



Review

Lymphocyte transformation test and cytokine detection assays: Determination of read out parameters for delayed-type drug hypersensitivity reactions

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ABSTRACT

Drug hypersensitivity reactions (DHRs) occur in certain people and are often not predictable. DHRs can be classified as immediate and delayed reactions regarding to onset of clinical manifestations. Both reactions are considered to be an important public health problem because they can lead to life-threatening conditions; however, this review article will focus on delayed DHRs. The most important points for diagnosis of delayed DHRs are the recognition of drug hypersensitivity characteristics and culprit drug identification. While it is usually difficult to identify a culprit drug; clinical evaluation using the causality assessment method, a non-invasive process, can identify the culprit drug without the need for intensive investigation. Delayed DHRs can cause life-threatening conditions; therefore, *in vivo* skin tests, as well as drug provocation tests, have to be cautiously performed by a drug allergist and have not been recommended in uncontrolled conditions. ENDA/EACI has recommended that *in vitro* tests (if available) be performed prior to any *in vivo* tests. Therefore, *in vitro* diagnostic tests can be alternative methods to identify a culprit drug for delayed DHR diagnosis as there is no or very low risk for patients under investigation. There are many testing approaches to identify causative agents for delayed DHRs such as: the lymphocyte transformation test (LTT), cytokine/mediator detection assays (i.e. ELISA and flow cytometry-based bead assays), multiplex bead-based immunoassay and ELISpot. The LTT is the most standardized method whereas it has been available in medical schools affiliated with university hospitals. Other *in vitro* tests, like cytokine detection assays, have also been used, even though they are still being evaluated. They could supplement LTT results that would provide drug allergist's with documentary evidence and prevent risk to patients by avoiding *in vivo* or drug provocation testing. Hence, the *in vitro* tests have been promising tests contributing to the management of the delayed DHR work-up process in clinical practice.

1. Introduction

Drug hypersensitivity reactions (DHRs), belonging to type B adverse drug reactions, are often mediated by immune responses, which occur in response to drugs taken in therapeutic doses, which are normally tolerated by the great majority of the population. Their clinical manifestations vary from mild to severely life-threatening conditions (Schrijvers et al., 2015). DHRs can be classified into four types according to Gell-Coombs classification as described in the article "Lymphocyte transformation test: History and current approaches" (Sachs et al.,

2021). Additionally, Type IV DHRs have been further sub-categorized as type IV a, b, c, and d according to their cytokine production and activation of inflammatory immune cells as described by Pichler et al. (Pichler, 2007). Type IV DHRs or delayed DHRs occur in certain people and are often unpredictable. The most common tissue involved in delayed DHRs is skin with maculopapular exanthem (MPE) being the most common clinical manifestation of cutaneous delayed reactions (Brockow et al., 2019). Nevertheless, delayed DHRs can cause severe cutaneous adverse drug reactions (SCARs) including: acute generalized exanthematous pustulosis (AGEP), drug rash with eosinophilia and

Abbreviations: AED(s), anti-epileptic drug(s); AGEF, acute generalized exanthematous pustulosis; ALP, allopurinol; DRESS, drug rash with eosinophilia and systemic symptoms; IFN, interferon; IL-, interleukin-; MPE, maculopapular exanthem; ND, not determined; OXP, oxypurinol; PBMC, peripheral blood mononuclear cell; RT-PCR, real time polymerase chain reaction; SJS/TEN, Stevens-Johnson syndrome with toxic epidermal necrolysis; TNF, tumor necrosis factor.

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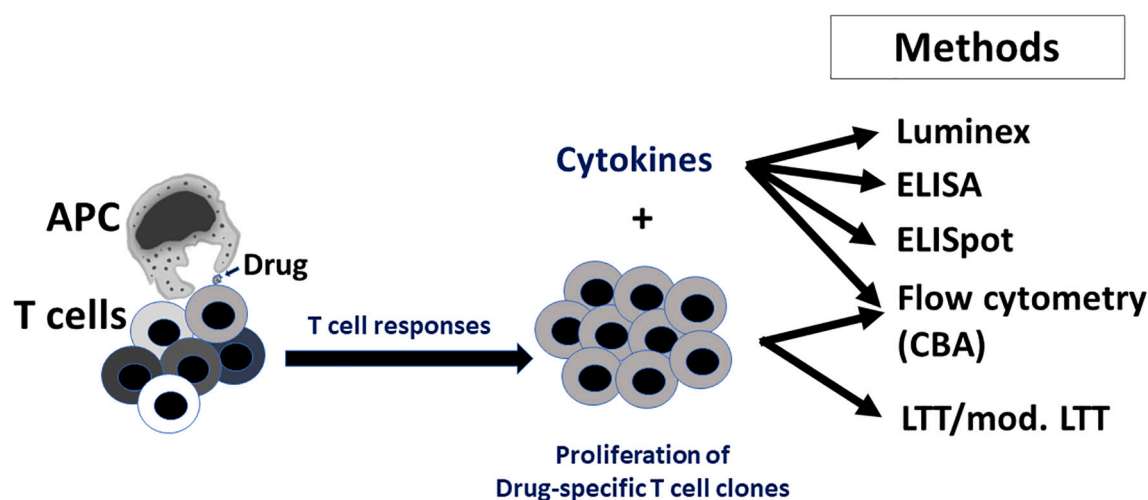


Fig. 1. Detection of drug-specific T cell responses.

systemic symptoms (DRESS) and Stevens-Johnson syndrome with toxic epidermal necrolysis (SJS/TEN) (Duong et al., 2017). Even though SCARs occur only in certain people and their prevalence is low, they can cause prolonged hospitalization with high morbidity and mortality as reviewed elsewhere (Borchers et al., 2008; Speeckaert et al., 2010; Cacoub et al., 2011; Srinoulprasert et al., 2012). Given that SCARs can cause life-threatening conditions and are considered to be an important public health problem, they will be a focus of this article.

This article focuses on the utility of common *in vitro* tests in culprit drug identification in cases of delayed-typed drug hypersensitivity such as the lymphocyte transformation test (LTT) and cytokine detection assays. Although LTT has been widely used to assess DHRs for more than three decades, its efficacy is variable and dependent on various factors, including clinical phenotypes, tested drugs, immunosuppressive drug interference, and assessing time intervals. (Pichler and Tilch, 2004; Srinoulprasert et al., 2020). Detection of cytokine production as a means to assess drug-induced immune responses has recently been proposed (Bienvenu et al., 2000; Sachs et al., 2002; Naisbitt et al., 2007; Kumkamthornkul et al., 2018; Chu et al., 2020). Although the sensitivity and specificity of cytokine detection assays are variable, the gradual usage of cytokine detection assays to diagnose drug-induced hypersensitivity has been reported. This article aims to summarize information relating to the efficacy of LTT and cytokine detection assays in the identification of culprit drugs in cases of DHR, especially SCARs.

2. Diagnostic tools for delayed DHRs

While recognition of delayed DHR characteristics is important, it is not enough for further management in severe cases in the absence of culprit drug identification. Accurate identification of the culprit drug(s) is important for correct labeling and subsequent patient education and avoidance. Unfortunately, definite identification of culprit drugs still remains difficult in practice as the majority outcomes of non-invasive assessment are not accurate enough to allow an unambiguous determination to be made as to whether the patient is susceptible to the suspicious drug (Naranjo et al., 1981; World Health Organization-Uppsala Monitoring Center, Last accessed on December 1st, 2020). Therefore, other tools are needed to identify culprit drugs and many new diagnostic tests have been launched. As T cells are an important player in mechanism of delayed drug hypersensitivity reactions, the existence of drug-specific T cell clones is essential evidence to reveal the pathogenesis of drug hypersensitivity (Neukomm et al., 2001). Many *in vivo* functional tests have been proposed to give a diagnosis for drug hypersensitivity; however, re-exposure to a suspicious drug during the process of *in vivo* tests may cause not only physical burden but also psychological

apprehension in patients. Many reports have demonstrated that these tests confer risk to the patient and may not be recommended in some situations (Romano et al., 2011; Heinzerling et al., 2013; Barbaud, 2014). Hence, many modalities with minimal risk, like venipuncture of *in vitro* readout systems, such as cytokine measurement, T cell proliferation and T cell clone detection, have been proposed as a way to detect drug-specific T cells as a means of elucidating the cause of delayed DHRs (Hausmann et al., 2010; Mayorga et al., 2016). *In vitro* tests are not only non-invasive procedures, they are also the safest methods when compared to *in vivo* tests, especially DPT (Porebski et al., 2011; Mayorga et al., 2017). Hence, this review article will mainly focus on *in vitro* tests launched for diagnosis of delayed DHRs.

3. *In vitro* tests for culprit drug identification in delayed drug hypersensitivity

In vitro tests with various read-out systems for culprit drug identification in cases of delayed DHRs, such as the lymphocyte transformation test (LTT) and cytokine detection assays, have been reported (Merk, 2005; Porebski et al., 2013; Kumkamthornkul et al., 2018). The reliability of a test depends on its sensitivity and specificity. In this regard, sensitivity is defined as the ratio of the positive outcomes of the test and the correctly identified positives, while specificity refers the ratio of negative tests to actual negative. As explained in "Lymphocyte transformation test: history and current approaches" by Sachs et al., LTT was developed to detect T cell proliferation and it has been used with a variety of readout systems, including the incorporation of nucleotide materials (^3H -thymidine and BrdU), fluorescent dye dilution, and flow cytometry enumeration of activated T cells (Nyfeler and Pichler, 1997; Pichler and Tilch, 2004; Sachs et al., 2021). The LTT has been widely used to identify culprit drugs for different types of delayed drug allergy reactions for more than three decades. The sensitivity and specificity of LTT ranges from good to excellent. Additionally, radioactive exposure is an inevitable part of the process of classical LTT. These points may have helped trigger the search for further read-out systems, such as ELISA, ELISpot, flow cytometry, flow cytometry-based bead assays, and multiplex bead-based immunoassays, to detect cytokine production as a means to assess drug-induced immune responses as illustrated in Fig. 1 (Bienvenu et al., 2000; Sachs et al., 2002; Naisbitt et al., 2007; Kumkamthornkul et al., 2018). Given that both LTT and cytokine detection assays involve cell cultivation and LTT has already been employed for drug allergy diagnosis, LTT is introduced in more detail because the principles involved can be applied to cytokine detection assays.

Table 1

Sensitivity and specificity of LTT for identification of culprit drug in different delayed drug hypersensitivity.

Clinical manifestation	Drugs	Sensitivity (%)	Specificity (%)	References
MPE	Beta-lactam	83.3	100	Sachs et al. (2002)
AGEP	AEDs	60.0	100	Sachs et al. (2002)
	Beta-lactam	100	100	Srinoulprasert and Pichler (2014) ^b
	Antibiotics ^a	33	100	Schmid et al. (2006) ^b
		100	100	
DRESS	Beta-lactam	73	100	Cabanas et al. (2018) ^b
		50	100	
	Antibiotics ^a	100	100	Cabanas et al. (2018) ^b
		83	100	
	AEDs	56	96	Karami et al. (2016)
		100	100	Cabanas et al. (2018)
		75	91	Kumkamthornkul et al. (2018)
	ALP	33	ND	Kim et al. (2017)
SJS/TEN		73	ND	Porebski (2017) ^b
		20	100	
	Beta-lactam	100	99	Hari et al. (2001)
		100	100	Rozieres et al. (2009) ^b
		67	100	
	Antibiotics ^a	0	100	Kano et al. (2007)
		0	100	Srinoulprasert and Pichler (2014) ^b
		50	100	
	AEDs	64	96	Karami et al. (2016)
		40	94	Kumkamthornkul et al. (2018)
	ALP	33	ND	Porebski (2017) ^b
		67	ND	
	Misc.	86.3	70.5	Bellon et al. (2020)

AEDs, anti-epileptic drugs; ALP, allopurinol; OXP, oxypurinol, ND, not determined; Misc. = beta-lactam, antibiotics⁺, AEDs, OXP, bactrim, NSAIDs, steroids, antihistamine, antifungal drugs, antiviral drugs, central nervous system effective drugs, vitamins.

^a (Quinolone, co-trimoxazole, macrolide).

^b our unpublished data.

3.1. Lymphocyte transformation test (LTT)

The LTT has been widely employed to identify culprit drugs in the diagnosis of T cell-mediated drug hypersensitivity. The principle of LTT is based on the existence of drug-specific memory T-cells that are able to proliferate upon re-stimulation with the culprit drug (Pichler and Tilch, 2004). Once activated, proliferative responses of drug-specific lymphocytes are measured by DNA incorporation of a labeled nucleoside (typically ³H-Thymidine) as a consequence of cell division. The level of the cell proliferation is expressed as the ratio between lymphocytes stimulated with suspicious drugs and the spontaneous background of lymphocyte proliferation. This ratio is called the stimulation index (SI). Details of how to perform LTT are fully described in “Lymphocyte transformation test: history and current approaches” (Sachs et al., 2021). Utilization of LTT as a highly sensitive tool to discriminate drugs to which the patients are sensitized (Neukomm et al., 2001). Since then, the sensitivity and specificity of LTT has been reported in a number of studies and its efficacy is summarized in more detail for various constellations (clinical phenotype, drug etc.) in Table 1. While it seems that LTT is quite useful for the diagnosis of delayed drug hypersensitivity, results obtained from LTT have to be interpreted and applied, carefully.

LTT has been used to detect the existence of drug specific T cell populations in peripheral blood; however, their existence is not necessarily an indicator of the severity of clinical symptoms. Rather, the clinical severity of a drug hypersensitive reaction seems to be related to the type of effector function of the target tissue(s) reacting cells, as opposed to the number of these cells in the peripheral blood. LTT is usually performed after remission of disease. This is because the blood obtained during the acute drug-allergic stage may contain immune cells with bystander activation and high background proliferation, which might make it difficult to detect proliferation of drug-specific T cell clones. The minimal time interval between the remission stage and test performance has been recommended at 4–12 weeks after recovery depending on the type of drug hypersensitivity (4 weeks for AGEP and SJS/TEN, 12 weeks for DRESS). It has also been recommended that LTT be performed within the first year after a DHR event (Ogawa et al., 2012; Porebski et al., 2015; Srinoulprasert and Tuchinda, 2020). LTT can provide good sensitivity with excellent specificity, but evaluation of sensitivity and specificity is often subject to many biases (i.e. too low or too high SI, too stringent or too loose definitions of cases, use of inappropriate control groups such as unexposed controls) and false-positive results from some drugs, such as vancomycin, acetaminophen and RCMs (Pichler and Tilch, 2004).

3.1.1. Sensitivity and specificity of the LTT compared to skin tests

A major role for T cells in the immunopathology of allergic drug reactions is indicated by positive outcomes from either a delayed reading intradermal test (IDT) or patch test. Not all patients with clearly positive skin tests also have a positive LTT (Hari et al., 2001; Pichler and Tilch, 2004; Cabanas et al., 2018). Nevertheless, it has been revealed that LTT is superior to skin testing for non-immediate type reactions. Hari et al. showed that the sensitivity of LTT (66.7%) was higher than that of the skin patch test (46.7%) in patients suffering from MPE resulting from exposure to a variety of culprit drugs (beta-lactam, anti-hypertensive drugs, anti-epileptic drugs, and others) (Hari et al., 2001). Consistent with this, Cabanas and colleagues demonstrated superior sensitivity of LTT (57.4%) as compared with sensitivity of the patch test (29.5%) in DRESS patients tested with various drugs (beta-lactam, NSAIDs, antibiotics, anti-epileptic drugs, and others) (Cabanas et al., 2018). Recently, Bellon and colleague showed high sensitivity of LTT (86.4%), which was in concordance with ALDEN algorithm in SJS/TEN patients tested with: beta-lactam, antibiotics, anti-epileptic drugs, NSAIDs, steroids, antihistamine, central nervous system effective drugs, and vitamins (Bellon et al., 2020). However, when the patch test and LTT are combined, they can yield a sensitivity of 76%. In another study involving aminopenicillin identification argued that delayed reading IDT and patch tests could provide higher sensitivity than LTT (Trautmann et al., 2014). The authors, however, suggested that their results specific to aminopenicillin could not be transferable to other drug interpretations. A recent study by Ban and colleagues (Ban et al., 2019) revealed a consistent finding of poor sensitivity when using the patch test to identify anti-tuberculosis drug (ATB) allergy. They also demonstrated that the sensitivity of IDT and LTT was comparable and higher than that of the patch test for diagnosis of ATB-induced delayed DHRs. Nevertheless, more studies to compare the sensitivity between delayed reading IDT, patch test and LTT are still needed.

3.1.2. Sensitivity and specificity of the classical LTT: Different clinical phenotypes and drug tested

It seems that identification of true positives for the LTT relied on the provocation test, which is assumed to be the gold standard for a drug allergy test. However, there are many limitations of a provocation test that have to be considered and the test can give false positive and false negative results (Mayorga et al., 2017; Mayorga et al., 2019). Theoretically, LTT seems to be useful in all types of delayed hypersensitivity reactions; however, when the sensitivity of the LTT was evaluated in different series it ranged from 17% to 100% as summarized in Table 1.

Table 2

Sensitivity and specificity of cytokine detection assays (ELISA, flow cytometry-based bead assay, and multiplex bead-based immunoassay) for the identification of culprit drugs in different delayed drug hypersensitivity reactions.

Cytokine(s)	Methods	Clinical manifestation	Drug	Sensitivity (%)	Specificity (%)	References
IL-2	CBA	SJS/TEN	AEDs	55.6	98.0	Porebski et al. (2013)
	Luminex	DRESS, SJS/TEN		75.0, 15.0	97.1, 95.8	Kumkamthornkul et al. (2018)
IL-4	Luminex	DRESS, SJS/TEN	AEDs	68.8, 45.0	79.4, 85.4	Kumkamthornkul et al. (2018)
IL-5	ELISA	MPE	Beta-lactam	83.3	100	Sachs et al. (2002)
	ELISA	MPE	AEDs	100	100	Sachs et al. (2002)
	CBA	SJS/TEN		55.6	100	Porebski et al. (2013)
	ELISA			100	100	Martin et al. (2010)
	Luminex	DRESS, SJS/TEN	Beta-lactam	56.3, 0.0	100, 100	Kumkamthornkul et al. (2018)
	ELISA	MPE		66.7	100	Sachs et al. (2002)
IL-10	ELISA		AEDs	40.0	100	Sachs et al. (2002)
	ELISA	SJS/TEN	AEDs	0	100	Martin et al. (2010)
IL-13	Luminex	DRESS, SJS/TEN	AEDs	56.3, 5.0	79.4, 79.2	Kumkamthornkul et al. (2018)
IFN- γ	ELISA	MPE	Beta-lactam	50.0	60.0	Sachs et al. (2002)
	ELISA	MPE	AEDs	20.0	60.0	Sachs et al. (2002)
	CBA	SJS/TEN		33.3	93.3	Chu et al. (2020)
	Luminex	DRESS, SJS/TEN		44.4	95.0	Porebski et al. (2013)
	ELISA			75.0, 10.0	100	Kumkamthornkul et al. (2018)
	ELISA	MPE		66.7, 14.3	93.3	Chu et al. (2020)
Granulysin	ELISA	MPE	AEDs	63.6	96.7	Chu et al. (2020)
	ELISA	DRESS	AEDs	69.2	96.7	Chu et al. (2020)
	ELISA	SJS/TEN	AEDs	53.9	96.7	Chu et al. (2020)
	ELISA		ALP/OXP	85.7	91.2	Chung et al. (2015)
Granzyme B	ELISA	MPE	AEDs	21.4	100	Chu et al. (2020)
	ELISA	DRESS, SJS/TEN		77.8, 26.5	100, 100	Chu et al. (2020)
IL-2 + IL-5	Luminex	DRESS, SJS/TEN	AEDs	75.0, 15.0	97.1, 95.8	^a
	CBA	SJS/TEN		55.6	98–100	Porebski et al. (2013)
IL-2 + IL-13	Luminex	DRESS, SJS/TEN	AEDs	75.0, 20.0	97.1, 77.1	^a
IL-2 + IFN- γ	Luminex	DRESS, SJS/TEN	AEDs	75.0, 15.0	97.1, 95.8	^a
	CBA	SJS/TEN		66.7	95–98	Porebski et al. (2013)
IL-4 + IL-2	Luminex	DRESS, SJS/TEN	AEDs	81.3, 55.0	82.4, 85.4	Kumkamthornkul et al. (2018)
IL-4 + IL-5	Luminex	DRESS, SJS/TEN	AEDs	75.0, 45.0	82.4, 87.5	Kumkamthornkul et al. (2018)
IL-4 + IFN- γ	Luminex	DRESS, SJS/TEN	AEDs	81.3, 50.0	82.4, 85.7	Kumkamthornkul et al. (2018)
IL-5 + IFN- γ	ELISA	MPE	Beta-lactam	83.3	60–100	Sachs et al. (2002)
	ELISA	MPE	AEDs	100	60–100	Sachs et al. (2002)
	CBA	SJS/TEN		66.7	95–100	Porebski et al. (2013)
	ELISA			100	100	Martin et al. (2010)
	Luminex	DRESS, SJS/TEN		75.0, 45.0	82.4, 87.5	^a

AEDs, anti-epileptic drugs; ALP, allopurinol; OXP, oxypurinol; CBA, flow cytometry-based bead assay; Luminex, multiplex bead-based immunoassay.

^a Our unpublished data (Kumkamthornkul, 2018).

The LTT could provide a sensitivity of 78% in patients classified as having a well defined drug allergy, while patients with lower clinical likelihood of drug hypersensitivity had a respectively lower incidence of a positive LTT (Nyfeler and Pichler, 1997). Sensitivity has been shown to be frequently positive in maculopapular exanthem, DRESS and AGEP (Srinoulprasert et al., 2012). As summarized in Table 1, among SCARs, the frequency of a positive LTT was found to be more than 50% both in DRESS and SJS/TEN, which was higher than that in previous reports (Roujeau et al., 1985; Naisbitt et al., 2003a; Cabanas et al., 2018; Kumkamthornkul et al., 2018; Bellon et al., 2020). However, these findings have to be concerned depending on the drug studied.

The specificity of the LTT has been determined to be in the range of 85–100% in a number of studies (Schnyder and Pichler, 2000; Hari et al., 2001; Luque et al., 2001; Naisbitt et al., 2003b). Many reports showed that the varying specificities of LTT seemed to depend on the drug being evaluated, as certain drugs were able to induce spontaneous T cell proliferation with unknown mechanisms (Pichler and Tilch, 2004; Lochmatter et al., 2009). For example, ibuprofen and sulfamethoxazole can elicit unstable positive T cell proliferation in T cell lines from non-exposure donors (Pichler and Tilch, 2004). Therefore, LTT has to be performed with irrelevant drugs every time to determine the reliability of the test. The test should include some drugs without previous established concentrations. The LTT study should also include control subjects (such as non-exposed subjects and/or exposed subjects without allergic reaction). A timeline for each drug exposure during a DHR event has to be established and assessed with reliable algorithms such as Naranjo's criteria and ALDEN score (Cabanas et al., 2018;

Kumkamthornkul et al., 2018; Bellon et al., 2020). It is quite important, especially in polymedicated patients, because it could help to discriminate suspicious culprit drugs from tolerant/non-culprit drugs and enhance the reliability of the LTT. Nevertheless, some drugs seem not to significantly provoke T cell responses as demonstrated by a study of carbamazepine and lamotrigine hypersensitivity, which showed specificities of 100% (Naisbitt et al., 2003a; Naisbitt et al., 2003b). Also the specificity of LTT for β -lactam hypersensitivity was evaluated as 98% (Luque et al., 2001). Regarding the drugs mentioned above, retrospective analyses with and without the RegiSCAR system inferred that the specificity of LTT was 85% (Nyfeler and Pichler, 1997; Pichler and Tilch, 2004; Cabanas et al., 2018; Bellon et al., 2020). Taken together, the overall specificity of LTT is at least 85%, whereas a higher value can probably be expected depending on the drug investigated.

3.1.3. Utilization of the classical LTT for diagnosis of anti-epileptic drug induced delayed DHRs

Anti-epileptic drugs (AEDs) are the most common drugs causing delayed DHRs. Karami and colleagues reported that the sensitivity and specificity of LTT from patients allergic to AEDs without discriminating symptoms were 58.40% and 95.80%, respectively (Karami et al., 2016). Other studies by our group are also consistent with previous studies in that distinguishingly positive LTT results for phenytoin and carbamazepine were detected in AED allergic patients (Kumkamthornkul et al., 2018). Findings of our group also demonstrated that LTT could also discriminate patients from control groups (normal and non-allergic patients exposed with AEDs) and LTT provided good sensitivity at 75.00%

for DRESS and poor sensitivity at 40.00% for SJS/TEN. Excellent specificity for DRESS and SJS/TEN at 91.20% and 93.80%, respectively, was observed. These results suggested that LTT could be an appropriate test to identify AEDs as causative drugs in epilepsy patients with very low risk, good sensitivity, and excellent specificity. Additionally, the LTT could be applicable to many different drugs in different DHRs and a positive LTT may not only support the diagnosis of drug allergy, but can also pinpoint the responsible agent in cases where a patient has taken several drugs.

3.2. Cytokine detection assays

During antigen-specific activation, T lymphocytes undergo blastogenesis and produce various cytokines in response to stimulation by antigen presenting cells that have interacted with an antigen. The cytokines released from antigen-specific T cells direct the effector phase of the immune reaction. Therefore, cytokine detection has been considered as an alternative readout system in the diagnosis of drug hypersensitivity as mentioned in “Lymphocyte transformation test: History and current approaches” (Sachs et al., 2021). The measurement of cytokine levels in cell culture supernatant by ELISA, flow cytometry-based bead assays and multiplex bead-based immunoassays to identify culprit drugs for the diagnosis of delayed DHR reviewed in this article is summarized in Table 2. Additionally, the detection of cytokine production in cell culture based enzyme-linked immunosorbent spot (ELISpot) assays has been reviewed by Porebski and colleagues in this special issue. Production of Th1-type cytokine patterns (IL-2, IFN- γ , and TNF- α) and mixed Th1/Th2 cytokine patterns in PBMCs isolated from patients with delayed reactions has been reported (Gaspard et al., 2000; Cornejo-Garcia et al., 2007; Lochmatter et al., 2009). Lochmatter and colleagues reported that the levels of IL-2 and IFN- γ production from PBMCs derived from delayed drug allergy patients were higher than those of a control cohort. Furthermore, production of certain cytokines (IL-2, IL-5 and IFN- γ) was also reported in AED-induced delayed DHRs (Verrotti et al., 2001; Beeler and Pichler, 2006; Tsuge et al., 2007; Lochmatter et al., 2009). All of these cytokines could potentially be used as biomarkers for patients suffering from drug-induced delayed DHRs.

3.2.1. Utilization of IFN- γ as dominant read-out parameter

Detection of IFN- γ production corresponding to pathogenesis in drug-induced exanthemas has been used as a diagnostic marker in *in vitro* assays. Increased production of IFN- γ from peripheral blood mononuclear cells stimulated with drugs was observed in several studies of patients suffering from MPE, DRESS, and AGEP induced by various drugs (Posadas et al., 2002; Naisbitt et al., 2003a; Halevy et al., 2005; Tsuge et al., 2007). Hence, various read-out systems, such as semi-quantitative RT-PCR, flow cytometry, ELISA and ELISpot, have been used to detect IFN- γ production to diagnose delayed DHRs with reported sensitivities of 75.0%, 71.4%, 76.2%, 77.8%, respectively. (Gaspard et al., 2000; Tsuge et al., 2007; Halevy and Grossman, 2008; Khalil et al., 2008). In our study, production of IFN- γ , detected by multiplex bead immunoassay, provided comparable sensitivity (75.0%) consistent with the aforementioned studies suggesting that IFN- γ could be used as a diagnostic marker to identify culprit drugs in delayed DHRs (Kumkamthornkul et al., 2018). Interestingly, the sensitivity of the IFN- γ detection (75.0%) was comparable to the sensitivity of LTT (75.0%); however, its specificity (100%) was higher than that of LTT (91.2%). By contrast, detection of IFN- γ production provided poor sensitivity for identification of culprit drug in SJS/TEN patients, which was consistent with the finding of Porebski and colleagues. (Porebski et al., 2013).

3.2.2. Role of multiple cytokine detection as an alternative read-out parameter in reactions to antiepileptic drugs

As mentioned earlier, higher levels of IL-2 were produced by PBMCs from drug allergy patients as compared to those from control PBMCs. Our group found that the sensitivity of IL-2 detection was comparable to

that of IFN- γ detection when identifying AED culprit drugs in epilepsy patients suffering from DRESS. Therefore, detection of IL-2 has been proposed as another read-out parameter. Combined detection of drug-specific secretion of IFN- γ and IL-2 from cultures of PBMCs, derived from MPE patients allergic to penicillins, increased sensitivity and specificity to 87% and 100%, respectively (Khalil et al., 2008). Production of IL-5 was revealed as a promising marker due to the fact that *in vivo* and *ex vivo* release of IL-5, from patients who had drug-induced MPE and DRESS, was demonstrated (Yawalkar et al., 2000; Sachs et al., 2002). Increased sensitivity of *in vitro* tests for drug hypersensitivities was demonstrated when detection of IL-5 production was combined with IFN- γ measurement (Martin et al., 2010). Additionally, combined detection of IL-5 and IFN- γ or IL-2 production yielded higher sensitivity (Porebski et al., 2013). In our study, IL-5 detection associated with pathogenesis of DRESS was less sensitive (56.3%) and had higher specificity (100%) when compared with LTT (75.0%, 91.2%) and detection of IL-2 (75.0%, 97.1%) and IFN- γ (75.0%, 100%) (Kumkamthornkul et al., 2018). However, it was consistently found that combined detection of IL-5 and IFN- γ production could increase the sensitivity of detection from 10.0% to 45.5% for the identification of AEDs as culprit drugs in SJS/TEN patients, as shown in Table 2.

Higher production of IL-4 from PBMCs of DHR patients, when compared with that from control PBMCs, were reported as another parameter to identify culprit drugs (Polak et al., 2013; Haw et al., 2016). Production of IL-4 released from PBMCs of DRESS patients can indicate pathogenesis of DRESS, whereas it is of less utility in identifying pathogenesis in SJS/TEN patients for unknown reasons. It has been proposed that both CD4+ (T helper) and CD8+ (T cytotoxic) lymphocytes can differentiate into type 1 (producing IFN- γ) or type 2 (producing IL-4) cells (Gaspard et al., 2000). This proposal was revealed in a study by Haw and colleagues, in which they performed ELISpot and demonstrated that drug-specific T cells from SJS/TEN patients could produce significant amounts of IL-4 and IFN- γ (Haw et al., 2016). Our study consistently demonstrated that detection of IL-4 production was useful for culprit drug identification in cultures of PBMCs obtained from epilepsy patients suffering from DRESS and SJS/TEN with a sensitivity and specificity comparable to that of LTT (Kumkamthornkul et al., 2018). Interestingly, combined detection IL-4 along with either IL-2 or IFN- γ could yield a higher sensitivity of detection when compared to individual cytokine detection; however, the specificity of combined detection was slightly lower. The higher efficacy of the IL-4 combined cytokine detection was observed in cultures of PBMCs harvested from both DRESS and SJS/TEN epilepsy patients.

Hence, the detection of the production of multiple cytokines could be considered as a promising diagnostic tool in delayed DHRs, especially in SCARs. While detection of multiple cytokine production could also increase the sensitivity of identification of culprit drugs in SJS/TEN patients, it still yields poor specificity. Hence, many groups of researchers have tried to make use of other cytokines in order to obtain higher sensitivity and specificity relative to that of standalone methods in the case of SJS/TEN as summarized in Table 2. As granulysin and granzyme B have been well known as effector/mediators for SJS/TEN pathogenesis, their use may also help to further characterize the reactions involved in pathomechanisms. (Porebski et al., 2013; Chung et al., 2015; Chu et al., 2020).

In spite of their promise, cytokine detection assays have not been employed for routine service yet because of the lacking of standardization (*i.e.* different kits from different companies with different times to collect culture supernatants) and accepted consensus cut-off points (for each cytokine). With regard to the pathomechanism of delayed DHRs (MPE, AGEP, DRESS, SJS/TEN), how one can select suitable cytokine(s) corresponding to their immune mechanisms as well as insure the validity of results from different laboratories awaits consensus. These points of concern can be attributed to the varying specificity and unreliability of these tests. Many studies with a sufficient number of delayed DHR patients and proper controls are still needed to provide good

Table 3

Efficiency of LTT and cytokine detection assays for the identification of culprit drugs in different delayed drug hypersensitivity reactions.

Combined test	Clinical manifestation	Drug	Sensitivity (%)	Specificity (%)	References
LTT + IL-2	SJS/TEN	AEDs	55.6	98–100	Porebski et al. (2013)
	DRESS, SJS/TEN		75.0, 45.0	88.2, 89.6	Kumkamthornkul et al. (2018)
LTT + IL-4	DRESS, SJS/TEN	AEDs	68.8, 55.0	76.5, 83.3	Kumkamthornkul et al. (2018)
LTT + IL-5	MPE	Beta-lactam	83.3	100	Sachs et al. (2002)
	MPE	AEDs	100	100	Sachs et al. (2002)
	SJS/TEN		55.6	100	Porebski et al. (2013)
	DRESS, SJS/TEN		56.3, 30	91.2, 93.8	Kumkamthornkul et al. (2018)
LTT + IL-10	MPE	Beta-lactam	83.3	100	Sachs et al. (2002)
		AEDs	60	100	Sachs et al. (2002)
LTT + IL-13	DRESS, SJS/TEN	AEDs	56.3, 30	76.5, 77.1	Kumkamthornkul et al. (2018)
LTT + IFN- γ	MPE	Beta-lactam	83.3	60	Sachs et al. (2002)
	MPE	AEDs	60	60–100	Sachs et al. (2002)
	SJS/TEN		55.6	95	Porebski et al. (2013)
	DRESS, SJS/TEN		75.0, 45.0	91.2, 93.8	Kumkamthornkul et al. (2018)
LTT + Granulysin	SJS/TEN	AEDs	66.7	100	Porebski et al. (2013)

AEDs; anti-epileptic drugs.

validation before routine implementation.

3.3. Combined LTT and cytokine detection assays: An answer to an unmet need?

During T cell activation, T cells undergo functional differentiation and produce distinct cytokine patterns that depend on different activation signals, such as drug-MHC-TCR signals and bystander inflammatory signals. The cytokine patterns further determine the patterns of effector phase of T cell function. Particular patterns in the effector phase could be revealed by *in vitro* tests that could detect different parameters such as surface marker up-regulation, cytokine production, predominant proliferation, and cytotoxic mediator production, which reflect effector phase differentiation (Sachs et al., 2002; Porebski et al., 2013; Kumkamthornkul et al., 2018). Therefore, standalone methods might not be sufficient to provide corresponding parameters for different effector phases. The use of combinations of parameters provided by different detection assays could yield higher competency for culprit drug identification in different clinical manifestations as summarized in Table 3.

As mentioned in the earlier section, measurement of IL-5 production was demonstrated as a promising method for the diagnosis of drug hypersensitivity. Furthermore, the combination of LTT and IL-5 detection was demonstrated as a better modality of *in vitro* testing to identify culprit drugs when compared with standalone LTT (Sachs et al., 2001; Porebski et al., 2013). Additionally, studies by our group demonstrated that IL-4 detection using multiplex immune bead assays could enhance the sensitivity of culprit drug identification when it was combined with LTT for DRESS and SJS/TEN patients. This was consistent with the findings reported by Polak and colleagues using an IL-4 ELISpot assay instead (Polak et al., 2013; Kumkamthornkul et al., 2018). It was suggested that combined LTT and IL-4 detection assays might be more beneficial than standalone LTT when high sensitivity is required. Therefore, cytokine detection assays might be a promising tool to supplement LTT for the identification of culprit drugs in drug-hypersensitivity diagnosis because they measure effector cell functions that are distinct from proliferation.

4. Concluding remarks and future

Reaching the goal of definite diagnosis of delayed DHRs requires the recognition of the characteristics of drug hypersensitivity and the ability to identify a culprit drug. The characteristics of delayed drug hypersensitivity could be obtained from the patient's history and clinical manifestations, whereas, identification of the culprit drug is a tough task requiring in-depth investigation. Assessment algorithms provide only a rough result, while *in vivo* testing could confer more risk to patients as it requires that they be re-exposed to the suspicious drug. Therefore, *in*

vitro tests are promising assays that could be used to identify the causative drug in delayed DHRs. Selection of the appropriate *in vitro* tests corresponding to the mechanisms involved in delayed drug hypersensitivity is important. Additionally, the usefulness of these *in vitro* methods depends on performing the test in well-characterized patients with DHR. LTT has been in use for a long period and it is a quite useful assay to identify culprit drugs in drug allergy work-up processes that could allow patients to avoid *in vivo* investigation. The LTT is the best standardized *in vitro* method available and has excellent specificity. As described in the article "Lymphocyte transformation test: History and current approaches", the classical LTT has been modified with various proposes to increase sensitivity such as co-cultivation with drug-pulsed antigen-presenting cells, depletion of regulatory T cells, and pre-incubation with proinflammatory cytokines (IL-1/IL-15). (Sachs et al., 2021). Additionally, a flow cytometry-based assay for determining T cell proliferation using BrdU or a fluorescent dye, as well as enumerating activated T cells using activation markers (CD69 and CD 107a), could be a feasible alternative to the LTT (Porebski et al., 2011; Sachs et al., 2021). Nonetheless, the problem is that drug hypersensitivities rely on different and complex pathomechanisms, thus a single diagnostic test most likely will not cover all possible reactions, even if there were many approaches that enhance the sensitivity and specificity of LTT. It has therefore been suggested that different tests be combined to increase the overall sensitivity (Porebski et al., 2013; Kumkamthornkul et al., 2018). With regard to *in vitro* tests, LTT could be supplemented with various *in vitro* detection assays for evaluation of different cytokine/mediator production patterns that correspond to effector immune responses in delayed drug hypersensitivity. While these tests seem to be promising, they still need further evaluation.

Declaration of Competing Interest

The author declares no conflict of interest.

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When a drug (parent or metabolite) activates a drug-specific T cell clone and its activation signals can overcome the threshold of T cell, the

drug-specific T cell clone should respond in accordance with T cell functions, which include cytokine production and cell proliferation. There are numerous techniques for detecting T cell responses. Numerous modalities have been used to detect cytokine production when a drug-specific T cell clone is re-exposed to the culprit drug *in vitro*, including ELISA, ELISpot, flow cytometry-based bead assay (CBA), and multiplex bead-based immunoassay (Luminex). Proliferation, on the other hand, is another function of the T cell response that has been used to assess the responsiveness of drug-specific T cell clones. The classic lymphocyte transformation test (LTT) and a modified LTT were developed based on the T cell proliferation principle. Many techniques have been used to determine T cell proliferation, including ³H-thymidine consumption, bromodeoxyuridine (BrdU) incorporation, fluorophore diluting, and flow cytometry enumeration of activated T cells. Due to the low sensitivity of T cell proliferation methods under certain conditions, it has been proposed to combine the T cell proliferation test with the detection of specific cytokine production to increase sensitivity. Nonetheless, this approach has been tested in the laboratory.

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