Lymphocyte Transformation Test in Immune-Mediated Cutaneous Adverse Drug Reactions

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Adverse Drug Reactions and Allergic Classification

Adverse drug reactions (ADRs) have been classically categorized into type A and type B reactions.[1,2] The majority of ADRs, approximately 80%, are classified as type A reactions, which are related to the pharmacologic activity of the drug and are considered to be predictable. For example, the side effect of non-steroidal anti-inflammatory drugs (NSAIDs) treatment can lead to gastrointestinal bleeding or some first/second generation antihistamines cause drowsiness. A minority of side effects are type B reactions, which are normally unrelated to the pharmacologic activity of the drug and are considered to be unpredictable. They include idiosyncratic, drug intolerance and drug hypersensitivity (allergic) reactions.[3] The allergic reactions have further been subclassified into four categories based on the immune mechanisms involved: (1) IgE mediated reactions (2) antibody-mediated cytotoxic reactions (3) immune-complex mediated reactions (4) delayed type hypersensitivity reaction (DHR).[4] Recent immunological data indicate that T cells may play an essential role on DHR and that delayed type hypersensitivity reactions or type IV reactions can be further sub-categorized according to their cytokine profiles and activation of various inflammatory cell types into four groups.[5]

Type IVa: This involves Th1 cytokines, especially IFN-γ. It will be secreted by drug stimulated T cells to activate macrophages and stimulate the production of complement fixing antibody isotypes. It also induces the production of pro-inflammatory cytokines and boosts CD8+ T-cell responses. Consequently, there is often an overlap with cytotoxic T cell reactions.

Type IVb: This corresponds to Th2 immune responses with IL-4, IL-13 and IL-5 cytokine production. The Th2 cytokine profile will promote IgE and IgG4 production as well as mast cell and eosinophil responses. The characteristic eosinophilic inflammation due to high IL-5 production can be found in many drug hypersensitivity reactions.[6] An eosinophil-rich maculopapular exanthema is a common example of this reaction type.

Type IVc: T cells can act as effector cells and they can kill keratinocytes or hepatocytes in a perforin/granzyme B, granulysin and/or FasL dependent manner.[7] Often Th1 cells are also activated. Cytotoxic T cells play an immunopathological role in maculopapular or bullous skin diseases, in contact dermatitis and hepatitis or nephritis. Of note is the fact that such cytotoxic reactions can be found in most drug-induced delayed hypersensitivity reactions, mostly together with monocyte, eosinophil or neutrophil recruitment. Not only CD8+ T cells function as cytotoxic effectors cells, but also CD4+ T cells can mediate cytotoxicity, albeit to a lower degree than CD8 cells. In severe bullous skin reactions such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), keratinocytes are killed by activated CD8+ T cells, and a massive accumulation of CD8+ T cells are found in the blister fluid of SJS/TEN.[8,9]

Type IVd: Sterile neutrophilic inflammation is also driven by T-cells. Examples are acute-generalized exanthematous pustulosis (AGEP). It leads to sterile neutrophilic inflammations of the skin and is due to secretion of CXCL8 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) from activated T cells.[10] The chemokine CXCL8 will recruit neutrophils, and GM-CSF will prevent apoptosis of neutrophils. These T cell reactions are also found in Behçet disease and pustular psoriasis.[11]

Severe immune-mediated Cutaneous Adverse Drug Reactions and Allergic Reactions

Severe immunologically mediated cutaneous ADRs (SCARs) which include SJS/TEN, AGEP, and hypersensitivity syndrome (HSS; also called DiHS or DRESS) are on one hand separate diseases. Consensus from various groups outlined an algorithm for the standardization of SCARs. The exclusive goal of this algorithm is to facilitate a clinician to categorize patient recruitment with accurate diagnosis. However, a small percentage of patients may show clinically overlapping features, e.g. DRESS and SJS, or SJS and AGEP. The Phenotype Standardization Project was launched to improve and clarify those SCARs based on genetic typing.[12]

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TABLE 1. Sub-type of delayed type hypersensitivity, mechanisms and cutaneous manifestations

Type IV	T cell type	Immune mediators	Inflammation or Effector mechanism	Clinical symptoms (example)
IVa	Th1 cells	IFN-, TNF-	T cells, macrophage activation	Tuberculin reaction Part of contact dermatitis
IVb	Th2 cells	IL-5, IL-4, IL-13	Eosinophilic inflammation	Maculopapupar and bullous exanthema with eosinophilia
IVc	Cytotoxic T cells Fas/FasL	Perforin/Granzyme B killing/apoptosis	CD4+/CD8+ mediated T cell	Bullous eruption (SJS/TEN) Part of contact dermatitis
IVd	T cells	CXCL8, GM-CSF	Neutrophilic inflammation	Acute generalized exanthematous pustulosis Behçet disease

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Stevens-Johnson syndrome (SJS) and Toxic Epidermal Necrolysis (TEN)

SJS and TEN are considered to be the same condition of type IVc of allergic reactions, but different severities.[1] The degree of skin detachment is used to distinguish SJS and TEN.[12,13] When skin detachment with blistering affects between 1 and 10% of the body surface area (BSA), it is referred to SJS. If degree of skin loss with blistering is between 10-30% of BSA, one postulates SJS/TEN overlap syndrome, If the degree of skin loss with blistering affects over 30% of BSA, it is called TEN. In addition, the involvement of at least one mucosal membrane as well as appropriate temporal usage of implicated drugs are included in diagnosis. Other manifestations include fever, elevation of liver enzymes, involvement of internal organs and target lesions predominantly on trunk and face. The incidence of SJS is approximately 1-6 cases per million a year, whereas that of TEN is around 1-2 cases per million. The mortality rate in SJS is around 5-10%, but it is estimated to be as high as 50% in TEN.[14] Antimicrobial sulfonamides, anticonvulsants, allopurinol, nevirapine, and NSAIDs (such as piroxicam) are commonly prescribed drugs associated with SJS/TEN. The onset of SJS/TEN occurs mostly within 3 weeks (mean 17 days) after the initiation of drug treatment (67% of cases), but manifestations of SJS/TEN can also occur within the first 8 weeks of drug exposure. In vivo test (patch test) and in vitro test (lymphocyte transformation test, LTT) are rarely positive in SJS/TEN and help in the identification of the eliciting drug in only 20% of patients.

Acute generalized exanthematous pustulosis (AGEP)

AGEP was first described as exanthematic pustular psoriasis as a member of neutrophilic dermatoses in 1968.[15] Later it was distinguished and found to be mostly due to drugs, exposure to mercury or acute viral, bacterial or parasitic infections. However, approximately 90% of the cases are associated with drugs.[16] Antibacterial drugs, especially β-lactam antibiotics, and carbamazepine anticonvulsant are common etiologies.

An incidence is approximately 1-5 cases per million annually, with no distinction in any age group or gender. Nevertheless, a mortality rate of 1-2% has been reported in elderly patients with chronic diseases.[16] It is a self-limiting disease with resolution occurring within 5-15 days. It is an acute widespread edematous erythema followed by sterile small non-follicular, intraepidermal or subcorneal pustules (<5 mm) on an erythematous background. The pustules which are first seen are often localized in the neck, groin, and axillae and become widely disseminated.[12] Fever is common due to systemic inflammation.

The pathophysiology of AGEP is different from SJS/TEN. In AGEP drug-specific T cells produce interleukin-8/CXCL8 and GM-CSF, which leads to neutrophilia with blood neutrophil counts > 7×10^9/L.[13] Early diagnosis of AGEP and differentiation from other diseases (e.g. generalized pustular psoriasis) can prevent this subgroup from unnecessary treatment (including retinoids and immunosuppressive therapy). In vivo test, patch test, in vitro test and LTT may be helpful in identifying the culprit drug. Patch-testing may even elicit pustular skin alterations.

Hypersensitivity syndrome (HSS)

The terminology of this syndrome is complicated: HSS, drug-induced hypersensitivity syndrom (DiHS) and drug reaction with eosinophilia and systemic symptoms (DRESS) are the most common ones.[12] HSS is a severe adverse reaction associated with significant morbidity and mortality. The incidence of this syndrome was estimated to range from 1 in 1,000 to 1 in 10,000 drug exposures with a significant mortality rate up to 10%.[17] Anticonvulsants (carbamazepine, phenytoin, phenobarbital), allopurinol, salfasalazine, and some anti-retrovirals (nevirapine) are major culprit drugs associated with HSS.

This syndrome is quite different from SJS/TEN, although a) some drugs can cause both reactions, and b) 1-2% of patients show an overlap syndrome. The pathogenesis still requires more work. Skin is frequently involved, with variable manifestations. The most common skin presentation is an exanthematous eruption, whereas urticarial plaques, exfoliative, pustular eruptions, and facial edema also occur. The disease can develop in (2-6 often observed) 12 weeks. It persists or even aggravates the patient despite the discontinuation of the causative drug.[18] The recovery phase is prolonged despite early culprit drug withdrawal.[12] Fever is common with >38°C. Internal organs are often involved as well as hematological abnormalities such as eosinophilia and atypical lymphocytosis are typical. Herpes viral reactivation, especially HHV-6, has also been postulated as an associated factor.[18,19] Because of the highly variable presentation, some consensus criteria were elaborated.

The Japanese consensus group on DiHS defines DiHS as follows:

(1) Maculopapular rash developing >3 weeks after starting with a limited number of drugs,

(2) Prolonged clinical symptoms 2 weeks after discontinuation of the causative drug

(3) Fever (>38°C)

(4) Liver abnormalities (alanine aminotransferase >100 U/L)

(5) Leukocyte abnormalities (at least one present)
(5a) Leukocytosis (> $11x10^9$/L)
(5b) Atypical lymphocytes (>5%)
(5c) Eosinophilia (>$1.5x10^9$/L)
(6) Lymphoadenopathy
(7) HHV6 reactivation

This group suggested that diagnosis of typical HSS is confirmed if seven criteria are met and atypical HSS can be diagnosis if criteria 1-5 are present.[18]

The Phenotype Standardization Project also suggested employing positive LTT, positive patch test, or the evidences of viral reactivation to support the diagnosis.

Supportive Diagnosis

Although the diagnosis of SCARs is based on the history, clinical manifestation and criteria mentioned above, additional tests have also been used to identify the causative substance. This is particularly important, as in the case of severe reactions like DRESS/DiHS, SJS/TEN and AGEP, so the drug provocation test should be avoided.[16,20]

There is agreement that T cell reactions play an important role in these diseases. Consequently, many in vitro tests like cytokine measurement and LTT to detect T-cell sensitization to drugs have been applied to elucidate the cause of SCARs.[21] The following paragraphs describes the advantages and disadvantages of the LTT and its variants in the diagnosis of drug allergy.

Lymphocyte Transformation Test in Drug Hypersensitivity Reactions

In principle, this test is based on the existence of drug-specific memory T-cell precursors able to proliferate upon re-stimulating with antigen.[20] Practically, peripheral blood mononuclear cells will be obtained from a sensitized patient and cultured in the presence of the suspected drug. Drug-specific lymphocytes undergo a proliferative response, which can be measured by means of the incorporation of 3H-thymidine during blastogenesis. The level of cell proliferation stimulated with antigen/drug can be expressed as a stimulation index (SI) which is related to a spontaneous background of cell proliferation. The important point is that drug-specific memory T cells need to be present in sufficient amounts in the circulation, and that the drug is available in a stimulatory form in vitro.

Sensitivity and specificity

The reliability of a test depends on its sensitivity and specificity. Sensitivity measures the proportion of actual positives which are correctly identified as such. Specificity measures the proportion of negatives which are correctly identified (e.g. the percentage of healthy people who are correctly identified as not having the condition). The problem relies in the correct identification of true positives, less so in the identification of true negatives. Unfortunately, identification of true positive standards for the LTT have not been developed. As mentioned earlier, a provocation test is assumed to be the gold standard for a drug allergy test. However, many limitations of a provocation test have to be considered and the test can give false positive or false negative results. In particular, the timing and duration of provocation tests for drugs causing delayed reactions are unclear, or are too dangerous to be performed (hepatitis, SJS/TEN, etc).

Evidences for a major role of T cells in the immunopathology of allergic drug reactions are positive patch test and the LTT. Therefore, standardized skin patch test and clinical criteria could be employed to evaluate the diagnostic potency of LTT. Nevertheless, not all patients with clearly positive skin tests have actually also a positive LTT.[20,22] Hari et al., showed in a prospective study that the sensitivity of LTT (67%) was higher than that of the skin patch test (50%).[22] However, when the patch test and LTT are combined, they can yield the sensitivity of 76%. These data have suggested that a patch test with LTT can provide a better sensitivity than LTT alone, which nevertheless can provide substantial sensitivity. This study indicated that the LTT and patch test may share primary roles for determination whereas they are not completely overlapping. Another retrospective study, proposed a RegiSCAR scoring system which was employed to categorize well-documented patients and analyze with the results of LTT.[23] According to the scores, LTT gave a sensitivity of 78% in the highest probability cohort. They also observed that the group of lower likelihood patients had a lower level of positive LTTs as well as the lowest incidence of positive LTT was found in unlikely patients. This study demonstrated that the clinical imputability criteria significantly correlated with LTT results. Taken together, the clinical criteria may need either LTT, or LTT complimented with patch test to support each other for diagnosis.

As shown in many literatures, specificity has to be concerned with the pharmacological activity of certain drugs to induce spontaneous T cell proliferation with unknown mechanisms.[20,23-25] For example, sulfamethoxazole can elicit unstable positive T cell proliferation in T cell lines from non-exposure donors (our observation, unpublished data). Many reports showed that various specificities of LTT seemed to depend on certain drugs evaluated. Evaluation of carbamazepine and lamotrigine hypersensitivity showed specificities of 100% repeatedly.[26,27] Also the specificity of LTT for -lactam hypersensitivity was evaluated as 98%.[28] Regardless to the drugs, above-mentioned, retrospective analysis using the RegiSCAR system can infer that the specificity of LTT was 85%.[20,23] Taken together, the overall specificity of LTT is at least 85%, whereas a higher value can probably be expected which depends on the drug investigated.

Timing of LTT performance

Memory T-cell response is subjected for measurement in LTT, since it could be positive for 10-20 years after the original treatment with certain drugs. During the acute phase, the majority of immune cells, in particular T cells, are strongly activated. For that reason, it is better to perform the test after remission meaning 4-8 weeks after the reaction.[20] Kano et al., demonstrated that LTT should be performed within one week after the onset in cases of maculopapular exanthem and SJS/TEN.[29] However, this report omitted to show non-specific responses against unrelated drugs to exclude bystander effects of drugs in cell cultures obtained from the acute disease status. It postulated a defect in regulatory T cells in the acute period.[30] More studies are needed to support the performance of LTT in the acute state. Immunological memory can persist for a long time, although some patients seem to lose drug-specific T cell precursors within three or four years. Since it is uncertain how long T cell memory persists, it has been recommended to perform LTT within 2-3 years after the reaction.[20]

LTT and SCARs

The aim of LTT is to detect circulating drug-specific

TABLE 2. Frequency of positive LTT found in SCARs

Often positive (> 50%)	Occasionally positive	Rarely positive (< 10%)
AGEP	Urticaria	SJS/TEN
DRESS	Angioedema	Fixed drug eruption
Generalized maculopapular exanthema		Macular exanthema (without T cell infiltration)
Bullous exanthema		
Anaplyxis		

Modified from : Pichler WJ and Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. Allergy 2004;59(8):809-20.

T cells. It not a mirror of the precursor frequencies of drug specific T cells and the stimulations of T cells with distinct functions may lead to quite different SI. Therefore, the values of SI in the LTT are not always associated with the severity of clinical symptoms.[20] Theoretically, LTT seems to be useful in all types of delayed hypersensitivity reactions like generalized exanthema (maculopapular, bullous, pustular), and LTT has been shown to be frequently positive in DRESS and AGEP as shown in Table 2.[20] Additionally, LTT has been employed in cases of patients with immune-mediated inflammations of internal organs such as liver, lung, and kidney, to prove whether the internal organ damage is caused by drug induced immune-mediated pathogenesis.

A major concern is that some drugs may become immunogenic only due to organ specific enzymes, which can locally generate reactive metabolites, which are not present in the blood cells used for LTT. In SCARs (SJS/TEN), positive results of LTT are quite rare in normal routine investigations after remission.[20,31] Possibly the removal of Treg may enhance the reactivity in SJS/TEN patients.[30] In this context, it is interesting that we observed that removal of regulatory T cells yielded better (higher) results of LTT in non-SJS/TEN patients with clear histories, but negative or borderline LTT results in non-SJS/TEN patients (unpublished data). Treg cell removal also enhanced the reactivity to drugs in multidrug hypersensitive patients.[32] Negative results of LTT are quite consistent in patients with fixed drug eruption. One reason could be that the disease is not associated with many drug-specific T cells in the circulation. Interestingly, LTT can also be positive in patients with IgE-mediated reactions-like severe anaphylactic reactions, if the causative agent was a hapten having caused a drug specific *T- and B-cell* response. In conclusion, LTT is still being used as a diagnostic test to determine causative agents with high sensitivity and specificity in drug hypersensitivity.

Conclusion Remarks

Among many in vitro tests, LTT has been widely used to determine the causative agent in drug hypersensitivity syndrome. This test is able to detect T cell responses, which play a key role in delayed type hypersensitivity upon drug stimulations. LTT is a safe test when compared with a provocation test. Additionally, LTT can provide insights into the pathogenesis of drug reactions (cytokine release, cytotoxicity). It is more sensitive and has a comparable specificity compared to in vivo patch test. Reliability is underlined by substantial reproducibility.

However, the LTT has some drawbacks, because its sensitivity is highly dependent on optimal culture conditions. Laboratory personnel experienced with cellular immunology, expensive chemicals and equipment, and sufficient background information about pharmacology and immunology are needed to interpret the results, correctly. Some reactions are not well detectable in LTT (SJS/TEN), and alternative tests need to be developed. In spite of the limitation of this test, LTT is still a promising diagnostic test for drug hypersensitivity.

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