	carbamazepine	1-100 µg/ml	N/A	^{3}H-T IL-5	2/2 (100%)	**2002[32]**
Cohort Study	2	>3	carbamazepine	10 µg/ml	6-8 Wks	^{3}H-T	2/2 (100%)	**2001[67]**
Case report	1	0	Zonisamide	3,10 µg/ml	N/A	^{3}H-T	1/1	**2001[68]**
Case series	2	2	carbamazepine	1,10,100 µg/ml	4-6 Wks	^{3}H-T	1/1	**2001[50]**
Case report	1	0	Phenytoin	0.1-1000 µg/ml	101 D	^{3}H-T	1/1	**2000[69]**
Case report	1	0	Phenytoin	N/A	AFT REM	^{3}H-T	1/1	**2000[70]**
Case report	1	1	Phenytoin / Phenobarbital	N/A	3 & 16 D	^{3}H-T	1/1	**2000[71]**
Case report	1	2	Lamotrigine	0.1-100	3-4 mo	^{3}H-T	1/1[d]	**2000[72]**
Case report	1	7	carbamazepine	10	N/A	^{3}H-T	1/1	**1999[73]**

Type of study	No. of patients	No. of controls	Drug[a]	Conc. (% unless otherwise indicated)	Time[b]	Read-out	Frequency of positive result (%)	Year/ Reference
Case series	2	0	carbamazepine	1, 10, 100	N/A	^{3}H-T	1/1	
	1	0	Phenytoin	1, 10, 100	N/A	^{3}H-T	0/1	**1997[37]**
Case report	1	1	Lamotrigine	0.1-50 µg/ml	3Wks & 1 mo	^{3}H-T	1/1	**1997[74]**
Case series	65	21	carbamazepine	5, 10, 15	N/A	^{3}H-T	26/65 (40%)	
	64	21	Oxcarbazepine	5, 10, 15	N/A	^{3}H-T	12/64 (19%)	**1996[55]**
Case report	1	5	carbamazepine	42, 84, 168 µM	2 Wks	^{3}H-T	1/1	**1996[56]**
Case series	1	0	carbamazepine	10, 100	1 mo	^{3}H-T	1/1	
	1	0	Phenytoin	10, 100	1 mo	^{3}H-T	1/1	**1995[75]**
Case report	1	0	carbamazepine	N/A	Aft. Rec	^{3}H-T	0/1	
	1	0	10,11-carbamazepine Epoxide	N/A	Aft. Rec	^{3}H-T	1/1	**1995[76]**
Case report	1	6	carbamazepine	100 µg/ml	N/A	^{3}H-T	0/1?	**1994[77]**
Case series	1	1	Phenytoin	0.01-10 µg/ml	30 D	^{3}H-T	1/1	**1994[78]**
Case report	1	0	carbamazepine	25 µg/ml	N/A	^{3}H-T	1/1	**1993[79]**
Case report	1	0	Phenytoin	N/A	2 mo	^{3}H-T	1/1	**1989[80]**
Case series	9	41	carbamazepine	1-100	1-104 Wks	^{14}C-T	9/9 (100%)	

Type of study	No. of patients	No. of controls	Drug[a]	Conc. (% unless otherwise indicated)	Time[b]	Read-out	Frequency of positive result (%)	Year/ Reference
	6	41	10,11-carbamazepine Epoxide	1-100	1-104 Wks	^{14}C-T	1/6 (16.7%)	
	6	41	Oxcarbazepine	1-100	1-104 Wks	^{14}C-T	1/6 (16.7%)	**1988[58]**
Case report	1	8	carbamazepine	3,10,30 µg/ml	8 Wks	^{3}H-T	1/1	**1984[81]**
Case series	6	10	carbamazepine	3,10,30	13Wk-5Y	^{3}H-T	6/6 (100%)	
	1	10	Phenytoin	3,10,30	13Wk-5Y	^{3}H-T	1/1	**1977[57]**
Case report	1	0	carbamazepine	25 µg/ml	DUR	^{3}H-T	1/1	**1975[61]**
Case series	2	25	carbamazepine	10	2-10 mo	^{3}H-T	1/2	**1971[82]**
Case report	1	3	Phenytoin	20	10 mo	^{3}H-T	1/1	**1964[23]**

[a]: The suspected drug causing the reaction as suggested by at least the medical history.
[b]: Time elapsed between the reaction and the test.
[c]: In this particular study 26.5% of control was positive.
[d]: positive skin patch test
Abbreviations: **^{3}H-T** = ^{3}H-Thymidine incorporation assay, **AC** = anticonvulsant, **Ace =** acetone, **Aft. Rec** = after recovery, **AHS** = anticonvulsant hypersensitivity syndrome, **A.R**. = after remission, **CFSE** = caboxyfluorescein succinimidyl ester dilution assay, **Cr.Tab** = crushed tablet, **DUR** = during, **Eth** = ethanol, **mo** = month, **Meth** = methanol, **Petr** = petrolatum, **Sal** = saline, **Wat** = water, **Wk** = week, **Y** = year.

reactions are attributed to different agents and mediated by distinct immunological mechanisms. For instance, haptens and pro-haptens such as β-lactam antibiotics require processing before they are able to activate T-cells whereas other drugs such as CBZ, lamotrigine (LMG) and SMX and their metabolites are thought to directly activate T-cell through other mechanisms.[86-89]

Nevertheless, in most of the clinical syndromes associated with aromatic anticonvulsant use (e.g., generalized macula-papular exanthema, bullous reactions, multi organ DRESS syndrome, etc.) the LTT is frequently positive.[31] As mentioned earlier, severe bullous reactions such as TEN rarely yield a positive LTT, and the reason behind this is unknown. Romano *et al.*[64] tested 8 patients with a history of hypersensitivity reactions to CBZ (6 patients with macula-papular exanthema (ME), one with bullous exanthema (BE) and one with SJS). All 6 cases with ME yielded negative LTT results despite a positive patch test in 4 of them. On the other hand, the test was positive with the other two cases (BE and SJS). Six ME patients were tested within 2 years of the adverse reaction whereas the BE and SJS cases were tested 6 and 12 years later, respectively.[64]

The LTT detects circulating peripheral drug-specific T-lymphocytes. Such aromatic anticonvulsant reactive T-cells have been cloned and characterized in multiple previous studies.[14, 15, 51, 53, 63, 90] If, for whatever reason, the pathophysiology of the disease does not involve a high frequency of circulating drug-specific T-cells, the LTT will not confirm the diagnosis. The mediocre sensitivity of the LTT in the diagnosis of AHS (around 70%) has provoked uncertainty about the real pathophysiology of this disorder and whether it actually exists. There is increasing evidence on the heterogeneity

of AHS reactions and that they might be mediated by distinct yet unidentified pathophysiology mechanisms.[43, 44, 91, 92]

Recognition of the suspected drug by the isolated peripheral lymphocytes is another dilemma as antigen processing does not seem to be required for T-cell activation *in vitro*. This can be partially explained by a relatively recently introduced paradigm, the "p-i" concept. This term stands for "direct **p**harmacological interaction of drugs with **i**mmune receptors" and assumes that a chemically inert drug can non-covalently interact with receptors on the immune cells and activate them without being a full antigen.[87] This interaction that involves only T-cells may explain why drugs such as CBZ elicit only T-cell-mediated adverse effects while haptens (β-lactam antibiotics) are able to cause all sorts of idiosyncratic reactions including anaphylaxis. In fact, some investigators have found the LTT to be useless in the diagnosis of reactions such as pneumonia caused by minocycline, bucillamine, amoxicillin and clindamycine[93-95] or hepatitis due to herbal medicines.[94] This probably is because these reactions were mediated by other mechanism(s). However, in cases with multiple organ involvement including liver dysfunction, the LTT is more likely to yield positive results.[72] This suggests that several different immunological mechanisms underlie the apparent clinical manifestations.

3.3.1.3. The specific drug.

The LTT has given positive results with most aromatic anticonvulsant drugs including DPH, CBZ, oxcarbazepine (OCBZ), PHB, LAM and zonisamide (ZIM) with sensitivity ranging between 25% and 100%.[14, 53, 55, 63, 68, 96] However, issues related to the chemistry and pharmacology of the tested drug may limit the outcome of the LTT. One such problem may be the solubility of the tested drug in the incubation medium. The

majority of the lipophilic aromatic anticonvulsant are not water soluble and require solubilisation in an organic solvent (e.g., dimethyl sulfoxide, ethanol or propylene glycol). It is important to ensure that the final concentration of the solvent in the medium is not cytotoxic. In addition some researchers found that it is necessary to sonicate the drug solution to enhance its solubility.[60]

In addition, some drugs may cause non-specific activation or deactivation of PBMCs resulting in false positive or false negative LTT results. Higher concentrations of CBZ or DPH cause cytotoxicity and kill PBMCs, an effect that may mask any expansion of lymphocytes, resulting in low SI levels (false negative). Therefore it is essential to examine the effects of the used drug concentration on cells stimulated by the non-specific mitogen PHA.[31]

The effect of coadminstrated systemic corticosteroids on test results is controversial. Although some researchers state that systemic administration of more that 0.2mg/kg of prednisolone may interfere with the LTT,[31] others have found no such effect.[60]

3.3.1.4. Test procedure and read-out system.

The most evident pitfalls of this *in vitro* diagnostic test are its complicated procedure and lack of standardization.[31] Attempts to simplify the test procedure and improve its reproducibility have been described for decades[32, 34, 37, 58, 62] however, the long-sought simple and reproducible LTT is not yet achievable. The common challenge among aromatic anticonvulsants is their need to be enzymatically activated to more reactive metabolites to elicit their presumed HS reactions.[42, 49] This observation has led

some researchers to use liver microsomes to increase the test sensitivity.[97] Others have also used *ex vivo* serum from healthy volunteers taking the drug.[78] However, these approaches did not improve the sensitivity of the test.

Another important aspect of the LTT is the read-out method. Traditionally T-cell proliferation is measured by [^{3}H]thymidine uptake which has proven to be very reproducible. Other methods to detect T-cell activation including such synthesis and release of IL-5, IL-10, IFN-γ and CD69 have also been used and shown to improve the sensitivity of the test.[62, 98] Other workers have also used increased secretion of soluble Fas ligand (sFasL) as a read-out or biomarker and found this protein to be significantly increased in patients with CBZ-induced blistering diseases (SJS/TEN).[13, 99] Granulysin, a cytolytic and proinflammatory protein excreted by activated cytotoxic T lymphocytes (CTLs), natural killer (NK) and NKT cells. [100] It was shown to be a key mediator of keratinocyte apoptosis in severe bullous reactions and to be present in high concentrations in blister fluids in patients with CBZ- and DPH-induced SJS and TEN.[101] Expression of such mediators can be of clinical value in diagnosis or determining the prognosis of drug-induced bullous reactions

3.3.2. Lymphocyte toxicity assay (LTA):

The use of the LTA in diagnosing AHS dates back to the early 1980s.[40] However, except for 4 major studies,[1, 102-104] the lack of large scale application is quite obvious. Shear and Spielberg[1] studied 53 patients with a medical history suggesting AHS due to DPH, CBZ or PHB as well as 49 unexposed healthy controls and 10 DPH-exposed healthy controls. Symptoms included fever, skin rash (varied in severity from generalized exanthema to TEN), eosinophilia, atypical lymphocytosis and internal organ

involvement (liver, kidney, thyroid or lung). The performance of the LTA as a diagnostic test in this cohort of patients was excellent, with only 2 false positive and one false negative results in patients with hypersensitivity reactions to PHB. Naranjo *et al.*[104] tested 51 patients with highly likely diagnosis of AHS to DPH, CBZ or PHB and estimated the sensitivity and specificity of the LTA to be 99% and 75%, respectively. However, only 8 drug-tolerant patients were included in the study, rendering precise determination of the specificity of the test impossible. On the other hand, the lack of a gold standard against which results of the LTA can be validated has always made these numbers merely an estimate.

In another study by Neuman and colleagues the LTA was used to diagnose AHS in 86 patients, 62 of them developed an adverse reaction as a result of taking aromatic anticonvulsants.[102] Although the inclusion criteria for the cases were not well defined, the sensitivity and specificity of results of the LTA in the diagnosis of AHS were estimated to be 98% and 89%, respectively and the positive and negative predictive values were found to be 90% and 64%, respectively. To evaluate cross reactivity among old aromatic anticonvulsant (DPH, CBZ and PHB) and zonisamide (ZNS), a new aromatic anticonvulsant, Neuman *et al.*[103] tested 20 AAC-hypersensitive patients and 20 AAC-tolerant patients using the LTA. Tested patients had exhibited a broad spectrum of AHS manifestations including fever, skin rash, internal organ involvement, SJS and TEN). The authors estimated the sensitivity and specificity of LTA in this cohort of patients to be 92.9% and 99.1, respectively.

In a technician blinded, hospital based controlled study, Dwivedi *et al.*[105] tested 11 patients with AHS to DPH. Five patients had SJS, 4 had erythrodermic eruption, 1 had

morbilliform eruption and 1 lichenoid eruption. The authors also tested 11 healthy and DPH-tolerant volunteers as controls. All 11 patients gave positive LTA (% of cell death ranged between 7% and 26% as compared to 2.6% and 3.5% for controls). It is of note that in this study cells from patients with a severe form of AHS (SJS) exhibited a higher percentage of cell death (12-26%) than milder forms (7-13%) and that was related to a greater deficiency in expression or effectiveness of detoxifying enzymes.

The LTA is simpler and has a less complicated procedure than the LTT, not requiring radioactive reagents. However, the sensitivity and specificity of the LTA in determining the culprit drug in AHS have not been extensively evaluated, mainly due to a lack of strict inclusion criteria in the reported cases. A systemic rechallenge could be a definitive proof of drug culpability but this is ethically unacceptable because of the possibility of danger to the patient.

3.4.2.1. Technical considerations for the LTA

As outlined earlier, the basic principle of the LTA is to generate *in tube* the presumed cytotoxic metabolite(s) of the suspected drug in the presence of the surrogate cell model and to measure lymphocyte susceptibility to metabolite-induced cell death. This process depends largely on the test procedure and reagents used. Table (3) summarises the different steps to be considered in optimizing the performance of the LTA.

For example, contamination of isolated PBMCs with platelets can affect lymphocyte function and activity *in vitro* [109, 110] and may also modify the evaluation of cell death by the MTT method. Platelets have unusually high numbers of mitochondria (the source of the succinate dehydrogenase enzyme that converts MTT to blue formazan dye) and their

presence in the medium can compromise the signal (unpublished data). A number of methods have been introduced to prepare platelet-free PBMCs, [109, 111-114] although this increases the cost and complexity of the test. On the other hand, the usage of platelets as a surrogate model in place of PBMCs could be advantageous as platelets are easier to isolate and more abundant in peripheral blood than PBMCs. They were found to respond with the same way as PBMCs to different chemical insult and platelets from drug hypersensitive patients were found to be more susceptible to cell death induced by the suspected drug than platelets from healthy volunteers who have never exposed to the drug (under preparation).

In addition, the *in vitro* metabolic activation system (liver microsomes) plays an important role in the success of the test especially when testing drugs such as those aromatic anticonvulsants whose toxic metabolites are still unknown. Differences among species in terms of metabolic activation of drugs by CYP isozymes[115] and their relative levels expression in untreated and induced microsomal systems should be considered when performing the LTA. The incubation conditions of the surrogate cells with the suspected drug and liver microsomes should be standardized. The pH of the medium have a tremendous effect on the viability of PBMCs (unpublished data), therefore, the pH should be adjusted to 7.2-7.4 just prior to performing the test. Other additives such as fetal bovine serum (FBS) are also essential to the viability of the cells and should be standardized.

Table II. Summary of data from original work that used lymphocyte toxicity assay (LTA) to investigate idiosyncratic reactions to aromatic anticonvulsants.

Type of study	No. of patients	No. of controls	Drug[a]	Concentration	Time[b]	Liver microsome species	Induction with phenobarbital	Endpoint measurement	Frequency of positive result (%)	Year/ Reference
Cohort study	20	20	Phenytoin Carbamazepine Phenobarbital Zonisamide	N/A	N/A	mouse	+	MTT	SEN=92.9%	2008[103]
Case report	1	1	Lamotrigine	0-32 µg/ml	2 mo	N/A	?	N/A	**1/1**	2006[47]
Case series	11	11	Phenytoin	0-32 µg/ml	N/A	mouse	+	TB	11/11 (100%)	2004[105]
Case report	1	0	Phenobarbital	0-32 µg/ml	70 D	mouse	+	TB	1/1	2004[106]
Cohort study	62	24	Phenytoin Carbamazepine	N/A	N/A	mouse	+	MTT	SEN=98%	
	62	24	Phenobarbital Lamotrigine	N/A	N/A	mouse	+	TB	SEN=91%	2000[102]
Cohort study	51	0	Phenytoin Carbamazepine Phenobarbital	N/A	N/A	mouse	+	TB	49/51 (96.1%)	1994[104]
Case report	1	6	Carbamazepine	50 µmol/L	N/A	mouse	+	TB	1/1	1994[77]
Case series	3[c]	59	Phenytoin	62.5 µmol/L	N/A	mouse	+	TB	3/3 (100%)	1991[107]
Case series	7	17	Carbamazepine	50 µM	N/A[d]	Human	-	TB	7/7 (100%)	
	1	17	Carbamazepine Oxcarbazepine	50 µM	N/A	Human	-	TB	1/1	1991[108]
Cohort study	34	59	Phenytoin	62.5 µM	N/A	mouse	+	TB	34/34 (100%)	
	25	59	Carbamazepine	62.5 µM	N/A	mouse	+	TB	21/25 (84%)	

Type of study	No. of patients	No. of controls	Drug[a]	Concentration	Time[b]	Liver microsome species	Induction with phenobarbital	Endpoint measurement	Frequency of positive result (%)	Year/ Reference
	22	59	Phenobarbital	62.5 µM	N/A	mouse	+	TB	21/22 (95.5%)	1988[1]
Case series	2	20	Phenytoin	31-125 µmol/L	After recovery	mouse	+	TB	2/2 (100%)	1986[39]
Case series	3	17	Phenytoin	31, 62, 125 µM	After recovery	mouse	+	TB	3/3 (100%)	1981[40]

[a]: The suspected drug causing the reaction as suggested by at least the medical history.
[b]: time elapsed between the reaction and the test.
† : In this particular study 26.5% of control was positive.
[c]: siblings from the same family.
[d]: only two patients were tested acutely and there was no significant difference.
Abbreviations: E.P.= end point measurement, Ind.= induction with phenobarbitone, M = month, MIC Sp= liver microsome species, TB= trypan blue, Wk = week, SEN = Sensitivity, Y = year.

Table (3): Opportunities for technical improvement of the lymphocyte toxicity assay (LTA).

Isolation and purification of surrogate model cells (PBMCs)
Reduction of the number of contaminating platelets
Use of platelets as a surrogate model
The metabolic activation system
Species difference in liver microsomes metabolic activity (human vs animals).
Use of induced vs. non-induced microsomes
Incubation conditions
Incubation time
Buffers and additives
End-point and measurement methods
Method of measurement of cell death
Other end-points (e.g., expression of other cell injury markers)

PBMCs=Peripheral blood monocytes

Cell death is the ultimate response of vulnerable cells exposed to the offending drug as an insult. However, it is a gross measure of the cellular and sub-cellular events that occur and may vary depending on the tested drug and the incubation conditions.[116] In this regard, cell death is unlikely to be the best end point to indicate cell response to a toxic insult. Finally, defining and obtaining the ultimate *in vivo* toxic metabolite(s) of the offending drugs and their direct *in vitro* testing will have a strong impact on the predictability of the results of the LTA as the complicated metabolic activation step(s) will be eliminated. This has been proven to be true in testing patients hypersensitive to sulphonamides where the reactive metabolites can be tested directly to assist in test validation.[117]

3.4. Discussion

AHS is a rare but potentially lethal disorder. One of most challenging aspects of this disease is the difficulty to establish solid diagnosis in a timely manner.[2, 8, 118-121] Lack of or misdiagnosis may result in increased morbidity, mortality and extended hospitalization.[122, 123] Ten to 27% of patients with epilepsy discontinue their first anti-epileptic drug because of the development of adverse reactions.[124] Aromatic anticonvulsant drugs such as DPH, CBZ, PHB and LMG are linked to a relatively high risk of for development of hypersensitivity reactions.[125] CBZ was found to be the commonest cause of severe forms of AHS (i.e., SJS and TEN).[126]

The diagnosis of AHS entails two main processes: first, establishing diagnosis of the hypersensitivity reaction, usually from a series of clinically similar differential diagnoses, and second, identifying the culprit drug, potentially among a number of other

concomitantly prescribed innocent ones. Numerous diagnostic tests are available and have been attempted for the diagnosis of drug hypersensitivity reactions; however, their epidemiological qualities are dependent on the type of reaction (immediate versus delayed reactions) and type of drug, and choosing the best test for a specific drug or drug class can be quite challenging.[7, 8, 37]

The LTT and LTA are two different approaches for AHS diagnosis and may complement each other as a battery of diagnostic tests that can also include *in vivo* tests such as the patch test and systemic rechallenge. The LTA may predict the susceptibility of patients to develop AHS based on a genetic deficiency in their cellular defence systems against toxic reactive drug metabolite(s), whereas the LTT may confirm the development of AHS by detecting peripherally circulating drug-specific T-cells. It is evident that these tests lack standardization and large scale validation to determine their appropriations in terms of sensitivity and specificity in addition to positive and negative predictive values.[31, 37, 48]

The sensitivity and specificity of the LTT in diagnosis of drug allergy has been estimated to range from 56% to 78% and from 85% to 93%, respectively although these estimates are generally based on cases of allergy to β-lactam antibiotics and cannot be extended to other types of drugs.[37, 59] In the diagnosis of AHS due to aromatic anticonvulsants (AACs) the LTT has frequently shown a sensitivity between 71% to 100%[14, 51, 53, 57, 58, 62, 63, 67] but this range also as low as 19% to 40%.[55, 64, 65, 76] Estimates of specificity, however, seem to be quite good (close to 100%).[51, 53] Nevertheless, one must always keep in mind that these estimates have been calculated in the absence of a

diagnostic gold standard, which may explain the considerable variability in these numbers.

AHS has a broad range of clinical manifestations[127] reflecting differences in the underlying pathophysiology.[91, 128] If the LTT is able to detect circulating drug-specific T-cells, it is logical to expect that other "non-T-cell-mediated" reactions will not be detected using this approach. Ironically, the LTT gave positive results with IgE-mediated type I reactions which also imply a role for T-cells in these types of reactions.[67] *In vitro* tests detecting antigen-specific IgE antibodies are also available and might be expected to be more sensitive for these types of reactions.[8]

Reviewing publications on the use of the LTA as a diagnostic tool for AHS has revealed a range of sensitivity between 85 to 100% with well documented AHS cases, with satisfactory negative and positive predictive values. The LTA has also been shown to possess good sensitivity in cases involving AHS due to sulphonamides[117, 129-131] and valproic acid.[132]

3.5. Conclusion

Analysis of peripheral blood lymphocytes has been a "promising" diagnostic tool for HAS for several decades. It appears that without further understanding of the mechanisms underlying the pathophysiology of AHS and how specific drugs and metabolites differentially affect these mechanisms that the development of more reliable tools for AHS diagnosis will be compromised. Consequently, in the absence of further research the predictability of these tests will remain questionable and they are unlikely to be utilized on a large scale.

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Chapter 4: Predictive Value of the Lymphocyte Toxicity Assay in Diagnosis of Drug Hypersensitivity Syndrome

This chapter has been published previously:

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4.1. Introduction

The term adverse drug reaction (ADR) is defined as any noxious and unintended response to a drug that occurs at a dose normally used in man for prophylaxis, diagnosis or therapy.[1] Most ADRs are predictable, dose-dependent and related to the pharmacological action of the drug (Type A), accounting for 75-80% of all reported ADRs. The remaining 20 to 25% are defined as Type B-ADRs which are unpredictable, unrelated to the known primary pharmacological action of the drugs and do not have a clear dose-dependency.[2, 3] As they are patient-specific and unpredictable this type is also titled idiosyncratic drug reactions (IDRs). Typically IDRs occur in a small fraction of patients at the normal therapeutic dose and are unrelated to the primary pharmacological action of the drug.[4] Unfortunately, the underlying mechanisms of IDRs are poorly understood although a large portion of IDRs are believed to be immune-mediated, at least in part, as delay in onset and rapid occurrence upon rechallenge are typical.[5] Drug hypersensitivity syndrome (DHS) is considered one type of immune-mediated IDRs, characterized by constellation of signs and symptoms that develop in susceptible patients following exposure to certain drugs.[6] It is most commonly associated with the use of NSAIDs, aromatic anticonvulsants (AACs), antibiotics and sulfonamides antimicrobials.[7]

It has always been a challenge to establish the diagnosis of DHS due to lack of a safe and reliable diagnostic test and because of its variable clinical picture. Another challenge is the fact that, with the exception of fixed drug eruption (FDE), it can be mimicked by other non-drug related illnesses (e.g., bacterial or viral infections).[4, 8, 9] In addition to the importance of identifying susceptible patients to establish safe clinical

practice it is essential not to falsely label a patient as hypersensitive to a drug which could be otherwise therapeutically useful. This is especially important in cases where alternative therapy is more risky or less effective (e.g., first line therapy for epilepsy or allopurinol for gout). This warrants the development of safe and reliable test to confirm diagnosis and identify the culprit drug.

We have recently performed systematic reviews to evaluate the usefulness of different *in vivo* and *in vitro* diagnostic tests in the diagnosis of DHS due to aromatic anticonvulsant drugs.[10, 11] These tests included the skin patch test (PT), the lymphocyte transformation test (LTT) and the lymphocyte toxicity assay (LTA). It was quite evident that the negative and positive predictive values of these tests were not clear, their usefulness in clinical practice is controversial and that more research is needed to prove or disprove their clinical usefulness. Systemic rechallenge or provocation test is considered the 'gold standard' in diagnosis of DHS. However, this approach is not always ethically plausible due to potentially severe reactions that may develop.[8, 12, 13]

The lymphocyte toxicity assay (LTA) was first developed by Spielberg and colleagues in the 1980s to investigate patient susceptibility to DHS [14-17]. The test is based on the concept that DHS develops as a result of imbalance between activation (toxication) and detoxication of drugs *in vivo* (the reactive metabolite hypothesis).[18] This hypothesis has provided partial explanations on how some patients are 'genetically' predisposed to develop such reactions and others are not. Genetic polymorphism in enzymes involved in both activation and detoxifications of drugs has been demonstrated in DHS patients and healthy controls.[19-21] The test includes isolation of peripheral blood lymphocytes (peripheral blood monocytes, PBMCs) from blood samples from patients and healthy

controls and incubation of these lymphocytes with the culprit drug for 2 hours at 37°C in presence of metabolic activation system (e.g., rat liver microsomes) in suitable media. After overnight recovery period at 37°C cell viability is then measured using different methods (Trypan blue exclusion method or MTT[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method).[14, 22] It has been demonstrated that cells from hypersensitive patients are more susceptible to cell death induced by incubation with the culprit drug than cells from healthy controls. This observation has found a clinical application for the diagnosis of DHS and identifying the culprit agent usually among a group of suspected drugs.[17, 23, 24]

The main obstacle that hinders accurate determination of predictive values of this test is lack of a safe gold standard test to measure it against. As a result, presently there is very little clinical data to confirm the LTA after re-exposure.[10] In an attempt to close this gap we performed this cohort study on a group of patients who had been tested using the LTA for susceptibility to DHS. We aimed at identifying individuals who might have deliberately or inadvertently been exposed to the culprit drug(s) after having been tested. Our objective was to use these cases of re-exposure to calculate the negative and positive predictive value of the test.

4.2. Methods

4.2.1. Patient recruitment and data collection

One hundred and forty seven patients were included in the study. These patients had developed hypersensitivity reactions to different drugs and were tested using LTA between 1991 to 2008 in two Drug Safety Clinics [Sunnybrook Health Sciences Centre (SHSC), Toronto, ON and the Children Hospital of Western Ontario (CHWO), London,

ON, Canada]. The drugs assessed included aromatic anticonvulsants (phenytoin, carbamazepine, phenobarbitone, lamotrigine), valproic acid, sulfonamides (sulfamethoxazole, sulfasalazine and sulfapyrazine), β-lactam antibiotics (amoxicillin, and cefaclor), macrolide antibiotics (erythromycin and clarithromycin), tetracycline, codeine and accutane. Subjects were identified from their medical records at the two locations. The inclusion criteria were: (1) the patient developed DHS as evaluated by the clinic specialist; (2) the patient was tested for susceptibility to DHS using the LTA; and (3) consent was obtained from the patient or parent (in case of children) to participate in the study. Patients were excluded from the study if their files did not contain sufficient information (contacts or LTA results) or they did not consent.

Ethical approval was obtained from the Research Ethics Boards of SHCS and the University of Western Ontario and information letters were sent to each potential participant prior to contacting them. Verbal consents were obtained from participants or their parent (in cases of children patients) prior to inclusion in the study.

4.2.2. Preparation of rat liver microsomes

Adult Sprague Dawley rats (mean weight 200 g) were sacrificed by decapitation and their livers were quickly isolated under aseptic condition and washed with ice cold 0.5 M potassium phosphate homogenization buffer. Livers were then diced with scissors and homogenized in 3 volumes homogenization buffer using drill powered Potter-Elvehjem homogenizer. Homogenate were then centrifuged at 9000 g for 30 min at 4°C. Supernatants were then centrifuged at 100,000 g for 1 hr at 4°C. The pellets were then resuspended in homogenization buffer and stored at -80°C until used. Microsomal protein contents were determined by the method of Lowry et al.[25]

4.2.3. Blood collection and isolation of PBMCs

Blood samples from patients and healthy volunteers were obtained by venepuncture, collected into heparinized syringes and separated immediately. To isolate lymphocytes samples were diluted 1:1 with phosphate buffered saline (NaCl, 137 mM; KCl, 2.7 mM; NaH_2PO_4, 10 mM; KH_2PO_4, 2 mM; pH of 7.4) and layered on a Ficoll® - paque density gradient. Gradients were then spun at 500 g for 20 minutes, the aqueous-Ficoll interface layer was collected, washed twice with PBS and cell density was adjusted to 1×10^6 cell per ml in 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline (HEPES, 15 mM; NaCl, 125 mM; KCl, 6 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 1.0 mM; $CaCl_2$, 1.0 mM; glucose, 10 mM;pH 7.4). At this stage cell viability is determined using trypan blue exclusion method and is always greater than 95%. All steps are performed in an aseptic condition in a laminar flow hood.

4.2.4. The lymphocyte toxicity assay (LTA)

The LTA was performed as previously described.[14] Briefly, 100 µl of peripheral blood monocytes (PBMCs, lymphocytes) suspension at density of 1×10^6 cell/ml were placed in each well of 96-flat-bottom multiwell plates. Cells were incubated with the either the drug (at concentrations ranged between 6.5 to 500 µM, aromatic anticonvulsants; beta-lactam antibiotics) or its reactive metabolite (In sulfa cases at concentration range 50 to 800 µM) for 2 hrs in humidified atmosphere at 37°C and 5% CO_2 partial pressure. In experiments where the parent drug is used the metabolic activation system (rat liver microsomes, prepared as described above) was included in the incubation media. Microsomal protein was added at concentration of 0.25 mg/ml followed by addition of the NADPH generating system (NADP, 0.6 mM; glucose-6-

phosphate, 2.4 mM; glucose-6-phosphate dehydrogenase, 2 U/ml). Plates were then spun down at 500 g for 15 minutes and media were replaced with fresh RPMI 1640 media containing 10% FBS and penicillin/streptomycine cocktail. Cells were then let to recover for 18 hrs in humidified atmosphere at 37°C and 5% CO_2 partial pressure. At this point plates were spun down and resuspended in 100 µl HEPES buffer. 25µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 5 mg/ml were added each well and incubated at 37°C for 4 hrs and reactions were stopped by adding 100 µl stop solution (NN-dimethylformamide, DMF, 50%; 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 as generated by seeding the cells at 0, 25, 50, 75 and 100% of cell concentration in the corresponding buffer in quadruplicates. 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. A cut-off value of 15% increase in cell death over base line (control) was used to determine positive tests. Patients were tested either in the lab of MJ Rieder at the University of Western Ontario or in the Drug Safety Clinic at Sunnybrook Health Sciences Centre.

4.2.5. Follow-up interviews

Information obtained from patient medical charts included patient age, gender, drugs tested, signs and symptoms of the reaction and LTA results. Participants were then asked to answer a standard questionnaire through telephone interviews. The questionnaire was designed to identify if the patient was exposed to the drug after being tested using the LTA and to find out the clinical response to the re-exposure. Patients were also asked

whether they have had allergic reactions to any other drugs or any family history of such reactions. The data obtained was analyzed to estimate the test predictive values in the diagnosis of DHS.

4.3. Results

One hundred forty seven patients ranging between 3 and 89 years of age (mean 30.1 year) consisted of 81 (55.1%) female and 66 (44.9 %) male were recruited. The LTA was performed on these patients as following: for AACs (n=124), for sulfa drugs (n=72), for β-lactam antibiotics (n= 76), for macrolide antibiotics (n=10), for ciprofloxacin (n=3), for valproic acid (n=3), for accutane (n=2), and 1for tetracycline and codeine. The LTA results were found to be negative in 179 tests (61.3%) and positive in 113 tests (38.7%). The majority of patients avoided any use of the suspected drugs after the first incidence regardless of the LTA test result. A subgroup of 22 patients had been exposed to a tested drug out of 147 patients studied (15.0%). In total of 26 events of re-exposure 21 took place after a negative LTA and 5 after a positive LTA (Table 1).

The clinical data collected at the first incidences vary among patients, including skin rashes (erythmatous or blistering), fever, edema, GIT, hepatitis, lung diseases, lymphadenopathy and hematological abnormalities (Table 2).

Among the 26 cases of re-exposures in 22 patients 4 were true positive, 17 were true negative, 1 was false positive, and 4 were false negative as determined by syatemic re-exposure . Based on the limited number of re-exposure obtained the negative predictive value (NPV) of the LTA in the diagnosis of DHS vary according to the drug tested. Among 13 cases of re-exposure to β-lactam antibiotics 2 were true positives, 8 were true negatives and 3 were false negatives. This suggests low sensitivity and high

specificity of the test in diagnosis of DHS due to these drugs. On the other hand, sensitivity of the LTA in the diagnosis of DHS due to sulfonamides may be higher as no false negative results were identified among the 7 cases of re-exposure. Five re-exposures to aromatic anticonvulsants revealed 4 true negatives and 1 false negative. The overall sensitivity and specificity of the LTA in the diagnosis of DHS based on our 22 cases are estimated to be 40% and 90%, respectively.

4.4. Discussion

Drug hypersensitivity syndrome (DHS) is a rare disorder with high rates of morbidity and mortality. It represents a challenging clinical problem and increases patient care cost. While early diagnosis of DHS is essential for patient safety, that is not always achievable due to lack of a safe and reliable diagnostic tests. The LTA has been a promising diagnostic tool for such reactions but its clinical value has not been determined. To our knowledge this is the first study to use a 'gold standard' test (systemic re-exposure) to validate the LTA. All previous attempts were based on correlation of suspected clinical manifestations and history of exposure with the LTA test. Clinical manifestations of DHS are quite variable and it is often difficult to associate a typical clinical picture to this disease.[26] Furthermore, temporal relationship between administration of the suspected drug and development of the disorder is known to be of poor predictive value especially in cases where multiple drugs are used.[14]

Previous studies have attempted to measure the diagnostic value of the test. Naranjo et al. (1994) evaluated the performance of LTA in diagnosis of drug hypersensitivity reactions using a Bayesian statistical instrument to determine causality

Table (1): Characteristics of patients with drug hypersensitivity syndrome (DHS).

Characteristics	Value
Sex (female/male;n[%]	81/66 [55/45]
Age (mean;y[range])	30.1 [3-89]
Type of reaction	
Skin rash only	45 [31]
Systemic involvement	83 [56]
None [a]	19 [13]
Drug tested [b]	
Sulfonamides	72 [25]
B-lactam antibiotics	76 [26]
Aromatic anticonvulsants	124 [43]
Others	20 [7]

a: These are either relatives to patients or their clinical data are unavailable.

b: patients may be tested for more than one drug.

Table (2): Characteristics of systemically re-exposed patients, their lymphocyte toxicity assay (LTA) results and results of re-exposure.

Patient no.	Age (y)	Sex	Drug tested	LTA result	Re-exposure
1	15	male	Amoxicillin	-	+
2	9	male	Amoxicillin	-	+
3	12	Female	Sulfamethoxazole	-	-
4	18	Female	Sulfamethoxazole	-	-
5	66	Female	Sulfamethoxazole	-	-
6	63	Female	Amoxicillin	-	-
7	23	Female	Cefaclor	+	+
8	62	Female	Sulfamethoxazole	+	+
9	10	Female	Amoxicillin	-	-
10	25	male	Sulfamethoxazole	+	-
			Sulfamethoxazole	-	-
11	17	male	Cefaclor	-	-
12	17	male	Cefaclor	-	-
13	13	Female	Amoxicillin	-	-
14	22	male	Accutane	+	+
15	9	male	Cefaclor	+	+
16	12	male	Amoxicillin	-	+
17	52	Female	Phenobarbital	-	-
18	14	male	Phenytoin	-	-
19	82	Female	Carbamazepine	-	-
20	14	male	Phenobarbital	-	+
			Lamotrigine	-	-
21	8	Female	Amoxicillin	-	-
22	11	Female	Sulfamethoxazole	-	-
			Cefaclor	-	-
			Amoxicillin	-	-

+ indicates a positive test result; - indicates a negative test result

on 51 patients with suspected drug reactions. They estimated that LTA has a specificity of 75% and sensitivity of 99% in their studied cases. However, their study still lacked a 'gold standard' measure such as systemic rechallenge.[27] In another study to validate the LTA in diagnosis of DHS Neuman et al. (2000) studied 86 patients with suspected reactions to sulfamethoxazole (SMX), 62 to anticonvulsants and 26 healthy volunteers. They estimated the negative predictive value of the test to be 64% and the positive predictive value to be 90% with sensitivity and specificity of 98% and 89%, respectively.[22] However, no re-exposure data were available. In cases due to sulfonamides we found that the test has high NPV. This is possibly due in part to the simplified procedure to test for SMX which does not include *in vitro* metabolic activation. The toxic metabolite of sulfa drugs has been identified and synthesized and was found to be adequately stable to be used experimentally for the *in vitro* testing.[28] This step has provided a more simplified and apparently more sensitive test. The PPV of LTA in highly suspected cases of DHS due to SMX has been estimated previously to be between 80% and 90% [29, 30]. However, in these studies the parent drug not the metabolite was used in the *in vitro* toxicity testing. In our study, for drugs such as β-lactam antibiotics (e.g., amoxicillin and cefaclor) however, the NPV was low which may be explained by the more complicated pathophysiology of hapten-mediated reactions caused by these drugs.[31] Previous studies have suggested that the performance of LTA in cases of hypersensitivity to cefaclor depends largely on the type of reaction.[32] Due to the retrospective nature of our study it was not possible to re-evaluate the patients clinically or to classify them according to the type of reaction. Having included cases in our study

with mixed types of reaction could be the cause for the relatively low NPV of LTA in β-lactam antibiotics cases.

The low sensitivity of the LTA test in β-lactam antibiotic cases is probably due to the complicated procedure of the test when using the metabolic activation system for drugs that need to be metabolized to a reactive (cytotoxic) metabolite(s). This is further supported by the good sensitivity of the test among SMX-induced cases.

Considering the lack of complete understanding of the pathophysiology underlying DHS, it is difficult to speculate on how patient cells would react with the suspected drug *in vitro*. Drugs such as β-lactam antibiotics are known to cause different types of reactions and they are classical hapten forming agents.[33] Different molecular mechanisms and requirements are thought to underlie each type of these reactions (i.e., Cell-mediated vs IgE-mediated) and some of them may not be detectable by the *in vitro* LTA test.[6] Furthermore, the variable clinical picture of the DHS may lead to difficulty in identifying affected patients. Among our cases 30.6% developed type of skin rash only and 56.5% developed other systemic signs that included fever, hepatitis, respiratory diseases, hematological abnormalities, gastroenteritis, facial edema, anaphylaxis, angioedema, lymphadenopathy and serum sickness-like reactions.

It is well established that 50% of all drugs in common use may cause some type of skin rash and that, in addition to fixed drug eruption, exanthematous (morbilliform, maculopapular or scarlatiniform) and urticarial rashes are the more common cutaneous adverse reaction.[34] Exanthematous and pustular eruptions are more commonly associated with DHS as oppose to urticarial rash, which is usually caused by immediate type (type I) hypersensitivity reactions. The pathophysiological mechanisms of both

types of reactions are thought to be different.[6] Taken together, one may expect that the LTA can have different predictive value in cases of both types of reactions; however, positive LTA tests were observed in this cohort in cases with all spectrums of cutaneous reactions (data not shown). This test detects the vulnerability of patient cells to the drug's reactive metabolite(s). According to the reactive metabolite hypothesis, drug activation is a pre-requisite for DHS to develop and this could be a common step in the cascade of events that lead to the development of different type of reactions. This is one of the LTA potential advantages; it detects the genetic susceptibility of the patient to develop DHS, thus the test can be also used for patient screening prior to prescribing a potential causative drug in high-risk populations.

Although the number of re-exposed cases was relatively low to allow comparative statistics, our data provided a fair evaluation of the test performance in the diagnosis of DHS based on the definite power of systemic re-exposure. There is no doubt that better controlled prospective studies using more defined inclusion criteria are required to decide on the true clinical value of this test; however, there are several technical and ethical obstacles that should be overcome before such studies can be conducted.

We have demonstrated anther critical aspect of the LTA that deserve discussion. From our data, it is evident that in most cases (88.3%) patients avoided reusing the suspected drug (as probably per their doctors' advices) despite reassuring negative results. This finding strongly suggests a situation of 'mistrust' of the clinical value of the test which is a direct result of the lack of evidence-based data on its predictive value. It is unlikely that a diagnostic test with unknown predictive value will contribute to a better clinical practice.

The LTA can be a valuable diagnostic tool for patient susceptibility to DHS. Although the currently used procedure is quite complex and requires specialized experience. Further research may yield a more simplified test that is adaptable for wide clinical use. Such research is driven by the urgent need for a safe and reliable diagnostic test for DHS.

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Chapter 5. The *In vitro* Platelet Toxicity Assay (*i*PTA): a Novel Approach for Assessment of Drug Hypersensitivity Syndrome.

This chapter has been published previously:

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5.1. **INTRODUCTION**

Adverse drug reactions (ADRs) account for 5% of all hospital admissions and occur in 10-20% of hospitalized patients.[1,2] Most ADRs (85-90%) are predictable, dose-dependent and related to the pharmacological action of the drug (type A), but 10-15% are unpredictable, unrelated to the pharmacological action of the drug and do not have clear dose dependency (type B).[3,4] A major category of the latter type is Drug Hypersensitivity Syndrome (DHS).[5,6] DHS is a rare but potentially fatal disorder that occurs in susceptible patients following exposure to the culprit drug. It is most commonly associated with aromatic anticonvulsants (e.g., phenytoin, carbamazepine, phenobarbital and lamotrigine) and antimicrobials such as sulfonamides.[5-7]

It has been difficult to establish a diagnosis of DHS due to its variable clinical presentation, overlap with other clinical conditions and the often delayed temporal relationship between administration of the culprit drug and the appearance of symptoms.[8] Lack of a reliable and safe diagnostic test plays a major role in the significant morbidity and mortality due to drug hypersensitivity.[9,10] Other than systemic re-challenge with the culprit drug, no gold standard diagnostic test is currently available. Unfortunately, systemic re-challenge is ethically problematic.[11] Currently available *in vivo* and *in vitro* tests are not well characterized and their sensitivity and specificity are unknown.[12,13] Attempts to develop safe and reliable *in vitro* diagnostic tests for DHS have been underway for decades; however, recent systematic reviews of both *in vivo* and *in vitro* diagnostic tests for DHS have documented a paucity of large scale studies to evaluate the usefulness of these tests.[14,15]

The lymphocyte toxicity assay (LTA) is an *in vitro* diagnostic test that was developed three decades ago to investigate patient susceptibility to drug hypersensitivity.[16,17] The usefulness of this test in diagnosing DHS is yet to be determined as its negative and positive predictive values are still unclear.[14] The main disadvantage of this test is the lengthy and complicated method used to isolate peripheral blood lymphocytes. This involves centrifugation of diluted blood samples over a gradient of synthetic high molecular weight polymer of sucrose (Ficoll®) and isolation of a narrow layer that contains lymphocytes, monocytes and a high number of attached and co-sedimented platelets.[18,19] This lengthy process complicates the assay, increases its cost and may contribute to its poor reproducibility.

In an attempt to simplify and improve the LTA procedure, we have explored the possibility to use another blood cell type as a surrogate cell model for the test. We hypothesized that platelets would be a suitable candidate for this role. Platelets are metabolically active non-nucleated cells derived from the cytoplasm of polyploid megakaryocytes. They are 2.0 to 5.0 μm in diameter and 0.5 μm in thickness and circulate in a density of $150\text{-}450\times10^9$ cell/liter in healthy individuals[20] Because of their small size and low density, platelets are very easy to obtain from peripheral blood samples using simple differential centrifugation methods[21,22] In addition to their well known pivotal role in blood homeostasis and thrombosis, a great deal of evidence has recently emerged on the role of platelets in inflammation, allergy and hypersensitivity reactions, as well as having effects on immunity[23-26] Platelets contain active mitochondria and have a complete machinery for apoptosis which suggests that they may be a good model to study cell toxicity.[27-29] Furthermore, being unable to proliferate, platelets

provide an extra advantage in accurately reflecting the degree of cell death upon exposure to chemical insult, an effect that can be masked by cell replication in other proliferating cell types (e.g. lymphocytes).

The present work is the first report on the use of blood platelets as surrogate cells for *in vitro* toxicity testing of drugs. Data described here suggest that using platelets as target cells may be a novel technique to predict the susceptibility of patients to develop drug hypersensitivity.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

The hydroxylamine metabolite of sulfamethoxazole (SMX) was synthesized as described previously,[30] and determined by HPLC, mass spectrometry, and nuclear magnetic resonance to be >99% pure. Carbamazepine, sulfamethoxazole, glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADP), glucose-6-phosphate dehydrogenase (type XV from bakers yeast) and the tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl) 2, 5 diohenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, Ontario, Canada). All remaining reagents were of analytical grade obtained from the usual commercial sources. Drugs were prepared as stock solutions in dimethyl sulfoxide (DMSO) and diluted in the used buffer to obtain the desired working concentrations. The final concentration of DMSO in the media was always less than 0.2 %.

5.2.2. Patients

Patients were selected according to the following criteria: 1) having a medical and clinical history highly suggestive of DHS due to carbamazepine (CBZ) or sulfamethoxazole (SMX) and/or 2) patients that had a positive LTA test to CBZ or SMX.

Blood samples were obtained from 8 individuals: two patients who had developed hypersensitivity reactions (DHR) to drugs as suggested by medical history and clinical manifestations; two patients who had never been exposed to the culprit drugs but were diagnosed to be susceptible to DHR by the conventional LTA; and four healthy volunteers who have denied any history of hypersensitivity reaction to the drugs or prior exposure to the drugs. The patient group consisted of 3 adults (mean age 42.3 years) and a child (9 years) who denied having been on any drug other than the suspected agent. The study was approved by the University of Western Ontario research ethics board and consent was obtained from participants or their guardians.

5.2.3. Blood collection and cells isolation

Blood samples from the patients and healthy volunteers were obtained by venipuncture, collected into heparinized syringes and separated immediately. To isolate lymphocytes, samples were diluted 1:1 with phosphate buffered saline (NaCl, 137 mM; KCl, 2.7 mM; NaH_2PO_4, 10 mM; KH_2PO_4, 2 mM; pH of 7.4) and layered on a Ficoll®-paque density gradient. Gradients were then spun at 500 x g for 20 minutes, the aqueous-Ficoll interface layer was collected, washed twice with PBS and cell density was adjusted to 1×10^6 cell/ml in 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffered saline (HEPES, 15 mM; NaCl, 125 mM; KCl, 6 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 1.0 mM; $CaCl_2$, 1.0 mM; glucose, 10 mM; pH 7.4). At this stage cell viability

is determined using trypan blue exclusion method and is always greater than 95%. All steps are performed in an aseptic condition in a laminar flow hood.

To collect platelets, blood samples were centrifuged at 200 x g for 15 minutes and the platelet rich plasma was then centrifuged at 900 x g for 15 minutes to pellet platelets. Platelets were then washed twice with modified calcium-free Locke's solution (NaCl, 154 mM; KCl, 2.6 mM; K_2HPO_4, 2.14 mM; KH_2PO_4, 0.85 mM; $MgCl_2$, 1.2 mM; glucose, 10 mM; and EGTA, 2.0 mM; pH 7.2) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and cell density was adjusted to 7.5×10^8 cell/ml in calcium-free Locke's solution.

5.2.4. Preparation of rat liver microsomes (RLM)

Adult Sprague Dawley rats (mean weight 200 g) were sacrificed by decapitation and their livers were quickly isolated under aseptic condition and washed with ice cold 0.5 M potassium phosphate homogenization buffer (pH 7.2). Livers were then diced with scissors and homogenized in 3 volumes homogenization buffer using drill powered Potter-Elvehjem homogenizer. Homogenate were then centrifuged at 9000 x g for 30 min at 4°C. Supernatants were then centrifuged at 100,000 x g for 1 hr at 4°C. The pellets were then resuspended in homogenization buffer and stored at -80°C until used. Microsomal protein contents were determined by the method of Lowry et al. (1951).[31]

5.2.5. The *in vitro* toxicity assay

The conventional LTA was performed as described previously.[6] Briefly, 100 µl of peripheral blood monocytes (PBMCs, lymphocytes) suspension at density of 1×10^6 cell/ml were placed in each well of 96-flat-bottom multiwell plates. Cells were incubated

with either the drug in question or its reactive metabolite for 2 hrs in humidified atmosphere at 37°C and 5% CO_2 partial pressure. In experiments where the parent drug was used, the metabolic activation system was included in the incubation media. Microsomal protein was added at a concentration of 0.25 mg/ml followed by the NADPH generating system (NADP, 0.6 mM; glucose-6-phosphate, 2.4 mM; glucose-6-phosphate dehydrogenase, 2 U/ml). Plates were then spun down at 500 x g for 15 minutes and media were replaced with fresh Roswell Park Memorial Institute (RPMI) 1640 media containing 10% FBS and penicillin/streptomycine cocktail. Cells were then let to recover for 18 hrs in humidified atmosphere at 37°C and 5% CO_2 partial pressure. At this point plates were spun down and resuspended in 100 µl HEPES buffer. 25µl of MTT at 5 mg/ml were added to each well and incubated at 37°C for 4 hrs and reactions were stopped by adding 100 µl stop solution (N,N-dimethylformamide, DMF, 50%; sodium dodecyl sulphate, SDS, 20% in DDH_2O) and allowed to set overnight at room temperature protected from light. Absorbance was measured at 590 nm by a Molecular Devices spectrophotometer (Beckman, Palo Alto, CA, USA). A standard curve was generated by seeding cells at 0, 25, 50, 75 and 100% of concentration in the corresponding buffer in quadruplicates. The data were analyzed using the Softmax™ Molecular Devices Group Analytical software version 2.35 and graphs were created using Microsoft Excel™ 2007 software.

The in vitro platelet toxicity assay (*i*PTA) was performed in a similar procedure except that calcium-free Locke's solution was used as a medium in the experiments. Platelets were incubated at density of 7.5×10^8 platelets/ml and plates were centrifuged at 900 x g in each step to pellet platelets.

5.2.6. Microscopic examination of the platelet preparation

Samples from isolated platelets were spun onto glass slides using a bench-top cytospin centrifuge, fixed in absolute methanol for 30 minutes and stained with Wright-Giemsa stain for 20 minutes. They were then washed several times with distilled water, mounted and visualized by phase-contrast under Olympus light microscope using oil immerged objective (100X, 1.3 aperture).

5.2.7. Statistical analysis of data

Data were analyzed using Microsoft Excel^{TM} 2007 software and differences between patients and controls were determined by t-test with Bonferroni correction for multiple comparisons. Values are expressed as percentage of control (vehicle only) and presented as mean ± standard error (SE).

5.3. RESULTS

5.3.1. Determination of platelets viability using MTT method

These experiments were carried out to determine whether cell death can be used as a measurable *in vitro* end point for platelet susceptibility to reactive metabolites. Platelets isolated from blood samples drawn from normal volunteers were incubated with different concentrations (50, 100 and 200 µM) of sulfamethoxazole hydroxyl amine (SMX-HA, the cytotoxic metabolite of SMX) for 2 hrs at 37°C and 5% CO_2. At the same time PBMCs isolated from the same samples were incubated under identical conditions side by side. After incubation, media were replaced with fresh RPMI 1640 media and allowed to recover for 18 hrs. The viability of both platelets and PBMCs was then determined using MTT as described above. SMX-HA induces concentration-dependent cell death in platelets *in vitro* in a similar manner as in PBMCs (figure 1). However, the cytotoxic

metabolite induced more cell death in platelets at 100 and 200 μM (percentage: 18.9±4.5 and 44.5±6.6; respectively, n=12) than in PBMCs (percentage: 5.4±4.3 and 16.7±3.9; respectively, n=12) ($p<0.05$). Platelets were able to convert the yellow tetrazolium to a spectrophotometrically measurable blue formazan. Conversion of the tetrazolium to the formazan depends on the activity of the mitochondrial succinyl dehyrogenase enzyme indicating viability.[32]

To rule out the possibility that our platelet preparations may be contaminated with other types of blood cells (e.g., erythrocytes), we performed microscopic examination of samples from the isolated platelets. Slides from samples taken from platelet preparations were prepared as described above and examined under a light microscope. At least 99.95% of cells observed were platelets confirmed by the characteristic morphology of platelets (figure 1, insert).

5.3.2. Comparison of induction of cell death between PBMCs and platelets from hypersensitive patients and healthy controls

We isolated PBMCs and platelets from 2 patients who had been diagnosed as susceptible to developing hypersensitivity reaction to SMX using the LTA. In parallel blood samples were withdrawn from 2 healthy volunteers with no history of exposure or hypersensitivity reaction to SMX. Platelets and PBMCs from both groups were incubated with increasing concentrations of SMX-HA (0-400 μM) and cell viability was then measured using the MTT method as described above.

Figure (1) Peripheral blood monocytes (PBMCs) (empty circles) and platelets (solid circles) from healthy volunteers were isolated and incubated with increasing concentrations of sulfamethoxazole hydroxylamine (SMX-HA). Cell death was then determined using the MTT method and expressed as percentage of control (vehicle without drug). Values are presented as mean±S.E. of at least 12 replications. Insert: Samples from platelets preparations were spun onto glass slides, fixed, stained with Wright-Giemsa stain and visualized under Olympus light microscope equipped with 100X objective lens (1.3 aperture). The figure shows a representative picture. Arrows indicate small platelet aggregates.*, $p<0.05$.

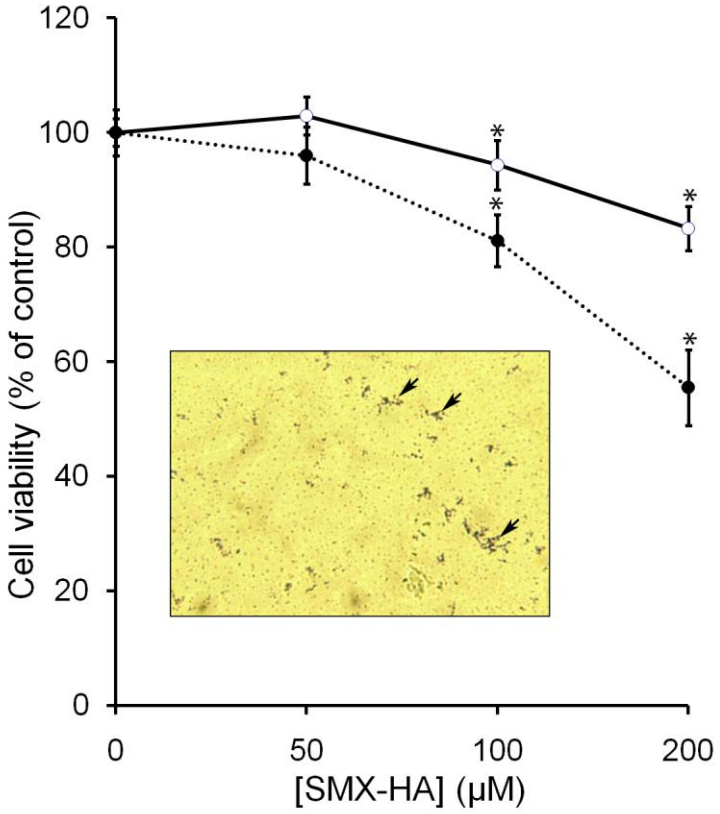
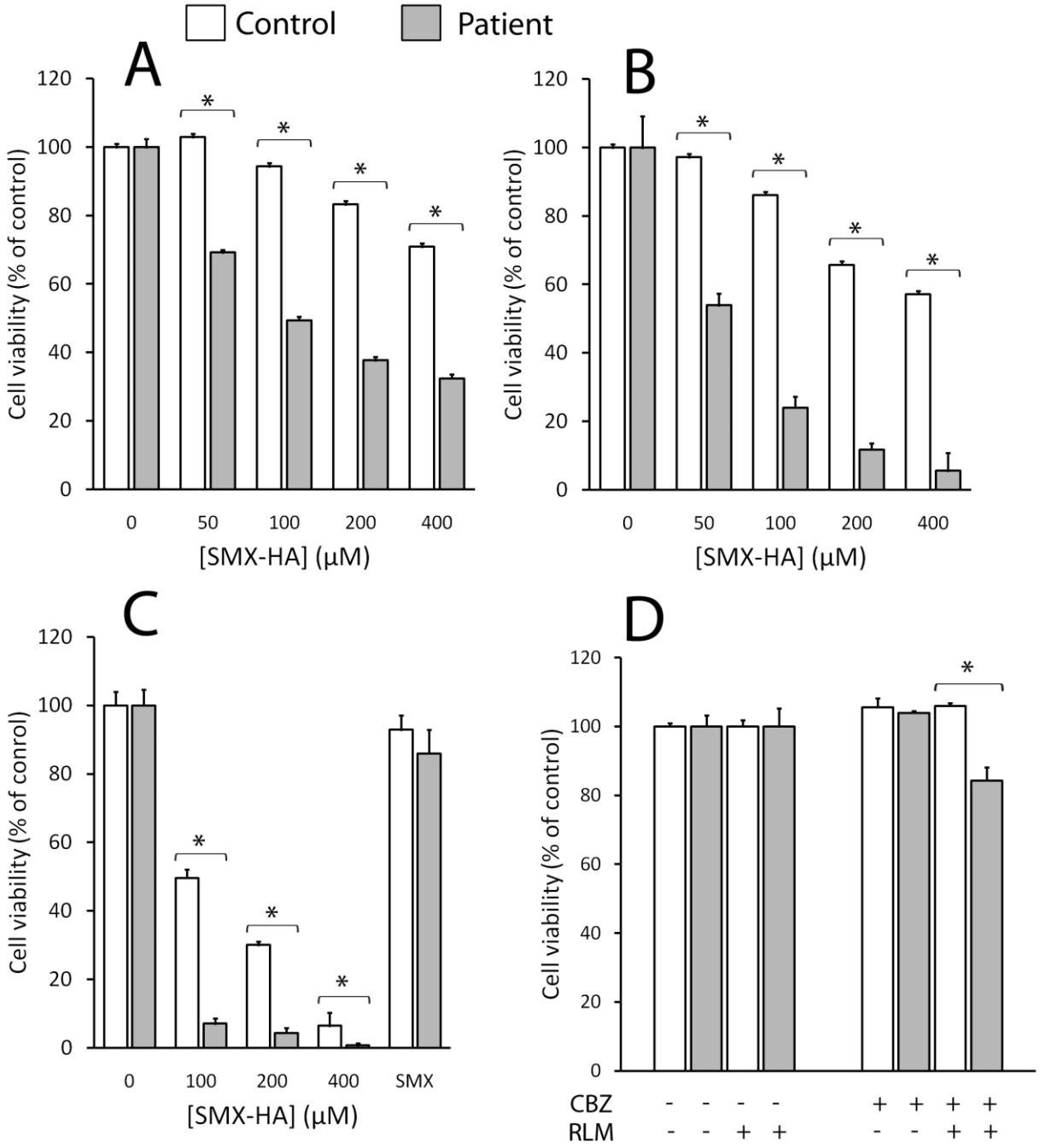

120
100
80
60
40
20
0
Cell viability (% of control)
0
50
100
200
[SMX-HA] (μM)
*
*
*

Figure (2) PBMCs (A) and platelets (B) from a healthy control (white bars) and from SMX-hypersensitive patient (shaded bars) were isolated and incubated with increasing concentrations of SMX-HA and cell death was then determined using the MTT method and expressed as a percentage of control (vehicle without drug). C: Platelets from a healthy control (white bars) and from a clinically suspected SMX-hypersensitive patient (shaded bars) were isolated and incubated with increasing concentration of SMX-HA or 800 µM SMX and cell death was then determined using MTT method and expressed as percentages of control (vehicle without drug). D: Platelets from a healthy control and a clinically suspected CBZ-hypersensitive patient were isolated and incubated with either 0 µM or 250 µM CBZ in presence or absence of rat liver microsomes (RLM). Values are presented as mean±S.E. of at least 6 repitations from each subject. PBMCs, peripheral blood monocytes; SMX, sulfamethoxazole; SMX-HA, sulfamethoxazole hydroxylamine; CBZ, carbamazepine; RLM, rat liver microsomes. *, $p<0.05$.


Control
Patient
A
B
C
D
Cell viability (% of control)
[SMX-HA] (μM)
0
50
100
200
400
SMX
CBZ
RLM
*

As anticipated, incubation of PBMCs from a patient with SMX hypersensitivity induced a concentration-dependent increase in cell death higher than in PBMCs isolated from a healthy volunteer which was statistically significant at all concentrations tested ($p<0.05$) (figure 2A). At 400 μM SMX-HA, for instance, the percentage of cell death was 29.1±1.0 (n=12) in PBMCs from a healthy volunteer vs. 67.6±1.2 in PBMCs isolated from an SMX hypersensitive patient with a difference of around 38.5% ($p<0.05$). When platelets isolated from the same hypersensitivity patient and healthy volunteer were incubated with increasing concentrations of SMX-HA (0-400 μM) the difference in induction of cell death was higher and significant at all concentrations tested ($p<0.05$) (figure 2B). As a comparison to PBMCs the percentage of induction of cell death at 400 μM SMX-HA in platelets from the patients was 94.4±5.2 (n=12) and in platelets from the healthy volunteer was 42.9±1.1 (n=12) with a difference of around 51.5%, suggesting that the platelet test is more sensitive than the conventional LTA.

5.3.3. Response of platelets from a clinically suspected hypersensitive patient

Blood samples were taken from a 58-year old woman who had developed high fever after taking a course of Septra™ (sulfamethoxazole/trimethoprim) in the past and from one healthy volunteer with no sulfonamide exposure. Platelets from the SMX hypersensitive patient exhibited more cell death than platelets from the healthy volunteer (figure 2 C) ($p<0.05$, with 6 samples from each). In fact, at 400 μM SMX-HA almost 100% cell death was observed in platelets from the hypersensitive patient (figure 2 C). In

contrast, incubation of platelets from the patient and control with up to 800 µM SMX (the parent drug) did not induce a significant amount of cell death ($p = 0.4$).

5.3.4. Response of platelets from a carbamazepine (CBZ)-hypersensitive patient to RLM-generated toxic metabolite(s) of CBZ

Platelets from a patient who had clinical symptoms suggestive of hypersensitivity reaction after exposure to CBZ, and from one healthy volunteer, were incubated with 250 µM CBZ in the presence (+RLM) and absence (-RLM) of rat liver microsomes and NADPH-generating system as described above. Each test was repeated on 6 samples to identify intra-test reproducibility. In the patient's platelets there was around 20% increase in cell death in the presence of microsomes and NADPH-generating system compared to platelets from the healthy volunteer (cell viability: 84±3.9 and 106± 0.73; respectively, $p<0.05$, $n=12$) (Figure 2 D). Neither platelets from the healthy volunteer under both conditions (-RLM or +RLM) nor platelets from the patient incubated without microsomes (-RLM) shown any increase in cell death compared to control (vehicle without drug).

5.4. Discussion

The pathophysiology underlying drug hypersensitivity is not well understood. However, several mechanistic frameworks have been introduced including the hapten hypothesis and the reactive metabolite hypothesis.[8,33] The latter hypothesis assumes that drug hypersensitivity reactions develop as a result of imbalance between bioactivation and detoxification processes of drugs in the body. Mechanistic studies using *in vitro* cellular model have shown that certain phase I oxidation enzymes appear to be responsible for generation of toxic metabolites which can then induce cell death *in vitro.*[34] It has been shown that PBMCs from hypersensitive patients and their family

members are more susceptible to cell death upon exposure to the culprit drug (or its metabolites) than cells from healthy subjects.[6,35]

A common property of most drugs implicated in eliciting DHS is their being extensively metabolized to more reactive and cytotoxic species, such as aromatic anticonvulsants (AACs) and sulfonamides.[36] Although the culprit metabolite(s) of AACs is/are yet to be identified, many *in vivo* and *in vitro* investigations have suggested arene oxide derivatives of these aromatic compounds are the culprits.[37-39] These species are chemically short lived, highly reactive and capable of modifying intracellular macromolecules (DNA, proteins) which can then act as haptens activating the immune system.[33] A major obstacle in *in vitro* toxicity testing of AACs is the unavailability of these suspected "reactive" metabolites for quantification and study due to their instability. Using the metabolic activation system (liver microsomes) to generate these metabolites *in vitro* is a complicated process which presently lacks both standardization and reproducibility.[40] The sulfonamide sulfamethoxazole (SMX) is metabolized *in vivo* to several primary and secondary metabolites including hydroxylamine (HA) derivative by cytochrome P 450 (CYP) 2C9 and myeloperoxidase (MPO) enzymes. This metabolite can undergo auto-oxidation to form the protein reactive nitroso species that is capable of covalently binding to cellular macromolecules, causing direct cell death and forming hapten complexes that can evoke immunological reactions.[41] Rieder *et al*. (1988)[30] synthesized and used sulfamethoxazole hydroxylamine (SMX-HA) for *in vitro* rechallenge of lymphocytes from patients to determine their predisposition to hypersensitivity reactions to sulfonamides.

Data presented here introduce and characterize for the first time an *in vitro* platelet toxicity assay (*i*PTA) which employs peripheral blood platelets as a surrogate model to test for patient susceptibility to DHS. The *i*PTA may have advantages and potential applications in clinical settings due to its low cost and simple procedure. Compared to PBMCs, we show that platelets from hypersensitive patients not only respond similarly, but the degree of cell death was greater and easier to detect than in PBMCs (figure 2 A and B). This observation can be explained by the fact that platelets do not proliferate as lymphocytes do, avoiding potential masking of cell death. In addition, platelets lack nuclei and capacity for protein synthesis that compromises their defense against reactive metabolite effects (e.g., glutathione S-transferase and glutathione contents can be easily exhausted).

We observed different degrees of cell death after exposure of platelets isolated from different individuals (figures 2 B and C). This can be attributed to difference in sensitivity of the target cells. Susceptibility of the cells to *in vitro* toxicity is probably not all-or-none but rather a graded response which is observed in testing different patients.[42] The question whether there is a certain threshold or level at which a reaction may develop is difficult to answer given that DHS involves several cascades of events leading to the clinical manifestations, and multiple mechanisms that may underlie the disease. A much larger study with more strictly defined clinical cases will be useful in answering this question.

A number of reports have recently documented a role for platelets in allergic inflammation, hypersensitivity reactions and modulation of leukocyte function.[23,25,43,44]

This provides further support for the rational of using platelets as a model cell for *in vitro* testing of hypersensitivity reactions. Historically, Comaish (1968) tested patients with suspected drug allergy using peripheral platelets and the increase in the release of radio-labeled serotonin (5-HT) as an end-point.[45] Abnormal cellular response was also observed after incubation of washed platelets from aspirin-sensitive asthma with NSAID drugs *in vitro*.[46]

In summary, our studies document for the first time that platelets react in a manner similar to PBMCs in response to AACs and sulfonamides. They are easy to obtain from small blood samples, and do not proliferate, which makes them ideal for cytotoxicity assays *in vitro*. As well, they contain the full apoptotic machinery to explore molecular mechanisms of cell death. More research with larger groups of patients is needed to confirm our findings. The new assay was established by us in single cases of CBZ and SMX severe ADRs showing excellent intra-assay reproduciability and significant differences from controls. We are now collecting multiple cases to validate the assay in the near future for both drugs.

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Chapter 6. Severe bullous hypersensitivity reactions after exposure to carbamazepine in a Han Chinese child with a positive HLA-B*1502 and a negative lymphocyte toxicity assay: Evidence for different pathophysiological mechanisms.

This Chapter has been published previously:

Elzagallaai AA, Garcia-Bournissen F, Finkelstein Y, Bend JR, Rieder MJ, Koren G. 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.J Popul Ther Clin Pharmacol. 2011;18(1):e1-9.

6.1. Introduction

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 *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 *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.

6.2. MATERIALS AND METHODS

6.2.1. 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 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.

6.2.2. 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).

6.2.3. In vitro Toxicity Testing

The lymphocyte toxicity assay (LTA) was performed as described previously.[9] Briefly, 100 µl of a peripheral blood monocyte suspension (PBMCs, lymphocytes), at a density of 1×10^6 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% CO_2 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% CO_2 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 7.5×10^8 cells/ml and plates were centrifuged at 900 x g, in each step to pellet platelets.

6.2.4. 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).

6.3. 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 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 allelle.

6.5. 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

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

Time of the *in vitro* toxicity assay[a]	Subject	*In vitro* toxicity testing	
		LTA	*i*PTA
3 months	CBZ-SJS Patient	NEG[b]	NEG
	Healthy control	NEG	NEG
9 months	CBZ-SJS patient	NEG	NEG
	Healthy control	NEG	NEG
3 months	CBZ-DHS patient	POS	POS
	Healthy control	NEG	NEG

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.

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 metabolic 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 CBZ-induced SJS (according to the international standard criteria[30,31]) in an HLA-B*1502 allele positive patient of Han Chinese origin represented a useful opportunity to investigate the latter assumption. We have also tested a typical 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]

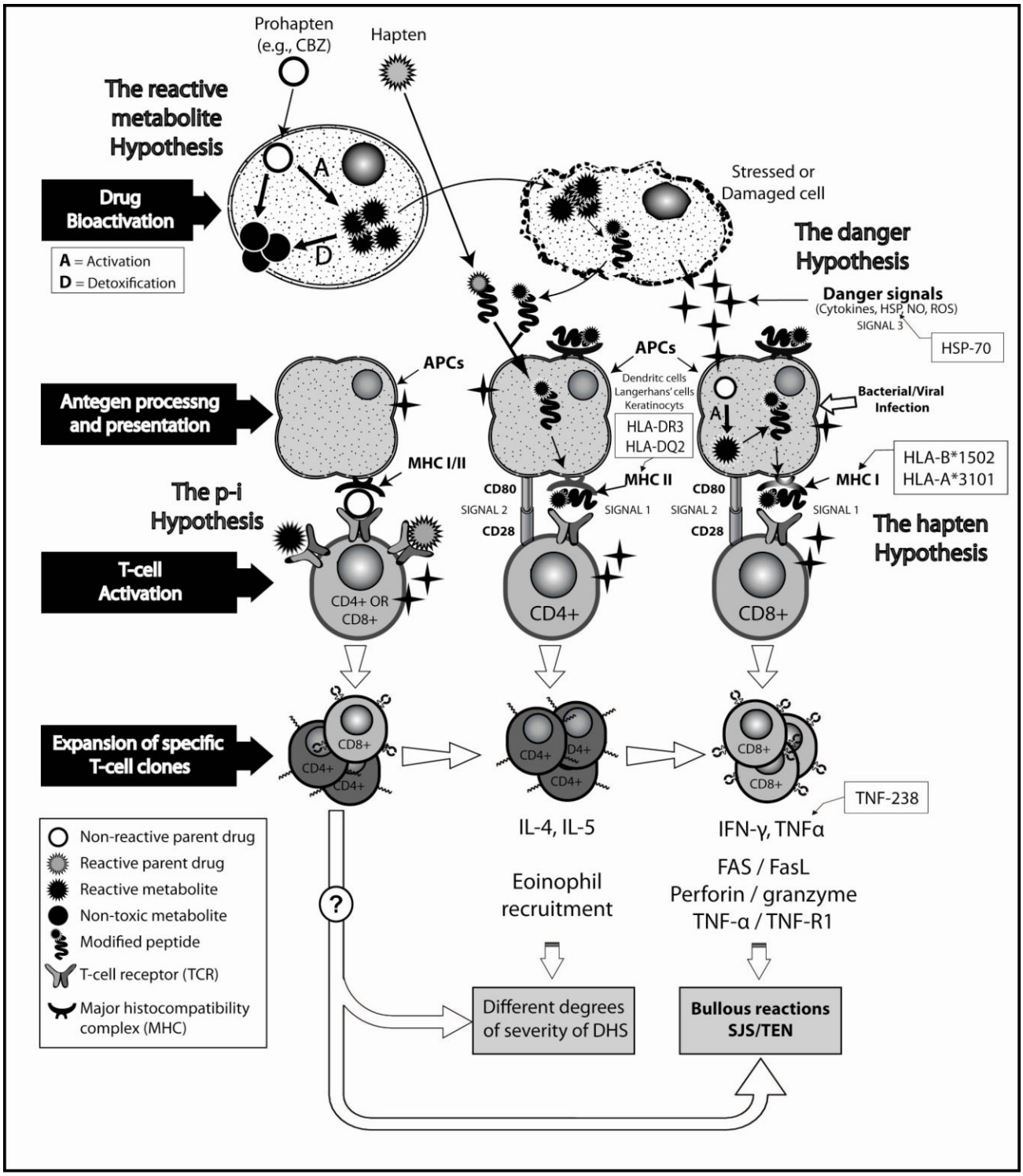
Enhanced *in vivo* concentrations of the toxic metabolites fit well with the suggested DHS mechanism. First, high level of cytotoxic reactive metabolites in either the

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 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).[34] 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.[38]

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.[39] 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.[23,39,40] Accordingly, 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.[41]

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 legend FasL or by a perforin/granzyme B dependent pathway.[42] These mediators are highly expressed in skin from SJS/TEN patients.[43] 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

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.


Prohapten
(e.g., CBZ)
Hapten
The reactive metabolite Hypothesis
Drug Bioactivation
A = Activation
D = Detoxification
A
D
Stressed or Damaged cell
The danger Hypothesis
Danger signals
(Cytokines, HSP, NO, ROS)
SIGNAL 3
HSP-70
APCs
Antegen processng and presentation
MHC I/II
APCs
Dendritc cells
Langerhans' cells
Keratinocyts
HLA-DR3
HLA-DQ2
MHC II
Bacterial/Viral Infection
HLA-B*1502
HLA-A*3101
MHC I
The p-i Hypothesis
T-cell Activation
CD80
SIGNAL 2
CD28
SIGNAL 1
CD80
SIGNAL 2
CD28
SIGNAL 1
The hapten Hypothesis
CD4+ OR CD8+
CD4+
CD8+
Expansion of specific T-cell clones
CD8+
CD4+
CD4+
CD4+
CD4+
CD8+
CD8+
TNF-238
IL-4, IL-5
IFN-γ, TNFα
Eoinophil recruitment
FAS / FasL
Perforin / granzyme
TNF-α / TNF-R1
?
Different degrees of severity of DHS
Bullous reactions SJS/TEN
Non-reactive parent drug
Reactive parent drug
Reactive metabolite
Non-toxic metabolite
Modified peptide
T-cell receptor (TCR)
Major histocompatibility complex (MHC)

these types of reactions.[44] 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)[45] whereas CBZ induces only type IV reactions.[46,47] 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.[48] 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 (bullous 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.[7,49] This has led some investigators to establish HHV-6 reactivation as one of the diagnostic criteria of DHS.[50]

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.[51] 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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Chapter 7. Challenges and Future Directions - *In Vitro* Testing for Hypersensitivity-Mediated Adverse Drug Reactions

This chapter has been accepted for publication

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7.1. Introduction

Drug hypersensitivity reactions (DHRs) are uncommon but potentially fatal adverse events. Their diagnosis and prediction is difficult due to variable presentation and overlap of symptoms with other clinical conditions. Systemic rechallenge is considered as a gold standard for the diagnosis of DHRs; however, this may have severe consequences. I*n vitro* tests are currently not sufficiently reliable to provide the basis for clinical decisions. This article summarises the challenges facing *in vitro* testing for DHRs.

Adverse drug reactions (ADRs) account for 6.5% of all hospital admissions and occur in 10-20% of hospitalized patients.(1, 2) Most ADRs (85-90%) are predictable, dose-dependent and related to the pharmacology of the drug (Augmented or Type A reactions), but 10-15% are unpredictable, unrelated to the known pharmacology of the drug and do not have a clear dose dependency (Bizarre or Type B reactions).(3) Drug hypersensitivity reactions (DHRs) or drug hypersensitivity syndrome (DHS) are a major sub-type of Type B reaction. The terms 'Type B ADRs', 'idiosyncratic drug reactions' and 'drug hypersensitivity' have been used to describe the same disease. DHS is a rare but potentially fatal disorder that occurs in susceptible patients following exposure to specific drugs. Classes of drugs most associated with DHS include β-lactam antibiotics, sulfonamides, dapsone, minocycline, terbinafine, azathioprine, allopurinol, NSAIDs and aromatic antiepileptics. DHRs include all immune-mediated drug hypersensitivity reactions (Types I to IV) while DHS refers more specifically to specific reactions characterized by delayed-onset constellation of symptoms including fever, rash and

multiple organ involvement. It has not been possible to accurately define the true incidence of DHRs due to difficulty in identification and classification as well as relatively few comprehensive studies, but it has been estimated to represent one sixth of all ADRs.(4)

Our incomplete understanding of the etiology and pathophysiology of DHRs has made it difficult to come to agreement on their classification and nomenclature. However, a general classification of 'allergic reactions' has been adopted to describe DHRs (Table 1).(5) The Gell and Coombs classification for immune-mediated reactions does not provide a mechanistically comprehensive classification system for DHR but it is a clinically relevant system that classifies DHRs into immediate, accelerated and delayed reactions related to the temporal relationship between introduction of the causative agent and appearance of the symptoms (Table 1). (6)

The clinical presentations and presumably the pathophysiology of DHRs are diverse and complex which make selection of an *in vitro* approach for their diagnosis or prediction a challenging task. Available *in vitro* tests are based either on detecting activation of the immune system (e.g., measuring immunoglobulins or immune cell activation) or detecting an enhanced ratio of metabolic activation to detoxication, an imbalance in drug metabolism (e.g., measuring cell susceptibility to *in vitro* chemical insult in metabolically-competent systems, Figure 1). The first category includes tests that measure drug specific IgE such as RAST (radioallergosorbent test), activation of basophils (BAT) or T-lymphocytes (e.g., the lymphocyte transformation test, LTT). The latter category includes the lymphocyte toxicity assay (LTA) as well as the recently

Table (1): Classification of immune-mediated hypersensitivity reactions (A) and classes of drugs most associated with causing delayed-type DHRs (B).

A): Classification of immune-mediated hypersensitivity reactions

Type	mediator	Pathogenesis	Clinical picture	Chronology
I	IgE	Degranulation of Mast cells and basophils	Urticaria; Anaphylaxis; Allergic rhinitis; Bronchospasm; Angiooedema;	Immediate (≤ 1 hr)
II	IgG/M	FcR dependent cell lysis	Blood Cell Dyscasia	Intermediate (5-14 days)
III	IgG/M	FcR-dependent immune complexes deposition	Serum Sickness; Vasculitis Arthus reaction	Intermediate (7-8 days)
IVa	T_H1 (IFNγ, TNFα)	Monocyte/macrophage mediated inflammatory response	Eczema	Delayed (1-3 weeks)
IVb	T_H2-IL4, IL5, IL13)	Eosinophils mediated inflammatory response	Maculopapular exanthema Bullous exanthema	Delayed (2-7 weeks)
IVc	Cytotoxic T cells (Perforine, Granzym B, FasL)	Cytotoxicity/Apoptosis	Maculopapular exanthema Bullous/pustular exanthema	Delayed (1-3 weeks)
IVd	T cells (IL8, CXCL8, GM-CSF)	Neutrophils mediated inflammatory response	AGEP Behçet's disease	Intermediate (≤ 2 days)

B): Drugs and classes of drugs most associated with delayed-type DHRs.

- β-lactam antibiotics
- Sulfonamides
- Aromatic antiepileptics
- Dapsone
- Minocycline
- Terbinafine
- Azathoprine
- Allopurinol
- NSAIDs
- Quinolones
- Abacavir
- Nevirapine
- Iodinated contrast media

AGEP, acute generalized exanthematous pustulosis; FcR, Fc receptor; DHRs, drug hypersensitivity reactions. Adopted from Pichler et al., 2010 (23); Coombs and Gell, 1975 (5); Levine and Ovary, 1961 (6)

described *in vitro* platelet toxicity assay (iPTA).(7) This article focuses on the current status of the *in vitro* diagnosis of immune-mediated DHRs by describing these tests their strengths and weaknesses, as well as possible future directions of this quickly evolving field.

7.2. Challenges in the evaluation of DHS

Factors that contribute to the difficulty of diagnosis of DHS include variable clinical picture, overlap with other clinical conditions (e.g., infection or malignancy) and the typical delayed temporal relationship between administration of the causative drug and the appearance of symptoms.(8) An active debate on whether this constellation of symptoms represent different degrees of severity of the same disease or distinct pathological entities is ongoing.(9, 10) Systemic re-challenge is not ethically acceptable because of the possibility of severe adverse effects to the patient. Currently, the diagnosis of DHS is based on the expertise of the treating physician and a comprehensive clinical work-up of individual cases. This includes detailed clinical and medical histories and thorough physical examination with laboratory data such as evaluation of liver function and blood counts as needed.

Developing an *in vitro* system for the evaluation of DHS would be of great advantage to researchers, drug regulators and clinicians. To appreciate potential approaches, it is important to first consider briefly the putative pathogenesis of DHS. Briefly put, immediate onset reactions typically are mediated by an antibody such as IgE. Delayed onset DHS appears to evolve as a result of the complex interplay of a number of variables (Table 1). This appears to begin with bioactivation, given that a common

characteristic of drugs causing DHS is the capacity to undergo metabolism to a reactive intermediate. While there are two competing theories as to the evolution of DHS, the most widely held hypothesis is that reactive drug intermediates alter the cellular environment and react with cellular macromolecules, following which these altered macromolecules are processed by the immune system. The immune response generated then determines the clinical expression of drug exposure – immune-tolerance or DHS. Thus, *in vitro* approaches to the evaluation of DHS would ideally evaluate key steps in the putative pathogenesis (Figure 1).

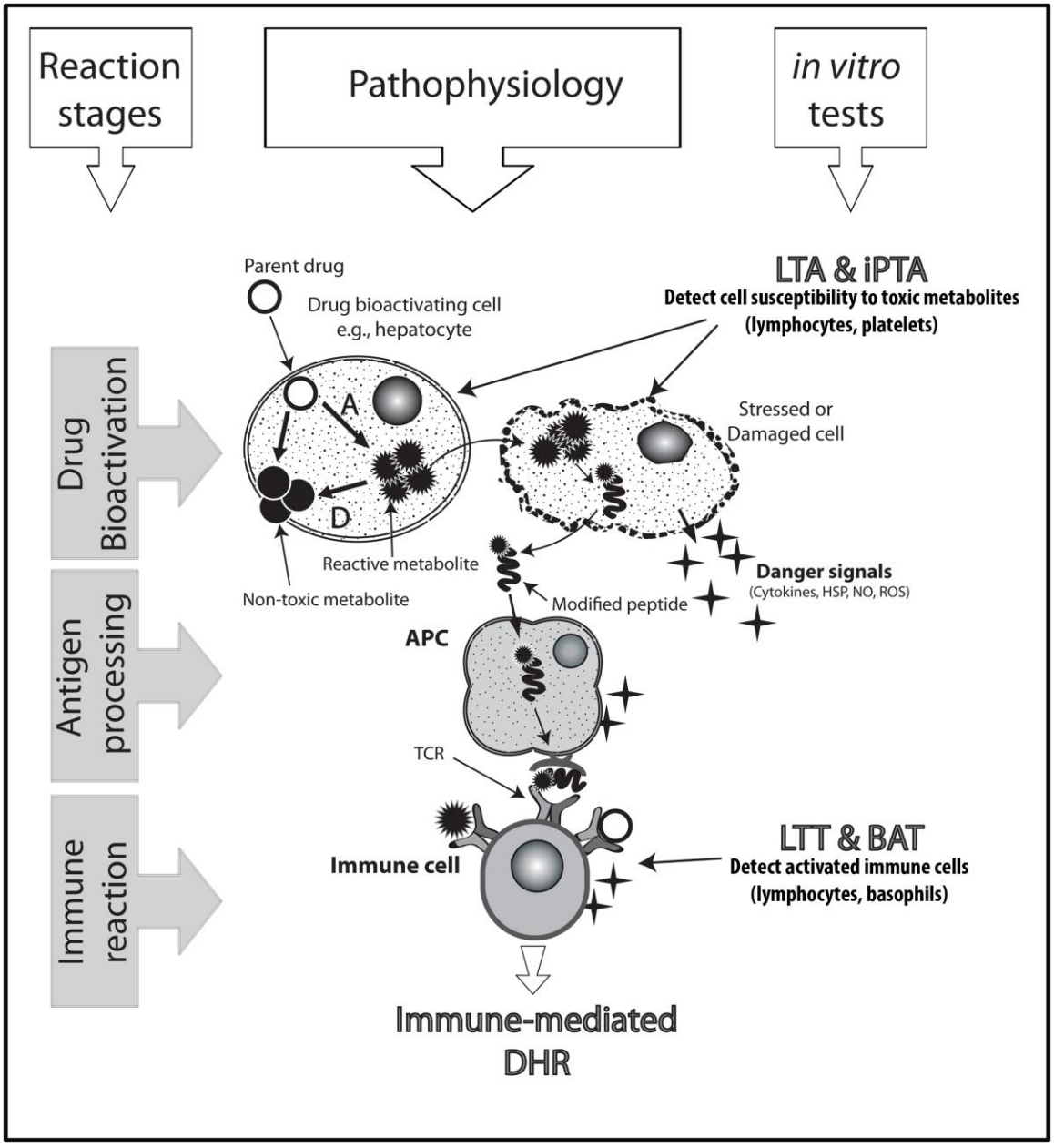
7.3. In vitro approaches to drug hypersensitivity reactions.

In vitro testing has the advantage of being safe. Selection of an *in vitro* diagnostic test for DHS depends on the type of reactions and the underlying pathophysiology predicted from the clinical picture and the natural history of the reaction. There are *in vitro* tests best used for immediate IgE-mediated reactions and those that are better for non-immediate or delayed reactions.

7.3.1. In vitro tests for immediate drug hypersensitivity reactions (DHRs).

In vitro tests for immediate hypersensitivity are based primarily on determination of specific IgE antibodies believed to be key mediators of Type I reactions. RAST, cellular fluorescent assay-IgE (CAP-IgE) and enzyme-linked immunosorbent assay (ELISA) are commonly used technologies which are known to have high positive predictive value (PPV) and low negative predictive value (NPV).(11) Thus, positive results strongly indicate immune mediation of the reaction but negative results do not exclude this possibility. Thus, either skin tests or rechallenge is required to determine

Figure (1): Pathogenesis and *in vitro* tests used for the diagnosis and prediction of immune-mediated DHRs (see text for details). **A**, activation; **APC**, antigen presenting cells; **BAT**, Basophil Activation Test; **D**, detoxication; **iPTA**, *in vitro* Platelet Toxicity Assay; **LTA**, Lymphocyte Toxicity Assay; **LTT**, Lymphocyte Transformation Test; **TCR**, T cell receptor.


Reaction stages
Pathophysiology
in vitro tests
Drug Bioactivation
Antigen processing
Immune reaction
Parent drug
Drug bioactivating cell
e.g., hepatocyte
A
D
Reactive metabolite
Non-toxic metabolite
LTA & iPTA
Detect cell susceptibility to toxic metabolites
(lymphocytes, platelets)
Stressed or
Damaged cell
Danger signals
(Cytokines, HSP, NO, ROS)
Modified peptide
APC
TCR
Immune cell
LTT & BAT
Detect activated immune cells
(lymphocytes, basophils)
Immune-mediated
DHR

safety of future therapy. As an example, one commercially available test (CAP-FEIA, Phadia®) was found to have sensitivity between 0 and 25% and specificity ranging from 83.3% to 100%, respectively, in diagnosis of immediate reactions to β-lactam antibiotics and the variation was reported to be dependent on the clinical manifestations.(12) Measurement of drug specific IgE antibodies is widely used for diagnosis of immediate reactions to β-lactam antibiotics, muscle relaxants and some NSAIDs.

Another cellular *in vitro* test for immediate hypersensitivity is the basophil activation test (BAT). Basophils respond to antigen stimulation *in vitro* by degranulation (e.g., release of histamine and leukotrienes) and expression of certain surface markers (e.g., CD45, CD11b, CD11c, CD62L, CD203c and CD63). Release of mediators including histamine can be measured in the media as an indicator of basophil activation although this method is limited by low sensitivity. Sensitivity was increased by the use of fluorescent antibodies for these surface markers coupled to flow cytometry. This latter test has been useful as an allergenic diagnostic approach and has been validated clinically for Type I reactions to muscle relaxants(13), β-lactam antibiotics(14), pyrazolones and certain NSAIDs(15, 16). The disadvantages of BAT include its relatively low sensitivity and its availability for only a limited number of drugs (Table 2).(17)

The lymphocyte transformation test (LTT) is a cellular test based on measurement of drug-specific T cells in samples of peripheral blood from patients with a suspected DHR. Although this test is mainly used for assessment of delayed T cell-mediated reactions (see below) positive LTT results also occur with type I (IgE-mediated)

reactions.(18) Although this may appear contradictory, it must be recalled that the production of drug-specific antibodies requires activation of T cells.

7.3.2. In vitro tests for non-immediate (delayed) hypersensitivity reactions

Delayed, presumably T-cell-mediated, DHRs are believed to be a result of a complex interplay of many different factors and pathways. Biochemical and genetic approaches have recently begun to shed some light on the pathophysiology of these types of devastating and potentially lethal ADRs. Understanding this pathophysiology is a prerequisite for development of evidence-based and biologically grounded approaches to manage this disease. Although some exceptions do exist, metabolic activation of drugs to metabolites normally represent the first step in the cascade of events leading to development of DHR(19) The 'reactive metabolite hypothesis' posits that DHR develops as a result of imbalance between metabolic activation or toxication and detoxication of drugs in the biological system leading to accumulation of one or more toxic reactive metabolites.(20, 21) DHS is always associated with either drugs known to be electrophilic or those readily bioactivated to electrophilic metabolites capable of covalently modifying endogenous macromolecules, including proteins and DNA.(22) It is important to understand that 'reactive metabolites' may not be the principle direct activator of the immune system as parent, non-reactive drugs can activate isolated T-cells *in vitro* without need for any bioactivation.(23) However, chemically reactive electrophilic metabolites seem to be the major, if perhaps not the only, products capable of supporting two important pathways in the immune system activation process: generation of haptenated endogenous proteins (act as antigens, signal 1) and generation of danger signals from stressed and dying cells (signal 2, Figure 1).(9) Signal 2 can also

be provided by factors such as trauma, bacterial and viral infections, or co-administered drugs and environmental pollutants. The clinical manifestations of DHS are probably primarily mediated by the immune system although in some cases a direct toxic effect of the reactive species generated from the drug during metabolism may be manifested clinically.(24) As an example, the immune response may be responsible for the skin reaction whereas enhanced formation of cytotoxic metabolites may result in liver or kidney dysfunction. It has been established for several decades that cells from DHS patients (peripheral blood mononuclear cells, PBMCs) are more susceptible to *in vitro* toxicity from the reactive metabolite(s) of the suspected drug than are cells from healthy individuals (controls) who have tolerated the drug.(25) These observations prompted the development of the lymphocyte toxicity assay (LTA).(26)

7.3.3. The lymphocyte toxicity assay (LTA)

The LTA is performed by isolation of PBMCs (lymphocytes) from blood samples withdrawn from patients and incubation of these cells with the suspected drug metabolite (if known and available) or the parent drug in the presence of a metabolic activation system (usually Phenobarbital-induced rat liver microsomes, RLM).(27) Following incubation with different concentrations of the tested drug for 2 h at 37°C, cells are allowed to recover for 16 hr and viability is then determined using different methods (e.g., trypan blue exclusion, MTT). Degrees of cell death are then expressed as percentage of the control and compared with percentage of cell death in cells from healthy individuals who do not experience an ADR with the same drug (controls). A pre-selected increase in the percentage of cell death of incubated patient cells (vs controls) is considered as an indication of patient susceptibility.(28) The predictive value of this test

remains difficult to define due to lack of a ‘gold standard’ test for comparison, and the technical complexity of the test.

We have recently performed a population survey on a cohort of pre-tested patients to evaluate the predictive values of the LTA for diagnosis of DHS.(29) In this study we included 147 patients who developed a DHS to primarily 3 different classes of drugs, β-lactam antibiotics, sulphonamides or aromatic anticonvulsants. It is clear from our evaluation that the performance of the LTA test is different in cases involving each one of these drug classes.(29)

The most complicated and non-standardized step in the test is the metabolic activation system using RLM. In some cases a murine system will not generate the same metabolite(s) of a particular drug at the same concentration(s) as would be generated in humans *in vivo* over the course of therapy. There are many pharmacokinetic and pharmacodynamic factors in humans that are unaccounted for in the *in vitro* activation systems, including lack of several functional detoxication pathways. This is supported by the observation that use of the synthetic reactive metabolite (as in case of sulfonamide drugs) resulted in increased test sensitivity and a positive predictive value of 100% (29). Another factor limiting the more routine use of the LTA is the requirement for careful isolation of white blood cells. Therefore, we have explored the possibility of using blood platelets as an alternate cell model for *in vitro* toxicity testing.(27)

7.3.4. The in vitro platelet toxicity assay (iPTA)

In order to overcome many of the limitations of the LTA test and to simplify the procedure to encourage wider clinical use, recent research in our lab focused on

development and validation of peripheral blood platelets (thrombocytes) as a surrogate cell model for *in vitro* toxicity testing.(7) Thrombocytes are metabolically active non-nucleated cells of 2.0 to 5.0 μm in diameter, 0.5 μm in thickness and abundance of 150-450×10^9 cells/L.(30) Due to their small size and low density, they are readily collected from peripheral blood using differential centrifugation.(31) In addition to blood homeostasis, their role in inflammation, allergy and hypersensitivity reactions has recently been recognized.(32-35) Thrombocytes have active mitochondria and complete apoptotic system, suggesting they can serve as a cellular model for studying drug toxicity. Furthermore, they do not proliferate which is an advantage in studying the degree of cell death. We have found that platelets from hypersensitive patients respond to *in vitro* chemical insult in a similar fashion to PBMCs; however, the degree of cell death is greater and easier to detect.(7) We speculate that platelets have lower capacity for detoxication of reactive metabolites.

Validation of any novel diagnostic test requires the availability of a 'gold standard' for comparison. Unfortunately, this is lacking in DHS because systemic rechallege data are generally unavailable. To validate the novel iPTA we used two approaches: (i) inclusion of rigorously identified DHS cases known to be caused by treatment with sulphonamide drugs (sulfa-DHS); and (ii) the use of the LTA, which we showed to have a positive predictive value of 100% in cases of DHS due to sulfa drugs in patients that had been clinically re-exposed.(29) Using a 20% increase in cell death as a cut-off value, there was 85% agreement (11 out of 13) between the LTA and the iPTA results in the 13 sulfa-DHS cases we tested.(36) In the two cases where the two tests did not agree the LTA was negative and the iPTA was positive, and of importance, these two

cases were clinically confirmed as sulfa-DHS cases. We think that this disagreement between the LTA and the iPTA results from the higher sensitivity of the platelet test to detect patient susceptibility.

The iPTA offers a simplified procedure for *in vitro* toxicity testing for DHS with higher sensitivity than the LTA. We believe that the iPTA is more suitable as a diagnostic procedure for DHS for wider clinical use than the LTA.

7.3.5. The lymphocyte transformation test (LTT)

A frequent feature of DHS is the presence of drug specific T-cell clones in the circulation of affected patients. These drug specific T-cell clones can be isolated and cloned *in vitro*; typically, they respond to incubation with the culprit drug with proliferation and expression of certain surface markers. Cell proliferation and surface markers can be measured using different methods and have been used as a measure of specific immune system response to the drug. This technique also involves isolation of PBMCs using differential gradient centrifugation and is prone to the same technical complexity as the LTA, a limitation that confines its use to well equipped research labs rather than labs designed for clinical diagnosis.(37) For a detailed description of this test procedure and its history please refer to Elzagallaai *et al.*, 2009.(27)

The LTT has been useful in defining clonal responses to drugs implicated in DHS. While these clones have been helpful in studying the immune response to drugs and drug metabolites, the LTT suffers from the same difficulty in extrapolating *in vitro* responses to *in vivo* clinical conditions as does the LTA.

Table (2): Advantages and disadvantages of *in vitro* tests used for diagnosis and prediction of DHRs.

	Lymphocyte transformation test (LTT)	Lymphocyte toxicity assay (LTA)	Basophil activation test (BAT)	*In vitro* platelet toxicity assay (iPTA)
Advantages	1. Positive results have been obtained with both immediate and delayed (T-cell-mediated) DHRs. 2. A reasonable experience with clinical use is available.	1. Detects genetic predisposition to DHRs; therefore, it can be used for prediction as well as diagnosis. 2. A reasonable experience with clinical use is available.	1. Recent adaptations using flow cytometric methods have increased its sensitivity.	1. Procedure is simplified with no need for special reagents such as Ficol™. 2. Smaller volume of blood sample is needed. 3. Has been shown to have a higher sensitivity compared to the LTA.
Disadvantages	1. Procedure is time consuming and demand special skills, resources and reagents. 2. Predictive value is not well defined. 3. Detects activated T-cells; therefore, it may not be used for prediction of DHRs prior to exposure. 4. Currently has a limited use mainly in special research centers.	1. Procedure is time consuming and demand special skills, resources and reagents. 2. Currently has a very limited use mainly in special research centers. 3. Predictive value is not well defined.	1. Can detect a limited number of types of reactions which involve basophil activation. 2. Prone to low sensitivity when conventional end-point detection methods are used (e.g., measurement of histamine release). 3. Available only for a limited number of drugs.	1. The test has not been validated for clinical use yet.

The sensitivity of the LTT for the diagnosis of DHS has been estimated to be from 56% to 78% and its specificity to range from 85% to 93%.(27) There are many factors that were found to affect the predictive ability of the LTT which include: (i) timing of the test in respect to the initial reaction; (ii) the clinical picture of the reactions; (iii) the type of drug involved; and (iv) the test procedure and read-out systems used.

7.4. Conclusion and future directions

Evaluation and management of immune-mediated DHRs require a great deal of clinical and laboratory experience and expertise.(38, 39) Advantages and disadvantages of the currently used *in vitro* tests for DHRs are summarized in table (2). We believe that the major current challenge for better *in vitro* approaches is incomplete understanding of the underlying pathophysiology of these complex ADRs. Genetic testing for predisposing alleles for DHRs has recently increased exponentially as the methodology to study the genome becomes more sophisticated while simultaneously per-test cost has declined. Genetic analysis has linked a few specific ADRs with certain polymorphisms for certain drugs in specific ethnic groups (e.g., HLA B*-1502 for carbamazepine-induced severe bullous reactions in the Han Chinese and HLA B*-5701 and abacavir hypersensitivity)(40). However, these studies have also made it clear that much more work is required in both basic and clinical science to enable us to better predict, manage and prevent this type of ADRs. Further research is required to elucidate the pathophysiology of DHS as well as rigorous trials to determine which of the available *in vitro* evaluations is most suitable for the assessment of patients or research subjects with possible DHRs.

Conflict of interest/Disclosure

The authors declare no conflict of interest directly related to the contents of this manuscript.

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Elzagallaai AA, Garcia-Bournissen F, Finkelstein Y, Bend JR, Rieder MJ, Koren G. 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.J Popul Ther Clin Pharmacol. 2011;18(1):e1-9.

Thank you very much

Abdelbaset (Baset) Elzagallaai, BSc. Pharm., MSc. (Pharmacology)
PhD graduate student

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Appendix 2. Patient questionnaire form.

Western

Children's Hospital of Western Ontario

The Ivey Chair in Molecular Toxicology

Schulich MEDICINE & DENTISTRY

Project: Defining the negative Predictive value of the LTA for diagnosis of DHS:

Telephone questionnaire form:

(I) Patient's information and medical history:

(A) Code number:	(B) Name:	© DOB: dd/mm/yyyy	(D) Sex: ☐M ☐F
(E) Street address:		City:	Provence:
(F) Telephone: (home)	Telephone (work)		Postal code:
(G) Referral date: dd/mm/yyyy	(H) Reason for referral:		

(I) Types of drugs used before test:	(J) Type of reaction: ☐Mild ☐Moderate ☐Severe **Symptomes**: ☐fever ☐skin rash; ☐articarial, ☐non-articarial, ☐blisters, ☐facial edema, ☐GIT, ☐jaundice, ☐haematouria, ☐hepatomegally, ☐liver function abnormality, ☐eosinophilia, ☐spleenomegally, ☐hematological abnormalities, ☐eosinophilia, ☐lymphadenopathy, ☐Others
(K) LTA Results	(L) Date of the test: (dd/mm/yyyy)
Drug / *Result*	(M) Advice of the managing physician:
_____ ☐+ve ☐-ve ☐not clear	☐ Discontinue therapy/ Change drug
_____ ☐+ve ☐-ve ☐not clear	☐ Continue using the same medication
_____ ☐+ve ☐-ve ☐not clear	
_____ ☐+ve ☐-ve ☐not clear	

☐ Any other health problems: ☐ None

_____

_____

(II) Contact log:

Contacted on: / / 2007 Time: _____	☐ No answer ☐ Wrong number ☐ Disconnected ☐ Message left	**Notes:**
Participation: ☐ Agree ☐ Refuse	**Preferred time to call:** Day: _____ Time: _____	

(III) Questionnaire:

1) After having received the test, have you had ……..……?	
2) If yes, have you experienced any hypersensitivity reaction? ☐Y ☐N	
3) Any other medications used: (Chronically)	5) Are you aware of any other hypersensitivity reaction toward any other drug? ☐Y ☐N If yes, what is it?

1

5) Do you know if any of your close family members has such a reaction? ☐Y ☐N	☐F ☐M ☐S ☐B ☐s ☐d

Patient's doctor contacts:

Name:			
Inistitution:			
Street address:		City:	Prov.
Phone:	Fax:	Postal code:	

Further notes:

Appendix 3. Research Ethics Board (REB) approval.

Office of Research Ethics
The University of Western Ontario
Room 00045 Dental Sciences Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. G. Koren
Review Number: 13124E **Revision Number:** 0
Review Date: March 7, 2007 **Approval Type:** Protocol
Protocol Title: Defining negative predictive value of lymphocyte toxicity assay for anticonvulsant hypersensitivity syndrome
Department and Institution: Paediatrics, University of Western Ontario
Sponsor:
Ethics Approval Date: April 4, 2007 **Expiry Date:** March 31, 2008
Documents Reviewed and Approved: UWO Protocol, Telephone Script
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. John W. McDonald
Deputy Chair: Susan Hoddinott

Signature

Ethics Officer to Contact for Further Information		
☐ Jennifer McEwen ( )	☒ Denise Grafton ( )	☐ Ethics Officer ( )

cc: ORE File
LHRI

This is an official document. Please retain the original in your files.

UWO HSREB Ethics Approval - Initial
2007-04-01 (rptApprovalNoticeHSREB_Initial) 13124E Page 1 of 1

Curriculum Vitae

Name: Abdelbaset A Elzagallaai

Qualifications:

BSc. Pharmacy (1991) Faculty of Pharmacy, Al-Fateh University, Triploi, Libya

MSc. Pharmacology, (2000) Department of cellular and Molecular medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada. Thesis title "*Presence of Myristoylated Alanine Rich C Kinase Substrate (MARCKS) in Platelets and its Role in Secretion"*

Work Experience and Academic positions:

1993-1995: Demonstrator (teaching assistant), Department of Toxicology, Faculty of Pharmacy, Garyounis University, Benghazi, Libya.

1996-2000: MSc Graduate student, Department of cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada.

2000-2004: Assistant lecturer and Chairman, Department of Toxicology, Faculty of Pharmacy, Garyounis University, Benghazi, Libya.

2004-2006: Lecturer and Chairman, Department of Toxicology, Faculty of Pharmacy, Garyounis University, Benghazi, Libya.

2001-2006 Director, Faculty of Pharmacy researches centre, Faculty of Pharmacy, Garyounis University, Benghazi, Libya.

2007-present PhD graduate student and teaching assistant, Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada.

Technical training:

Advance workshop on atomic absorption spectroscopy, ***Analytikjena Group***, June 21st - 27th, 2004, Jena, Germany.

Training on the operation of nuclear magnetic resonance (NMR) spectroscope, September 12th -15th, 2004, Benghazi, Libya. Organized by ***Bruker Biospin***, France.

The 3rd Hong Kong University (HKU)-Pasteur Immunology Course, October 31st – November 12th, 2010. Hong Kong, China.

Awards:

The Libyan Ministry of Higher Education MSc Scholarship award (1995-2000).
The Libyan Ministry of Higher Education PhD Scholarship award (2006-Present).
American Society for Clinical Pharmacology and Therapeutics ASCPT Presidential Trainee Award, 2011.

Publications:

1. **Elzagallaai AA**, Rieder MJ and Koren G. The In vitro Platelet Toxicity Assay (iPTA): validation of the novel diagnostic test for drug hypersensitivity syndrome. Under preparation.

2. **Elzagallaai AA**, Koren G, Bend JR and Rieder MJ. Challenges and Future Directions for In Vitro Testing for Hypersensitivity Mediated Adverse Drug Reactions. J Clin Pharmacol & Therap. In press.

3. **Elzagallaai AA**, Garcia-Bournissen F, Finkelstein Y, Rieder MJ and Koren G. Severe bullous hypersensitivity reactions after exposure to carbamazepine in Han Chinese child with positive HLA-B*1502 and negative lymphocyte toxicity assay: A window to different pathophysiological mechanisms. J Pop Ther Clin Pharmacol. 2011;18(1):e1-9.

4. **Elzagallaai AA**, Rieder MJ and Koren G. The In vitro Platelet Toxicity Assay (iPTA) - a Novel Approach for Assessment of Drug Hypersensitivity Syndrome. *J Clin. Pharm,* 2010 Apr 16.

5. **ELzagallaai AA**, Jahedmotlagh Z, Del Pozzo-Magañ BR, Knowles SR, Prasad AN, Shear NH, Rieder MJ, and Koren G. Predictive Value of the Lymphocyte Toxicity Assay in Diagnosis of Drug Hypersensitivity Syndrome, *Mol Diagn Ther, 2010 Oct 1;14(5):317-22.*

6. **Elzagallaai AA**, Knowles SR, Rieder MJ, Bend JR, Shear NH, Koren G. in vitro testing for the diagnosis of anticonvulsant hypersensitivity syndrome: a systematic review. *Mol Diagn Ther*. 2009;13(5):313-30.

7. **Elzagallaai AA**, Knowles SR, Rieder MJ, Bend JR, Shear NH, Koren G. Patch testing for the diagnosis of anticonvulsant hypersensitivity syndrome: a systematic review. *Drug Saf.* 2009;32(5):391-408

8. **Elzagallaai AA**, Rose SD, Brandan NC, Trifaro JM. Myristoylated alanine-rich C kinase substrate phosphorylation is involved in thrombin-induced serotonin release from platelets. *Br J Haematol*. 2001;112(3):593-602.

9. Lejen T, Skolnik K, Rose SD, Marcu MG, **Elzagallaai AA**, Trifaro JM. An antisense oligodeoxynucleotide targeted to chromaffin cell scinderin gene decreased scinderin levels and inhibited depolarization-induced cortical F-actin disassembly and exocytosis. *J Neurochem.* 2001;76(3):768-77.

10. Trifaro J, Rose SD, Lejen T, **Elzagallaai AA**. Two pathways control chromaffin cell cortical F-actin dynamics during exocytosis. *Biochimie.* 2000;82(4):339-52.

11. **Elzagallaai AA**, Rose SD, Trifaro JM. Platelet secretion induced by phorbol esters stimulation is mediated through phosphorylation of MARCKS: a MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. *Blood.* 2000; 95(3):894-902.

12. Marcu MG, Zhang L, **Elzagallaai AA**, Trifaro JM. Localization by segmental deletion analysis and functional characterization of a third actin-binding site in domain 5 of scinderin. J Biol Chem. 1998; 273(6):3661-8.

Invited Speaker:
Drug Information Association (DIA) workshop: Personalized Medicine: Biomarkers and Diagnostics in Drug Development, Regulatory Approval, and Access to Patients: October 15-16, 2009, Toronto, ON, Canada.
American Society for Clnical Pharmacalology and Therapeutic (ASCPT) annual meeting, Drug Safety, Vaccines: Challenges and potentials. March 2-5, 2011, Dallas, TX, USA..

Peer-reviewing activity:
Reviewer for the Journal of Therapeutic Drug Monitoring
Reviewer for the Journal of Clinical Pharmacology

Affiliations:
Member of the Libyan Pharmaceutical Society (LPS)
Member of the Libyan Society of Biological Technology (LSBT)
Member of the Canadian Society of Pharmacology and Therapeutics (CSPT)
Member of the Canadian Pharmacists Association (CPhA)
Member of the American Society for Clinical Pharmacology and Therapeutics (ASCPT)
Member of the European Academy of Allergy and Clinical Immunology (EAACI)