

Review article

The lymphocyte transformation test in the diagnosis of drug hypersensitivity

Diagnosis of drug hypersensitivity is difficult, as an enormous amount of different drugs can elicit various immune-mediated diseases with distinct pathomechanism. The lymphocyte transformation test (LTT) measures the proliferation of T cells to a drug *in vitro* – from which one concludes to a previous *in vivo* reaction due to a sensitization. This concept of the LTT has been confirmed by the generation of drug-specific T-cell clones and the finding that drugs can directly interact with the T-cell receptor, without previous metabolism or need to bind to proteins. In this review, technical aspects and usefulness of this test for the diagnosis of drug hypersensitivity are discussed. The main advantage of this test is its applicability with many different drugs in different immune reactions, as drug-specific T cells are almost always involved in drug hypersensitivity reactions. Its main disadvantages are that an *in vitro* proliferation of T cells to a drug is difficult to transfer to the clinical situation and that the test *per se* is rather cumbersome and technically demanding. In addition, its sensitivity is limited (for β -lactam allergy it is in the range of 60–70%), – although at least in our hands – it is higher than of other tests for drug hypersensitivity diagnosis. Consequently, drug hypersensitivity diagnosis needs to rely on a combination of history and different tests, as none of the single tests available has *per se* a sufficiently good sensitivity. Within this setting, the LTT has proven to be a useful test for the diagnosis of drug hypersensitivity reactions and helped to better understand these reactions. Further work on the simplification of this test and systematic evaluation of its sensitivity and specificity in some main groups of drugs are necessary to make this test more widely available.

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Drug hypersensitivity reactions account for one of six of drug-induced adverse side-effects. They are due to different mechanisms, which can lead to a great variety of distinct diseases such as anaphylaxis, maculopapular, bullous, pustular or urticarial exanthemas, Stevens Johnsons-syndrome (SJS) and toxic epidermal necrolysis (TEN), interstitial lung or kidney disease, hepatitis, pancreatitis and different forms of blood cell dyscrasias and autoimmune reactions (1–4). While the role of drug-specific IgE in anaphylaxis and related diseases as well as the role of drug-specific IgG and IgM antibodies in immune-mediated blood cell dyscrasias has been well-established, the pathomechanism underlying many drug-induced diseases affecting the skin, liver, kidney, lung etc. has for a long while been enigmatic. A role for T cells has been inferred by analogy to contact dermatitis and some immunohistological data, where a preponderance of T cells was found. However, how T cells recognize drugs and how they contribute to the pathology has only recently been better understood (1, 3–10).

T cells take a central role in organizing the immune defence and are practically involved in all types of immune reactions either by orchestrating the type of immune response or as effectors themselves: antigen-specific T cells secrete cytokines and thus participate in IgE-mediated reactions by secreting the cytokines IL-4 and IL-13, in eosinophilic inflammation by secreting IL-5, in neutrophilic inflammation by secreting IL-8 (CXCL-8) and GM-CSF and in monocyte/macrophage-rich inflammation by secreting IFN γ , TNF α and other cytokines (2, 11, 12). All these effects can also be observed in drug hypersensitivity or were even discovered there (1, 2, 6). In addition, in many drug hypersensitivity reactions T cells are actively involved as cytotoxic effector cells by killing tissue cells-like keratinocytes or hepatocytes (2, 7, 8).

In this review, we will focus on the value of the lymphocyte transformation test (LTT, synonyms are lymphocyte proliferation or stimulation test), and its variants in the diagnosis of drug hypersensitivity reactions. The review is based on published studies, some case

reports and our own experience with ~ 8000 LTTs performed during the last 20 years.

General problems of diagnosing drug hypersensitivity

The diagnosis of drug hypersensitivity reactions addresses three questions:

- Is it a drug hypersensitivity reaction?
- Which type of immune reaction is involved? and
- What is the eliciting drug?

In this review, we will mainly focus on tests to pinpoint the relevant drug. This aspect of drug allergy diagnosis is thought to be difficult as:

- Some drugs might affect the innate immune system and/or effector cells such as basophils directly (so-called pseudoallergic or nonimmune-mediated hypersensitivity reactions), without demonstrable involvement of the specific immune system. Such reactions are by definition test negative, but the symptoms are similar to reactions triggered by the involvement of the specific immune system.
- Different mechanisms may underlie the reaction [antibody- or T-cell mediated (1–4)]. Diagnostic tests are dependent on the type of immune reaction, e.g. in contact dermatitis, prick tests detecting drug specific IgE have a doubtful value, and in an IgE-mediated reaction like anaphylaxis a positive patch test, which demonstrates a delayed, inflammatory response to the drug, is difficult to interpret, since it does not fit to the clinical picture of immediate reactions. On the contrary, one has to be aware that different immune mechanism may occur simultaneously in drug hypersensitivity reactions: Th1 and Th2 reactions can occur together (10, 13), and detection of drug-specific IgE may be found in a patient with maculopapular drug eruption and a strong patch test reaction. Such findings are difficult to interpret in the individual patient and indicate only that a sensitization has occurred.
- A Pandora's box of different drugs can elicit many different symptoms. Moreover, one has to consider that sometimes not the drug but some component within the tablet or a metabolite might be responsible for the reaction (14). It is practically impossible to have available standardized and validated tests for all drugs causing hypersensitivity reactions. Indeed, there are only very few commercial tests on the market to detect a sensitization to a drug. The few tests used regularly are mainly focusing on IgE-mediated reactions, but do not detect T-cell or IgG-mediated reactions.
- Last but not least, drug hypersensitivity reactions are a very common problem in general, but are still rare for each single drug. Thus, clinical experience with a specific class of drugs and side-effects is difficult to achieve – with the exception of β -lactam antibiotics.

T-cell recognition of drugs

T cells are able to be stimulated by small chemicals such as drugs. As drugs were considered to be too small to be immunogenic *per se*, immunogenicity was thought to rely on their ability to bind to larger molecules-like proteins or peptides (1–4, 14). This implies that the drug acts like a hapten and binds covalently to a peptide or protein, which makes it immunogenic. For a long time it was an enigma and the main reason for a certain scepticism against the LTT that a chemical inert drug, unable to act as hapten, cannot stimulate T cells in a specific way. Thus, positive reactions were seen as an *in vitro* artefact because of some stimulatory action by the drug. However, this could not explain that the drug was only stimulatory in certain patients. Moreover, newer findings proved that the LTT detects drug-'specific' T cells:

- Persons tolerating the drug do normally not show an enhanced proliferation to the drug. In sensitized individuals, only a small fraction of T cells are reacting to the drug as revealed by double-staining T-cell subsets and activation markers after drug stimulation (15, 16).
- We and others were able to clone drug-specific T cells from patients with drug hypersensitivity reactions and positive LTT, demonstrating the specificity of the T-cell reaction to the drug and structurally related compounds (5, 16–18).
- Recently we found that the drug itself can interact with the T-cell receptor directly (pharmacological interaction of drugs with immune receptors, so-called p-i-concept) (19–21). This would explain the feasibility to perform LTT with peripheral blood mononuclear cells (PBMC), where the metabolizing potential and the ability to transform an inert drug (prohapten) to a chemical reactive drug (hapten) may be absent for most drugs.

Read out system for T-cell reactivity – patch testing

There exist *in vitro* and *in vivo* tests to detect a T-cell reaction: *in vivo*, drug hypersensitivity reactions can be detected by epicutaneous, so-called patch tests. Positive reactions rely on the development of a localized inflammatory response based on activation of drug-specific T cells acting as cytotoxic effector cells and recruitment of inflammatory cells (2, 6, 9, 22–24).

The test is actually done like a test for contact sensitivity: in analogy to contact sensitizers, the drug in solution or petrolatum is put on the skin for 24–48 h (9, 22). However, one should be aware that the drug causing the reaction was taken up orally or parentally and did not sensitize-like a contact sensitizer. The nontoxic concentration has to be established previously by testing

nonallergic persons, including some exposed but not sensitized individuals (22, 25). In highly sensitized individuals a positive reaction develops, which can lead to erythema, cell infiltration, sometimes even vesicular or pustular reactions.

The test relies on a cascade of events (drug penetration through the skin, drug presentation and T-cell recognition of the drug, T-cell infiltration into the skin, recruitment of effector cells into the skin, etc.) with formation of papules, vesicles, pustules. It is actually quite astonishing that the application of a drug to the skin can elicit such a localized inflammation – and it is probably only positive if the immune reaction to the drug is rather strong. Its overall sensitivity may thus vary dependent on the type of drug tested (ability to penetrate the skin, etc.) as well as on the type of immune reaction. The more inflammatory components the hypersensitivity reaction had (e.g. generalized maculopapular eruption, bullous eruption, etc.) the higher is the chance to detect a reaction with this skin test. A mainly vascular response (e.g. only transient erythema with or without urticaria, but without T-cell infiltration) will in most instances not be associated with a positive test result, even if it appeared delayed. Moreover, if the drug needs to be metabolized in the liver to become immunogenic, the test may remain negative as well.

There might be some difficulty to differentiate allergic from toxic reactions, but in general this test is in good agreement with drug reactions with high imputability and with other test systems [LTT (13, 26)]. This test may reach a sensitivity of about 50%, sometimes even higher with certain drugs (22), if done in patients with severe reactions and if a high imputability is taken as ‘gold standard’, as provocation tests with skin test positive patients are rarely done. However, this test can clearly be false negative because of the above-mentioned reasons. This relatively low sensitivity, the lack of standardized test reagents and thus of experience with this test in many centres are the main reasons why this test is still not widely used.

At present, a positive patch test is a highly reliable indicator of a sensitivity leading to an inflammatory reaction in the skin, while a negative test does not exclude a hypersensitivity reaction.

Read out system for T-cell reactivity – the lymphocyte transformation test

The detection of drug-specific T cells *in vitro* has a different meaning than the elicitation of a localized, T-cell-mediated inflammatory response by patch testing *in vivo*. The *in vitro* test is simply measuring the activation of T cells by different means. Interleukin (IL)-2 secretion and proliferation is a key feature of many types of T-cell reactions (Th1, Th2, Th0). A particular advantage of such an *in vitro* system measuring T-cell reactions to drugs is its potential to detect both the ‘conductor’ as well as the

key players of the ‘orchestra’ (=the immune system). The disadvantage of this rather general approach to hypersensitivity is, however, that the clinical relevance of detecting drug reactive T cells in a certain drug hypersensitivity reaction is often unclear because its relationship to the actual clinical picture is rather indirect, e.g. what means the detection of proliferating T cells *in vitro* if the clinical picture was anaphylaxis, where IgE antibodies mediate the effector phase?

The encounter of a T cell with its relevant antigen (peptide or drug) presented by MHC-molecules is a complex process which leads to a cascade of events, which can be measured by different means reflecting the different steps needed for full T-cell activation: using immunofluorescence analysis, up-regulation of surface markers-like CD69, CD25, HLA-DR and others can be measured with simultaneous identification of the reactive cell type (15, 16). Activated T cells produce cytokines, which can be measured intracellularly or in the supernatant of stimulated cell cultures by enzyme-linked immunosorbent assays (ELISAs). It has been postulated that drug-specific T cells secrete a high amount of IL-5 (18, 27), and that measurements of IL-5 might be more sensitive than measurements of proliferation (28). Under certain circumstances, specific cytotoxicity can be analysed (19, 29, 30).

The test most widely used to detect a T-cell sensitization to drugs is the proliferation test, which measures ³H-thymidine uptake of dividing cells. It has been in use for more than three decades. It relies on the observation that specific T cells divide and expand after encountering the antigen. However, the usefulness of this test for drug hypersensitivity diagnosis has been debated for various reasons (see above). In particular, many laboratories do not obtain a sufficient sensitivity of this test, and only few groups use it routinely. Since we use this test for many years both for drug allergy diagnosis as well as for a better understanding of hypersensitivity reactions to drugs, we would like to summarize our experience with this test.

Technical aspects of LTT

The blood has to be anticoagulated: we do it with heparin [e.g. Liquemin (Roche, Basel, Switzerland) 5000 U, 0.1 ml/10 ml blood]; ethylenediaminetetraacetic acid (EDTA) or another anticoagulation is also working for rapid processing, but we have no experience with the stability of such anticoagulated blood probes.

The principle of the LTT follows the rules of a simple proliferation test with a protein antigen. Peripheral blood mononuclear cells are separated over a density gradient (Ficoll/Hypaque, Amersham Bioscience, Uppsala, Sweden). Care should be taken to avoid a too high content of macrophages (> 25%) within this population of mononuclear cells, as they might produce high amounts of PGE₂, which may suppress T-cell proliferation (31).

The cells in a density of $2 \times 10^6/\text{ml}$ are put in flat-bottom wells of microtitre plates (100 μl). We use RPMI-1640 medium supplemented with HEPES-buffer and AB-serum (20%) or autologous plasma (10%): The AB serum has been screened beforehand for its capacity to support the proliferation to protein antigens, and only batches with good supportive quality are used. The autologous plasma is obtained from the density-gradient centrifugation. It is advisable, to culture the cells in both AB-serum as well as autologous plasma. The proliferation to the drug may differ significantly using these two serum supplements – the reason for this is unknown (Table 2).

The drug should be available as a pure substance. Most companies provide the pure substance, other, mainly the USA-based companies are reluctant to do so. Alternatively, one may obtain the substance from Sigma (Buchs, Switzerland) or another provider of chemicals. One should always perform dose-response curves, and rather frequently a clearly positive response is observed with one concentration only. We normally use doses of 1, 10 and 100 μg of the drug. Occasionally, a lower or higher concentration (0.01 μg or 200 μg , 500 μg and 1 mg) can be used as well. The appropriate drug concentration (and its solubility) must be evaluated previously in a so-called 'toxicity tests': different concentrations of the drug were added to phytohaemagglutinin (PHA)-stimulations of three donors: only concentrations, which do not inhibit the PHA-induced proliferation by more than 15% will be used.

It is a tricky issue and exceeds the scope of this review to describe all the solvents used: some drugs are quite lipophilic and difficult to solve in normal aqueous buffer/medium: they have to be solved in dimethyl sulphoxide (DMSO), some drugs are solved only after addition of 1 M NaOH. Thereby stock solutions are prepared and then further diluted in culture medium to adapt the concentration and to dilute the solvent.

If the pure substance of the drug is not available, one can use the content of a capsule or an injectable form of the drug. We used occasionally pills, crushed and solved in medium, but positive results are difficult to interpret and may be due to an artefact. Therefore, positive results need confirmation with the pure substance.

The cell culture lasts for 5 days in a CO_2 ventilated (5%) incubator at 37°C . Thereafter, ^3H -thymidine is added for 10–14 h (=overnight) and the cells will be harvested on day 6 with a cell harvester. We still use the scintillation fluid to detect the β -radiation, but newer, scintillation-free systems are also possible.

Interpretation of LTT-results – stimulation index

Normal, nonallergic individuals as well as drug exposed but nonallergic individuals do not show a proliferative response to a drug. This has been demonstrated by

various groups (26, 32–34) for a variety of drugs and is the control if a new drug is tested in the LTT (minimum three individuals). In addition, the lack of reactivity to an exposed drug is often proven by the results of LTT from patients exposed to many different drugs, where one observes a selective reactivity against only one drug, but not other drugs taken as well (Table 2). But there might be some drugs, which increase ^3H -thymidine uptake by an unknown mechanism (see below).

All tests are regularly performed in triplicates, sometimes quadruplicates. The standard error of these values should be $< 30\%$. The most important part of the test is the control culture without drug: it is the reference culture, as the proliferation of the drug-stimulated cultures has to be put in relation to the background proliferation. Most results are given as stimulation index (SI): the proliferation is measured as ^3H -thymidine uptake, counts per minutes (cpm). This SI is calculated by proliferation (cpm) with drug/proliferation (cpm) without drug. The spontaneous proliferation (cpm values) obtained with different donors differ enormously, which make SI values better comparable and easier to communicate than cpm results.

The proliferative values obtained should always be interpreted by someone familiar with the pitfalls of proliferation tests. If the spontaneous proliferation is high (e.g. > 2000 cpm), it is likely that already activated cells are present in the peripheral blood and divide. This makes it more difficult to increase the SI by adding an antigen. For example, an increment of the ^3H -thymidine incorporation from 2000 (background-control) to 4000 cpm (plus drug) results only in a SI of 2, but adding the antigen lead to an increment of 2000 cpm. Therefore, an SI of 2.0 may mean more than an SI of 3, if the control proliferation was only 400 cpm and the addition of the drug-antigen resulted in 1200 cpm (reflecting an increment of 800 cpm, but yielding an SI of 3.0). In general, it is advisable to rely only on tests, where cpm's in the drug-stimulated culture exceed values of 1000 cpm.

The test is considered positive if a certain SI is achieved. Which SI should be considered as indicative for a sensitization is rather controversial. The SI depends on certain variables-like the number of precursor cells for a drug, the type of reaction (Th1 cells seem to proliferate more than Th2) and the affinity of the T-cell receptor for antigen (TCR) for the drug – as a better fitting TCR may lead to a faster and stronger signal for the T cell and the type of drug analysed.

We use in general an SI > 2 to classify the test as positive, based on negative values in exposed but not allergic individuals (Table 1). Values between SI 2 and 3 are considered as weakly positive. The relevance of such a low proliferation is hard to judge without additional support for a sensitization by other tests or a clear history. But we have already cloned drug-specific T cells from cell cultures which had only a moderate SI of 2.1 (6), which indicates that also a moderate enhancement of

Table 1. Cut-off values – stimulation indices (SI)

β-Lactams	
Negative	SI < 3
Positive	SI > 3
Other drugs	
Negative	SI < 2
Marginally positive/doubtful	SI 2–3*
Positive	SI > 3†

* T-cell clones could be generated from a lymphocyte transformation test (LTT)-culture with an SI of 2.1 (6).

† Frequently false positive: vancomycin, radio-contrast media, tablets.

proliferation could be indicative for a sensitization. If the SI is > 3, the LTT is considered positive, particularly if the test is positive in more than one concentration. We observe quite frequently that the proliferation is strongly enhanced in only one concentration, which might be due to the peculiar binding of drugs to TCR (21) and which emphasizes the need of dose–response curves to obtain a sufficient sensitivity. We have observed an SI of > 60 quite frequently (penicillin G, lidocacin, carbamacepin, phenytoin, sulfonamide, etc., Table 2).

Some drugs are able to elicit an enhanced proliferation even in nonsensitized individuals. Remarkable examples are vancomycin, possibly paracetamol as well as certain radio-contrast media, which might elicit a slightly enhanced proliferation (SI 2–4) in PBMC of certain individuals previously not exposed to it. The reason for this is at present unclear. Some nonsteroidal anti-inflammatory drugs (NSAID) do also slightly enhance the proliferation, which is normally explained by their ability to inhibit PGE₂ synthesis (31). However, this effect is not seen consistently. Some compounds such as diclofenac and pyrazolones might cause ‘real’ allergies as well with a clearly positive LTT (Table 2).

Penicillins like amoxicillin are typical haptens: they modify proteins and these might be stimulatory for some T cells of nonsensitized donors as well and thus elicit a slightly enhanced proliferation in exposed but not sensitized individuals. Thus, for β-lactam reactions we ask for an SI > 3 to be judged as positive (Table 2), and LTT to RCM should have an SI > 4 to be positive.

Cell cultures are rather variable and need to be put in relation to the proliferation to a control antigen. In Switzerland, we use tetanus toxoid (TT, obtained from Berna-Biotech, Bern, Switzerland), a protein antigen, as practically all Swiss are immunized to tetanus. A strong TT-induced proliferation indicates that the proliferative capacity of the isolated cell population is good and it shows that the cells are alive. It also puts in relation the SI obtained with the incriminated drug, e.g. if TT induces an SI > 100, what means an SI of 2.5 against a drug? Alternatively, if TT elicited only an SI of 5, the SI of 2.5 to a drug may mean a rather substantial sensitization, as the culture conditions might be suboptimal.

Interference by drug treatment

Treatment with immunosuppressive drugs might suppress the proliferation *in vitro*. Therefore, it is important to have a positive control included (TT, 5 µg/ml), which normally gives an SI > 5. Corticosteroids are those drugs most frequently interfering with the test results: they cause lymphopenia and the composition of mononuclear cells after density-gradient centrifugation is often suboptimal (low lymphocyte number, many monocytes/macrophages). Moreover, corticosteroids inhibit cytokine synthesis in T cells as well. We therefore perform the test only with blood from patients who take < 0.2 mg/kg prednison-equivalent/d. Other potentially immunosuppressive drugs-like, e.g. methotrexate or azathioprim are less interfering with lymphocyte proliferation and we perform the test if there is no lymphopenia.

Reproducibility

The reproducibility of the LTT can be analysed by different means:

- Different batches of the same drug can be analysed simultaneously with the same blood probe (Table 3);
- cells can be frozen and thawed at different time-points and tested with the identical drugs; and
- one can analyse fresh blood samples of the same donor at different time-points (Table 3).

The comparison of different batches of the same drug gives similar results, indicating that the test at a given time-point in itself is reproducible (Table 3) and that different batches of drugs are comparable. The analysis of frozen cells at different time-points has the advantage that the same batch of cells could be analysed, thus avoiding differences in cell composition at different time-points. We have frozen cells from drug allergic individuals with a strong proliferation to the drug (SI > 20) and repeatedly thawed them over a time period of 3 years: the drug specific proliferation in AB-serum varied considerably (between SI 60 and 35, data not shown). It was also possible to clone drug-specific T cells from frozen batches repeatedly. Nevertheless, the variability of such analysis are large and are influenced by the time of storage at –80 or –196°C, the thawing procedure, some differences in the culture medium and probably other factors.

If the LTT is compared at different time-points with fresh blood probes, the test procedure may have even more variables like, e.g. the level of spontaneous proliferation; the cell distribution (monocyte–lymphocyte ratio), the composition of the autologous plasma (e.g. content of lipids), the length of time before the blood has been worked up, natural course of the immunity, in particular a lower precursor cell frequency due to the time interval from drug allergy diagnosis to work up etc. Still, the analysis of separate blood probes of

Table 2. Instructive examples of lymphocyte transformation test stimulation index (LTT SI)

		Amoxicillin (µg/ml)			Clavulanic acid (µg/ml)			Ciprofloxacin (µg/ml)			Tetanus toxoid (TT) (µg/ml)			
Symptoms		100	200	500	10	50	100	0.01	0.1	1.0	5.0			
EEM*	AB-S-20%	7.8	7.2	13.8	2.8	5.3	2.6	1.2	0.4	1.5	105			
	Autol.PI. 20%	2.5	3.0	1.7	1.2	1.5	1.1	1.4	1.7	2.0	111			
		Cefuroxim (µg/ml)									TT (µg/ml)			
		1			10			100			200			
Anaphylaxie*	AB-S-20%	1.0			1.7			0.5			0.2			
	Autol.PI. 20%	2.9			4.9			2.9			0.4			
		Amoxicillin (µg/ml)			Ampicillin (µg/ml)			Clavulanic acid (µg/ml)						
		100	200	500	200			500			1	10	100	
MPE†	AB-S-20%	2.5	1.6	1.0	1.9	1.1			5.0			10.9	10.4	
		Phenytoin (µg/ml)			Amoxicillin (µg/ml)			Sulfamathoxazol			Trimethoprim (µg/ml)		TT (µg/ml)	
		1	10	50	10	25	10	50	100	200	10	25	5	
DRESS and erythro-dermia‡	AB-S-20%	26.0	72.5	52.1	1.4	1.8	1.4	7.1	24.6	22.7	1.4	1.8	37.8	
		Nimesulid (µg/ml)						Atorvastatine (µg/ml)						
		1			10			50			1	10	50	
Drug-induced hepatitis§	AB-S-20%	1.4			0.8			0.8			6.3			
	Ambol.PI. 20%	1.0			0.7			0.7			6.8			
		Penicillin (µg/ml)			Ampicillin (µg/ml)			Azitromycin (µg/ml)			Paracetamol (µg/ml)			
		200	500	1000	200	500	0.1			1	10	1	10	100
Anaphylaxie	AB-S-20%	21.0	48.7	48.3	13.0	4.7	2.0	0.6	1.4	0.5	0.5	0.4		
		Diclofenac (µg/ml)						MTX (µg/ml)						
		10			100			200			0.01	0.1	1	
Exanthem**	AB-S-20%	63.2			80.9			11.6			1.2	0.9	1.5	

EEM, erythema exsudativum multiforme; MPE, maculopapular exanthema.

* Two patients with clearly different reactions in AB serum or autologous plasma.

† Selective reactivity to one compound only – good tolerance of amoxicillin alone.

‡ typical example of multiple drug hypersensitivity with drug hypersensitivity syndrome with eosinophilia and systemic symptoms (DRESS) to phenytoin and erythrodermia to amoxicillin.

§ Very narrow dose–response curve – seen in AB serum and autologous plasma.

¶ Positive LTT 20 years after life-threatening anaphylaxis and strict avoidance of β-lactams.

** Strong reaction to diclophenac, helping to differentiate the anaphylactic reactions from a ‘pseudoallergic’ reaction to NSAID.

patients with RCM-hypersensitivity in a time interval of $\frac{1}{2}$ year revealed rather stable SI-values (Table 4).

The complexity of the test and the many variables influencing it show that one cannot expect the high reproducibility of a serum test (for example, of an ELISA) for a cellular test such as the LTT. The LTT is able to detect a strong sensitization over many years, as positive results both to drugs but also to a protein antigen-like TT remain positive over years (35). However, the degree of positivity may change quite much and a SI of 20 reflecting a strong sensitization may be followed by

a SI of 6. Thus, a weakly positive result (e.g. an SI of 2.5) might rapidly become negative – simply because of the variability of the test.

Timing of LTT performance

The LTT measures a memory T-cell response. It can be positive for many years. We and others observed positive reactions 10–20 years after the original treatment with β-lactams or carbamazepine, which originally had caused

Table 3. Reproducibility of the lymphocyte transformation test (LTT)

Patient number	Time from reaction to LTT (months)	Tetanus toxoid control (5 µg/ml)	Stimulation index																			
			Iodixanol (batch number 10042717)				Iodixanol (batch number 10003226)				Iohexol (batch number 803104)				Iopentol (batch number 706098)				Iopamidol (batch number 195331)			
			concentration (mg/ml)				concentration (mg/ml)				concentration (mg/ml)				concentration (mg/ml)				concentration (mg/ml)			
			0.1	1	10	100	0.1	1	10	100	0.1	1	10	100	0.1	1	10	100	0.1	1	10	100
A	1.5	53	1.2	1.1	1.6	2.7	0.8	0.8	1.6	2.8	0.9	1.0	0.6	0.6	1.2	1.1	0.9	0.5	0.6	1.0	0.7	0.4
A	7.5	20	1.3	1.2	1.6	4.8	2.2	2.6	2.1	2.9	0.9	0.4	0.3	0.3	0.9	0.4	0.4	0.4	0.3	1.0	1.6	0.6
B	2	197	1.7	1.4	4.7	4.7	0.6	0.7	1.7	3.0	1.5	1.8	1.5	0.7	1.2	1.1	1.5	1.7	1.4	1.4	0.5	1.0
B	8	408	1.2	1.6	1.7	4.7	1.2	1.5	2.0	5.2	1.6	1.5	0.4	1.2	0.8	1.3	1.0	0.7	1.3	0.9	1.2	1.4

Two patients were with delayed reactions (exanthema) to radiocontrast media (RCM) were repeatedly analysed.

Note that

(A) The batch-to-batch variability was low, and that the same drug gave similar values in the same concentration using the same cells.

(B) The same RCM elicited an enhanced proliferation also after a time interval of c. 6 months. Interestingly, the biggest difference of the two time-points was seen with the control cultures to tetanus toxoid.

Table 4. Sensitivity of the lymphocyte transformation test (LTT)

Imputability	Retrospective		Prospective
	LTT‡ (n, %)	LTT (n: %)	
Definite	74/100†: 74%	31/50§, 62%	14/21: 67%
Highly probable	211/450: 47%		
Not probable	89/271: 33%		
Negative†	15/102: 15%		1/93: 1%

* LTT > 2.

† Corresponds to a specificity of 85 and 99%.

‡ Provocation or re-exposure positive; or clear history, corresponding to the experience with the drug (38).

§ Provocation positive (32).

a delayed or even anaphylactic reaction (Table 2, 32). On the other hand, some patients appear to loose reactivity in a time frame of 3–4 years. At present, one cannot predict whether the drug reactivity in an individual patient will persist or not, and whether those which have lost its reactivity will tolerate the drug again. Thus, it is recommend to perform the test within 2–3 years after the reaction.

As during the acute drug hypersensitivity reactions the immune system, in particular T cells is strongly activated (26) the test should not be done in the acute stage. We perform it after remission, which means normally 4–8 weeks after the reaction.

As the test relies on living cells able to respond to an antigen the heparinized blood needs to be processed as soon as possible. Survival is best if the blood is stored at room temperature (c. 20°C). Separated PBMC survive longer if kept in serum supplemented medium (incubator at 37°C).

Comparison of freshly processed blood to blood stored for 24 h revealed similar results (< 20% difference of cpm, data not shown). We thus accept a delivery time of the heparinized blood at room temperature of ~ 24 h until

processing (=Ficoll/Hypaque). Analysis reveals normally < 15% dead cells and the positive control (TT) gives good results. However, after 24 h the viability of mononuclear cells in unseparated blood rapidly deteriorates. Thus, one should always screen for viability of the cells after density centrifugation and perform the test only if cell viability is > 70%, and consider the test as interpretable if the TT driven cell culture gives SI values > 5.

Sensitivity

Diagnostic sensitivity of a test refers to the level, which detects a 'positive' compared with a true positive control. Such positive standards do not yet exist for the LTT. Preliminary data analysing the precursor frequency of drug-specific T cells within PBMC from sensitized individuals suggest that the LTT is positive if > 1/20 000 T cells is reacting.

Clinical sensitivity

The clinical sensitivity of an allergy test indicates to what extent a test detects a clinically relevant (=symptom-causing) sensitization. The golden standard for sensitivity (and specificity) of an allergy test is normally a positive provocation test. However, one has to be aware of certain limitations of provocation tests in drug hypersensitivity (36):

- They are mainly done with the intention to rule out an allergy, as the intentional reproduction of a drug hypersensitivity is thought to be unethical. Thus, patients with severe reactions or positive skin tests are usually not re-exposed.
- Provocation tests detect not only a sensitization, but also the clinical manifestation of it. The latter is a complex process, which may rely on the presence of

cofactors, which are not present during the time of provocation – thus also provocation tests can be false positive or false negative (36). More importantly, *in vitro* tests-like the LTT or the detection of specific IgE by, e.g. CAP-methodology can only detect a sensitization, which may or may not be associated with clinical symptoms, e.g. in Switzerland 30–35% of the population have specific IgE to various ubiquitous environmental allergens (e.g. pollen), but only 15–20% report symptoms in spite of allergen-exposure (37). Thus, *in vitro* tests-like the LTT differ in principle from *in vivo* tests-like provocation or patch tests, as they can only detect a sensitization but cannot predict whether the sensitization leads to symptoms. However, a strong immune reactivity is frequently associated with clinical symptoms.

- A further difficulty arises from the abundance of drug-allergens – it is impossible to standardize each drug-specific skin- or *in vitro*-test by provocation.

In view of these considerations, the sensitivity and specificity analysis of the tests most frequently used in drug hypersensitivity is based on:

- a) a prove of concept of the test, e.g. by cloning drug-specific T cells from blood or the patch test site (6, 24);
- b) a correlation of different tests methods (e.g. *in vitro* and *in vivo*), which confirm independently a sensitization (13, 26, 32);
- c) a relation to a history of drug hypersensitivity reaction with high imputability (38); and
- d) a collection of patients with accidental or intended re-exposure (=provocation) (26, 32).

Ad a: Many different T-cell clones, mainly CD4+, TCR $\alpha\beta$ + were cloned against β -lactams, lidocain, mepivacain, sulfamethoxazole, celecoxib, lamotrigine, carbamazepine, *p*-phenylendiamine, ciprofloxacin etc. (5, 6, 16–18, 24, 39).

Ad b: Most but not all patients with clearly positive skin tests have actually also a positive LTT (26). We have collected patients with different manifestations of drug hypersensitivity elicited by different drugs and positive LTT; 41 of 44 had positive skin tests as well (13).

In 21 prospectively analysed patients with documented drug hypersensitivity 14 of 21 had a positive LTT (67%); in seven of 15 a patch test was positive. Five of these seven skin test positive patients had a positive LTT as well, while six of the 14 patients with positive LTT had a negative patch test. Combining patch and LTT yielded a sensitivity of 76%. The data indicate a better sensitivity for the LTT compared with the patch tests, and suggests that both tests supplement each other, as they are not completely overlapping.

Ad c: In a retrospective study, 923 patients were classified according to the imputability of drug reactions (38). A high imputability was assumed if the patient was challenged, (accidentally) re-exposed and showed the

same symptoms or if the patients history was clear and typical (e.g. exanthema after 8 d of amoxicillin treatment, no other medication, no viral infection). Using these criteria (Table 4), 100 patients with a high probability were identified: 78 of them had a positive LTT, thus the LTT has a sensitivity of 78%. In agreement with this finding was the observation that those patients classified to have a lower likelihood of drug hypersensitivity had a lower incidence of a positive LTT and patients with an unlikely history had the lowest incidence of positive LTT (Table 5). In agreement with our prospective study, the skin tests showed a lower sensitivity: they were only positive in 64% of the patients.

In a similar study of Luqué et al. (32) of 50 patients with well-documented delayed or immediate β -lactam hypersensitivity, the overall sensitivity of the LTT was found to be 62% and it was superior over skin testing (delayed reading of intradermal tests) for nonimmediate reactions, which was positive in 37% only, while those with immediate reactions had in 68% a positive immediate skin test.

Ad d: There are many case reports of well-documented drug hypersensitivity reactions with positive skin and *in vitro* tests, occasionally even with positive provocation tests. However, some data do also indicate that certain drugs may elicit either a positive skin test or a positive LTT only, dependent on the type of drug and may be also influenced by the clinical picture, e.g. abacavir, which may elicit a severe generalized hypersensitivity reaction affecting skin and various organs (lung, liver, etc.) may elicit a positive delayed type skin test reaction, but consistently failed to elicit a proliferative response *in vitro* (40, own experience). Thus, dependent on the drug,

Table 5. Diseases, in which the lymphocyte transformation test (LTT) has been found to be positive

Frequently positive (>50%)
Generalized maculopapular exanthema
Bullous exanthema
acute generalized exanthematous pustulosis (AGEP)
DHS/drug hypersensitivity syndrome with eosinophilia and systemic symptoms (DRESS)
Anaphylaxis (generalized, severe symptoms)
Occasionally positive
Hepatitis (dependent on type of drug)
Nephritis (dependent on type of drug)
Urticaria, angioedema
Interstitial lung disease*
Pancreatitis*
Rarely positive (<10%)
Toxic epidermal necrolysis (TEN)
Vasculitis
Macular exanthema (without T-cell infiltration)
Guillain-Barré*
Blood dyscrasia-like idiopathic thrombocytopenic purpura (ITP), haemolytic anaemia
Fixed drug eruption

* Rarely investigated.

different tests systems might be required to pinpoint the causative drug for a hypersensitivity reaction (Table 5), emphasizing the necessity to combine *in vivo* and *in vitro* tests to best identify the causative drug.

In conclusion, the LTT has an general sensitivity of 60–70%, whereby this specificity is mainly based on the analysis of β -lactam hypersensitivity. Many of these patch- and LTT-negative patients were provoked by the incriminated drug, but a systematic analysis is still lacking. Thus, a positive LTT helps to define the incriminated drug, but a negative tests cannot rule out a drug hypersensitivity.

Specificity

In the frame of the retrospective analysis the specificity of the LTT was also evaluated. Using the above described criteria, 15 of 100 patients with an hypersensitivity reaction were thought to have a false positive LTT. Almost all 15 had reacted to NSAID with cutaneous symptoms, which was interpreted as nonallergic side-effects and a positive LTT was thus considered false positive (38). However, this interpretation of the LTT might have been false as some NSAID like diclofenac may elicit true allergic reactions. Indeed, other analyses repeatedly showed a good specificity for the LTT using various drugs (26, 32–35). In the above-mentioned prospective evaluation of the LTT of 21 patients, 93 control LTT with blood from either exposed but not allergic persons or not exposed individuals were performed: only one showed slightly elevated LTT to indapamide. Other groups have analysed the reactivity of LTT to certain compounds separately. They found specificities of 100% repeatedly, for example, in carbamazepine hypersensitivity and lamotrigine hypersensitivity, and of 93% for β -lactam hypersensitivity (32–34) as exposed, while nonallergic persons showed no or only very rarely some proliferation to the drug. Thus, the overall specificity of this test is at least 85% but probably higher for drugs such as lamotrigine, carbamazepine, and β -lactams.

Disease spectrum

The LTT aims to detect circulating drug-specific memory T cells, which proliferate upon drug (=antigen) stimulation. Such drug-specific T cells need to be present in sufficient amounts in the circulation to lead to a detectable response by the *in vitro* stimulation (but also for patch tests). This is normally the case after a strong immune response was generated, as in generalized hypersensitivity reactions where a strong T-cell activation can be observed during the acute stage of the disease (16, 26, 41). It has the advantage to detect T cells involved in different types of reactions, not only in classical delayed hypersensitivity reactions (Tables 2 and 5). High SI values in the LTT are

not associated with the severity of clinical symptoms as it is only reflecting a high precursor frequency of drug-specific T cells, which is not necessarily associated with severe clinical symptoms. A patient might have a severe anaphylactic shock, but only marginally positive LTT, while analysis of a rather harmless maculopapular exanthema might reveal a very strong proliferation (Table 2).

The LTT has been proven to be rather useful in patients with generalized exanthema of different types (maculopapular, bullous, pustular) (5, 6, 18, 19), in patients with drug hypersensitivity syndrome with eosinophilia and systemic symptoms (DRESS) (16, 33, 34) and also in patients with IgE-mediated reactions-like severe anaphylactic reactions (13, 32). In addition, the LTT has been found positive in patients with immune-mediated pancreatitis (42), interstitial lung diseases (own observation), isolated drug fever (43), later followed by vasculitis, and might also be positive in certain autoimmune diseases where cross-reactivity of the drug-specific cells with autologous structures may be involved (44).

The LTT was found to be occasionally positive in interstitial nephritis (even without skin involvement). We observed positive reactions with diclofenac and β -lactams. But the LTT was quite frequently negative with other compounds in spite of a very suggestive history.

In drug-induced hepatitis the LTT can be positive (45, 46) and was found useful to discriminate immune hepatitis from toxic reactions caused by antituberculous drugs (47). A positive LTT in nephritis and hepatitis may, however, depend on the type of eliciting drug, as some drugs might become immunogenic in the kidney or liver only – due to local generation of reactive metabolites. Culture of the drug with peripheral blood cells may not yield these immunogenic metabolites. Generalized hypersensitivity reactions involving the skin and liver (such as DRESS) show frequently a positive response in the LTT (16, 33, 34).

Rather seldom positive are the LTT in blood cell dyscrasias (e.g. haemolytic anaemia or aplasia), in TEN, where a positive LTT seems to be the exception (48) and in (small vessel, ANCA-negative) vasculitis. Rather consistently negative is the LTT in patients with fixed drug eruption, as this disease is not associated with many drug-specific T cells in the circulation.

Drugs

We have observed positive LTT to different group of drugs (Table 6): antibiotics (β -lactams, quinolones, sulfonamides, etc.), antiepileptics (lamotrigine, carbamazepine, phenobarbital, phenytoin), antihypertensives, local anaesthetics, various diuretics, vitamins, and many more. While some drugs stimulate the T cells by the haptens

Table 6. Drugs – suitable for lymphocyte transformation test (LTT)*,†

Antibiotics – β -lactams, quinolones, macrolids, sulfonamides, tetracycline, etc.
Antiepileptics – phenytoin, carbamazepin, lamotrigine, etc.
ACE-inhibitors – enalapril, etc.
Antituberculous drugs – isoniazid, rifampicin
Diuretics – hydrochlorothiazide, furosemide, indapamid, etc.
NSAID (Cox 1 and Cox 2 inhibitors) – diclofenac, celecoxib, mefenaminic acid, acetaminophen, etc.
Pyrazolones – propyphenazone, metamizol
Local-anaesthetics – lidocain, mepivacain, etc.
HMG-CoA-reductase inhibitors – acrivastatin
Morphin-derivatives – pethidin, codein, etc.
Radio-contrast media‡ – iohexol, iopamiro, etc.
Muscle relaxants – suxamethoniumchlorid, etc.
Vitamins – cyancobalamin (vitamin B12), folic acid, etc.
Contact allergens – <i>p</i> -phenylendiamine, nickelsulfate, etc.
Varia – allopurinol, domperidon, hydroxymethylcellulose, etc.

* List incomplete.

† Always as pure substance.

‡ Stimulation index (SI) should be >4 to determine it as positive, false positive values possible.

mechanism (β -lactams), the majority of T cell-mediated reactions to other compound may be due to the p-i concept (sulfonamides, local anaesthetics, lamotrigine, carbamazepine, quinolones, etc.).

Conclusion

The scientific basis for the LTT has been well-established in the last years, and its usefulness has been demonstrated in various diseases and with many different drugs. Thus, this test has already contributed a lot to a better understanding of drug hypersensitivity and new developments (application of cytometry to identify the reactive cells) might further booster our understanding of drug hypersensitivity.

If performed correctly, the LTT has clearly certain advantages:

- one can perform it with many drugs without the need to establish new test reagents;

- as an *in vitro* test it is not harmful to the patient;
- it is positive in drug reactions with different pathomechanism; and
- it is – at least in our hands – generally more sensitive than other tests.

Nevertheless, its application in routine diagnosis is still controversial which is actually the case for all other tests in drug allergy diagnosis. This limitation of drug hypersensitivity tests is due to the great heterogeneity of drug hypersensitivity reactions, where no single test is able to demonstrate a sensitization with sufficient reliability. Moreover, the LTT requires experience with cellular techniques, certain expensive equipment and profound background information on pharmacology and immunology by the interpreter.

A *positive* LTT is often a valuable contribution to the diagnosis of drug allergy, e.g. a positive test against only one drug within three candidate drugs is surely helpful to pinpoint the relevant drug (Table 2). But as the sensitivity of the LTT is limited, a *negative* LTT cannot exclude a drug hypersensitivity. It is also clear that more systematic research is required to further simplify this test and to better define the drugs and diseases where the LTT is best suited for.

In conclusion, as the pathogenesis of drug hypersensitivity reactions is complex – its diagnosis is complex as well, only a combined approach using:

- an exact history (which is the most important component);
- skin tests (immediate and delayed);
- LTT;
- determination of specific IgE, if available; and
- provocation tests (mainly to *rule out* an hypersensitivity), seems to be appropriate. If done correctly and put in context with the history, the LTT is in spite of its obvious limitations a useful test in the puzzle contributing to the diagnosis of drug hypersensitivity and its eliciting drug(s).

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