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Analysis of Viability as Readout of Lymphocyte Transformation Test in Drug Hypersensitivity Diagnostics

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Abstract: In vitro tests of cellular activity form part of the diagnostic algorithm of drug hypersensitivity reactions. Because of the wide range of pharmacological mechanisms, clinical symptoms, genetic components, and laboratory tests involved, it is important to know how a particular test performs in the diagnostic procedure. We carried out a detailed retrospective analysis of more than 6000 measurements of numerous drug compounds tested in 738 serum samples over the past 6 years. Our cell viability-based lymphocyte transformation had a coefficient of variation of 10% and showed similar performance over the whole range of tested ages. With an adequate number of parallel measurements, the test can identify modest increases in stimulation indices with high confidence. Similar percentages of analytically positive responses (11.4%, 13.5%, and 9.7%) were observed for the three most frequently tested drug groups, namely, antibiotics, non-steroid anti-inflammatory agents, and anesthetics. These results confirm that cell viability tests are suitable alternatives for proliferation assays in drug allergy testing.

Keywords: drug; allergy; lymphocyte transformation; hypersensitivity

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1. Introduction

Medicinal drugs are biologically active molecules introduced into the body with the aim of modulating biochemical events. While drugs are used to prevent or treat malfunctions of the organism, unintended effects may also be induced because of improper dosage, inadequate target specificity, drug interactions, the genetic and physiological variability of the human population, and the temporal variance of physiology, or combinations thereof [1,2]. When such adverse drug reactions are caused by the immune system [3–5], the term drug hypersensitivity is used, even though the exact mechanism is not necessarily conventional hypersensitivity [6]. If we consider only those adverse drug reactions where the mechanism is true hypersensitivity, the Coombs or modified Coombs categorization can be applied [7–10]. As a consequence of increasing medicine usage, the incidence of adverse reactions is on the rise [11].

Even if the clinical symptoms of adverse drug effects are unequivocally related to a single drug, which is not often the case, it is desirable to prove the causality for diagnosis. Repeated introduction of the drug (drug provocation test) is a straightforward approach but, depending on the nature of symptoms, may impose a high risk and require appropriate medical preparedness [12,13]. In vitro diagnostic tests are a possible alternative to provocation; however, it must be emphasized that these tests have limited sensitivity, as a sample of blood can never mimic the fate of a drug molecule that is introduced into the body. Yet,

if properly chosen, these tests can confirm that immunological mechanisms are responsible for the drug sensitivity [14,15]. Two main classes of tests are in use currently: immediate hypersensitivity reactions are tested for by basophil activation tests (BATs) [16,17] and their alternatives (mast cell tests) [18,19], while delayed-type hypersensitivity can, in general, be detected by a lymphocyte transformation test (LTT) [20–22], in which suspected drug molecules are mixed with the cells and biological responses are monitored.

LTT utilizes mononuclear cells isolated from peripheral blood by standard methods. Lymphocytes in this fraction of white blood cells are capable of responding to in vitro stimuli during cell culture by expressing activation markers, proliferation activity, and the secretion of cytokines [23–27]. Therefore, even though LTT originally referred to the blast transformation of lymphocytes and the measurement of proliferation [28], various techniques can be used to quantify the lymphocyte response [29]. Our laboratory uses a viability test based on enzyme activity [30], similar to those that have been successfully applied for drug allergy testing [31,32]. This approach basically quantifies the effects of a tested drug on the survival of the isolated blood cells; therefore, it gives a combined readout on proliferation and cell death. Since cellular activation is accompanied by the secretion of pro-survival and pro-proliferation factors, with the properly adjusted culture conditions (e.g., cell number, culturing time), viability is a useful readout.

From the clinical point of view, LTT (called blastogenic response at the time) was originally used to reveal immunodeficiency, because the lack of a mitogen-induced proliferative response indicated abnormal lymphocyte numbers or functions [33–36]. Whereas mitogens induce polyclonal responses, antigens induce responses in specific clones only. Such antigen-specific responses are restricted not only in the number of responding cells, but also in the quality and nature of the response, involving the subpopulation of lymphocytes responding and the cytokines and chemokines secreted. Nevertheless, these responses can also be detected by LTT, albeit with varying efficiency, so the test has gained acceptance as an in vitro diagnostic method for detecting hypersensitivity to specific antigens. It is especially suited for detecting delayed-type hypersensitivity (type IV per Coombs categorization), a major mechanism in drug hypersensitivity [8,9,37], metal allergy [38,39], environmental allergies, and autoimmune conditions [40]. In general, LTT has relatively low diagnostic sensitivity (50–80%) but high specificity (80–100%) [41–43].

The essence of LTT is to recognize when there is a difference between an untreated (negative control) and a drug-treated culture. This is a matter of statistics, where the number of parallels and the expected and measured variance crucially influence the results [44,45]. An important confounding factor is the huge variability in baseline cellular proliferation and responsiveness, even within a single individual [44,46]. This can be compensated for by increasing the number of parallel negative control wells.

The result of LTT is an index, the stimulation index (SI), which is the signal ratio of treated versus untreated cultures. For diagnostic purposes, either the culture conditions are adjusted so that a given SI value can be used as a cutoff, or a diagnostically relevant cutoff SI value is identified by metrology [44,47,48].

Here, we show that with the appropriate experimental design, cell viability measurements can reveal the responsiveness of circulating blood cells towards drugs with high confidence, and can therefore serve as an in vitro diagnostics procedure in drug hypersensitivity evaluation.

2. Materials and Methods

2.1. Source of Data

The results of 6 years of drug allergy testing were anonymized and used for this meta-analysis. The dataset included 738 blood samples from individuals (Table 1), who either brought their own medications or ordered the examination of pure active substances.

Table 1. Patient characteristics.

Parameters	Man	Woman
patient number	178	560
age (mean ± SD, years)	45.3 ± 15.1	47.1 ± 15.3
age range (years)	18–89	18–91

2.2. Preparation of Drugs for LTT

The tested medicinal drugs were obtained from the subjects who underwent drug allergy testing. Stock solutions were produced by pulverizing if necessary and dissolving the medicine in culture medium or distilled water. The concentrations of stock solutions were based on solubility properties and active substance content of the concerned medicine. Drug solutions were sterile-filtered to ensure aseptic conditions.

Pure pharmacologically active drug substances were purchased from Merck KGaA (Darmstadt, Germany). A set of 10 mM stock solutions was prepared with dimethyl sulfoxide (DMSO) for all experiments. The medium with the highest DMSO concentration (0.1%) did not exert any remarkable effect on cell viability and proliferation. The list of applied medicines and drug substances is provided in the Supplementary Materials. Phytohemagglutinin (PHA) from Phaseolus vulgaris was purchased from Merck KGaA, and a 1 mg/mL stock solution was prepared with PBS.

2.3. Lymphocyte Transformation Testing

Peripheral blood mononuclear cells (PBMC) were collected by means of Ficoll-Paque separation of whole blood [49]. After a washing step in PBS, PBMCs were transferred to RPMI 1640 media (Gibco) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, St.Louis, MO, USA), 1% sodium pyruvate solution (100 mM, Sigma-Aldrich, St.Louis, MO, USA), 1% L-Glutamine (200 mM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 1% MEM Vitamins Solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

PBMCs were cultured in the absence (negative control) or presence of the drug of interest, as well as in the presence of mitogen (positive control), for 72 h, and the viability of cells was then assessed using an MTT assay [30]. Cells were seeded onto 96-well microplates at a density of 150,000 cells/well. The wells were filled with medium containing the test compounds prior to the seeding. Untreated cells served as the negative control, while PHA was used as the positive control in all experiments. Samples were incubated at 37 °C in humidified air containing 5% CO_2. After 72 h, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution (5 mg/mL) was added to each well. After 4 h, formazan crystals formed by the living cells were solubilized in Hydrogen chloride–2-propanol solution and measured by spectrophotometry. Negative control wells were comprised of ten parallels; other measurements were performed with five parallel wells.

2.4. Meta-Analysis, Statistical Methods

Ten parallel measurements of negative control wells with no stimulant were used for the accurate estimation of background signals and for the calculation of the variance of the method. Five parallel wells were used for positive control and test substance measurements.

The number of parallel measurements required for distinguishing SI values with >95% confidence was defined by the equation:

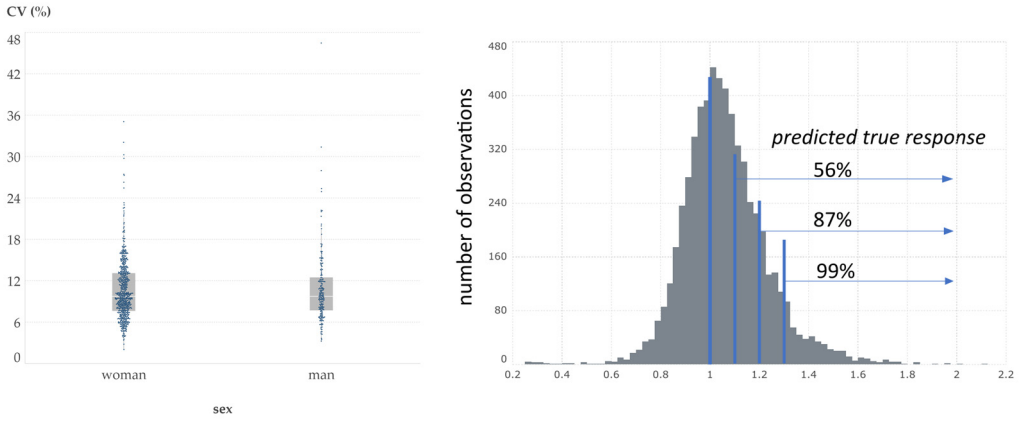
$$r \geq 2\frac{CV^2}{D^2}(t_1 + t_2)^2 \quad (1)$$

where r is the number of replicates, CV is the percentage of coefficient of variation, D is the difference required to be detected as a percentage of the mean, t_1 is the tabular value from the T-test with specified significance for Type I errors, and t_2 is the tabular value from the T-test with specified significance for Type II errors. Images were created with Scimago Graphica 1.0.45.

3. Results

3.1. Variance of Negative Control

The results of the LTT are expressed as the stimulation index: the ratio of the signal in the treated group to that in the untreated group. This unitless quantity is 1 in the absence of stimulation, increases upon stimulation of cells, and decreases if the cells die. Reliable discrimination of deviance from 1 requires statistics that characterize random, accidental deviation from 1 in the absence of stimulus. We characterized this property by the analysis of signals of parallel negative control wells. The coefficient of variation (CV% = standard deviation × 100/mean optical density) characterizes this deviation, and was found to be statistically identical in women (n = 560) and men (n = 178) (Figure 1a).



(**a**) (**b**)

Figure 1. Variance in signals and distribution of SI values. The distribution of CV% values is shown for women and men (**a**). Assuming a normal distribution for random signals and 10% CV, the percentage of truly increased events can be calculated (**b**).

Next, we examined the distribution of SI values from all the tests. The expected average of SI values if all tested substances were non-stimulatory would be 1 with an SD value of 0.1, corresponding to the 10% CV of the measurement. The histogram of obtained SI values, however, shows a distribution that is skewed towards greater SI values (Figure 1b). This skew is the result of stimulatory effects of some of the tested substances in responsive individuals. Observations beyond the normal distribution are non-negative results statistically. A sample with the lower limit of positivity (SI = 1.4) has a normal distribution, with the lowest SI values of its distribution overlapping with the negative test distribution (Figure 1b). These histograms demonstrate the behavior of the cut-off values that we used for classification of results: negative SI < 1.3, equivocal 1.3 ≤ SI < 1.4, and positive SI ≥ 1.4.

3.2. Effects of Age and Sex

The immune system changes throughout life, gradually transforming towards a memory cell-based protection system with the accumulation of immunological experience. To confirm that a viability measurement is applicable throughout adult life, we tested whether age has an effect on the variance in the background proliferative responses, as reflected by the standard deviation of signals in parallel wells. Age had no effect on the SD values of negative control measurements, irrespective of the individual's sex (Figure 2a). Age did influence, however, the responsiveness to the mitogen PHA that was used as a positive control (Figure 2b). In spite of this age-dependent decrease in PHA responses, we did not observe age-dependent responsiveness to the tested substances (Figure 2c).

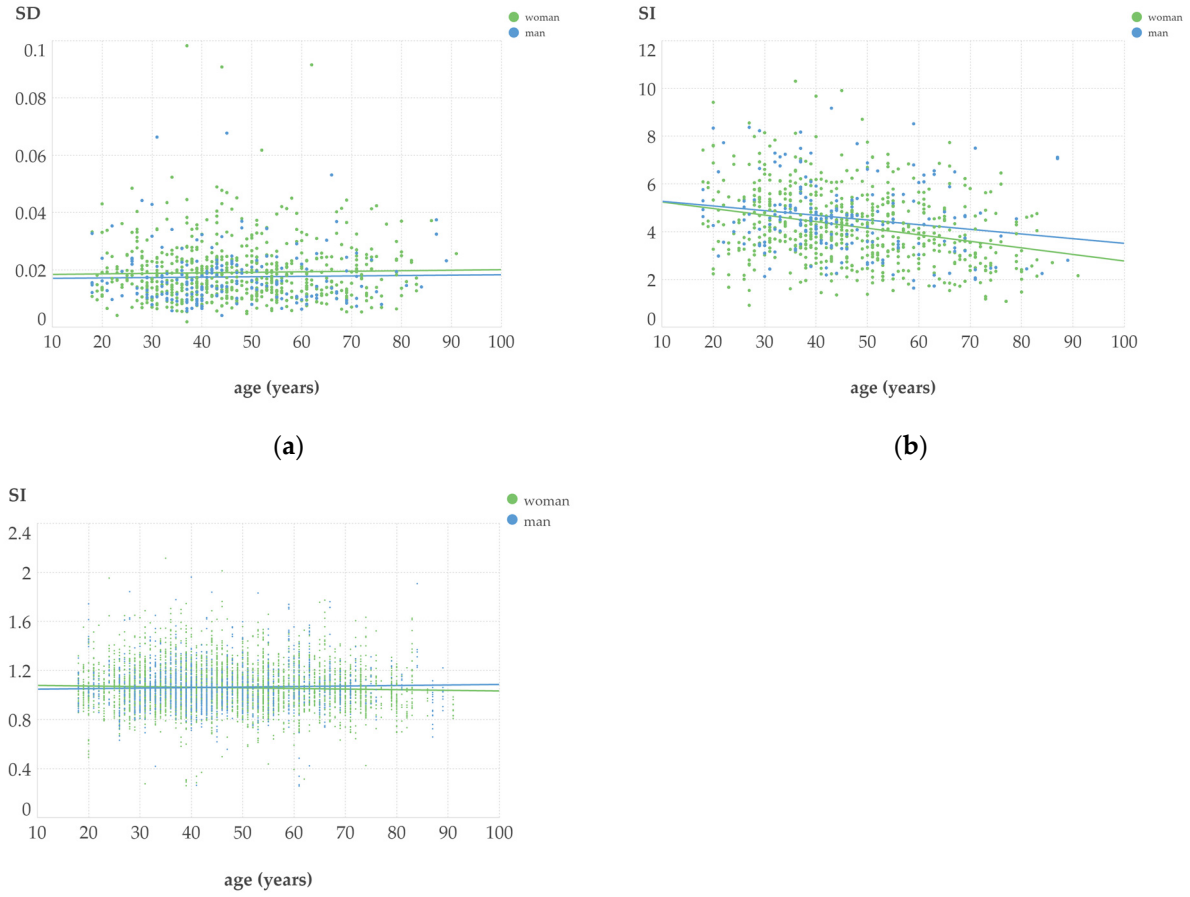
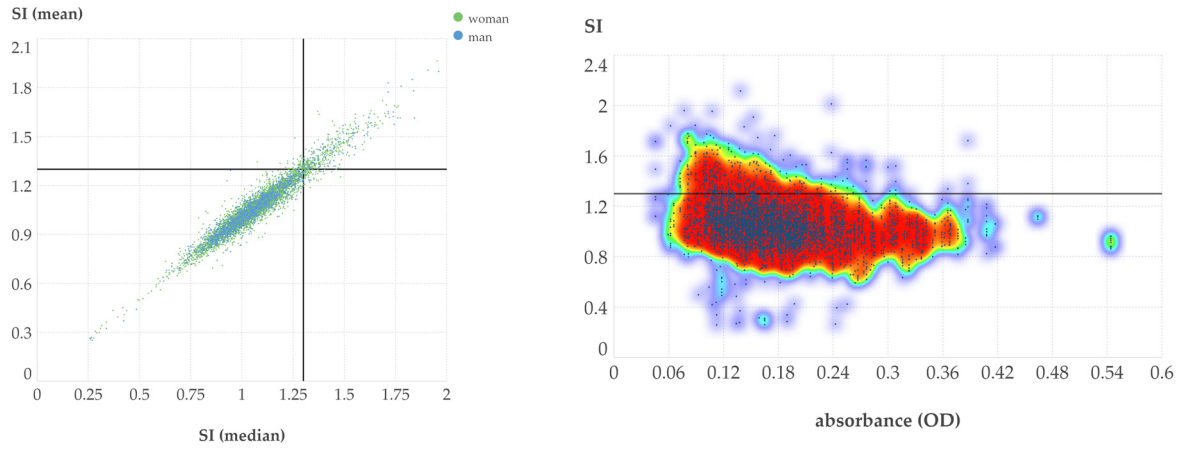


Figure 2. Effects of age and sex on test performance. (**a**) SD of negative controls; (**b**) SI of positive controls; and (**c**) SI of tested substances, all as a function of age. Lines represent the linear regression lines for the respective sexes.

3.3. Adjustment of Cut-Off for SI

Since cell cultures were set up in parallel to increase the precision of the measurements, the average of these parallels needs to be computed for the calculation of SI. Arithmetic mean and median are the usual choices for averaging in LTT tests. We compared the effect of using these values for SI calculations (Figure 3a). The results of the tests with SI values below or above the cut-off values by both calculations are unambiguous (lower left and upper right quadrants). A small percentage of the tests are only above the cut-off with either mean-based or median-based SI calculation (upper left and lower right quadrants).

These tests should be examined with regard to the values of the parallel wells and a decision should be made considering all potential confounding factors in the measurement.



(**a**) (**b**)

Figure 3. The effects of calculation and of background signals on SI values. (**a**) Mean and median values of parallels are strongly correlated, but result in slightly different classification in the grey zone; (**b**) density map of SI values as a function of negative control absorbance, representing the background cellular activity. Black lines represent the upper cut-off value (SI = 1.3) of a negative result.

When all the blood cells in culture are quiescent (resting lymphocytes), a lower signal for the negative control wells is expected, alongside increased cellular activity (blast formation, proliferation) due to ongoing immune responses increasing the background signal. To examine the effect of background signals, we analyzed the relationship between the median OD values of negative controls and the SI of tested substances (Figure 3b). An increase in background signals was associated with a diminishing frequency of positive test outcome.

3.4. Frequencies and Relationships of Positive Responses

We sorted the tested drugs according to their pharmaceutical effects and created categories reflecting the three most frequently tested groups (antibiotics, anesthetics, and non-steroid anti-inflammatory agents) and a miscellaneous group. The proportions of equivocal and positive results were comparable for the three major categories (Figure 4).

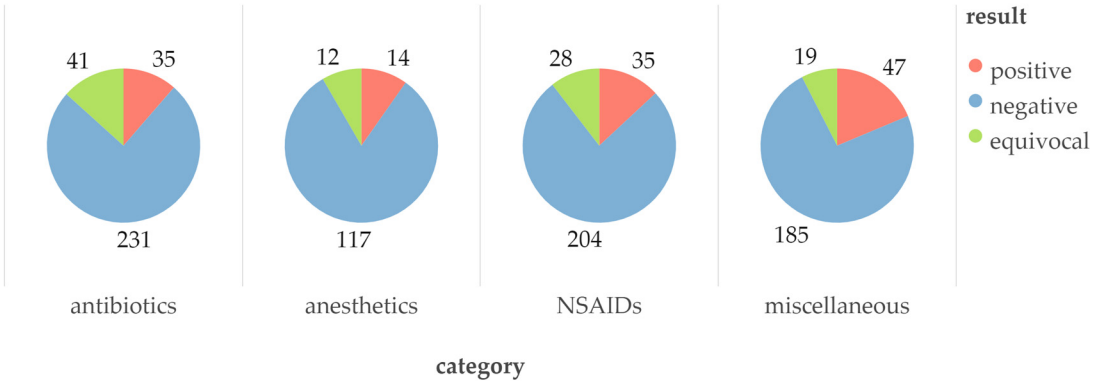


Figure 4. Number of patients with negative, equivocal, and positive results in the four tested drug categories. Note that the same patient may have been tested for several drug categories.

Next, we sorted the tested drugs according to the number of patient blood samples in which they were examined. Amoxicillin, which is generally used in combination with

the beta-lactamase inhibitor clavulanic acid, was the most frequently tested substance, in agreement with the common need for confirming penicillin allergy. The median values of the percentage of negative, equivocal and positive results for the most frequently examined drugs were 81.8%, 9.0%, and 9.5%, respectively (Table 2). While specific drugs showed notable deviations from these values, the tendency was toward a comparable percentage of equivocal and positive results, supporting the right-skewed distribution of SI values shown in Figure 1.

Table 2. Results for most frequently examined medicines and substances. The data represent the number and percentage of observed cellular responses (−, negative; +/−, equivocal; +, positive) for the listed investigated substances and medicines, containing these active substances alone or in combination.

Medicine/Substance	n Patient	−	+/−	+	− (%)	+/− (%)	+ (%)
amoxicillin	201	166	18	17	82.6	9.0	8.5
clavulanic acid	173	149	13	11	86.1	7.5	6.4
lidocaine	119	104	6	9	87.4	5.0	7.6
clavulanic acid * alone	96	89	3	4	92.7	3.1	4.2
metamizole	92	72	11	9	78.3	12.0	9.8
articaine	90	74	9	7	82.2	10.0	7.8
diclofenac	85	78	0	7	91.8	0.0	8.2
paracetamol	81	70	4	7	86.4	4.9	8.6
phenoxymethylpenicillin	78	71	5	2	91.0	6.4	2.6
acetylsalicylic acid	72	53	9	10	73.6	12.5	13.9
ibuprofen	69	55	7	7	79.7	10.1	10.1
cefuroxime	32	25	3	4	78.1	9.4	12.5
benzylpenicillin	27	22	3	2	81.5	11.1	7.4
sulfamethoxazole & trimethoprim	24	19	2	3	79.2	8.3	12.5
ciprofloxacin	22	18	1	3	81.8	4.5	13.6
azithromycin	21	17	2	2	81.0	9.5	9.5
levofloxacin	19	13	4	2	68.4	21.1	10.5
tolperisone	17	14	0	3	82.4	0.0	17.6
perindopril	17	15	1	1	88.2	5.9	5.9
levothyroxine	13	6	3	4	46.2	23.1	30.8
enoxaparin	10	7	2	1	70.0	20.0	10.0

* Pure drug substance.

4. Discussion

LTTs are traditionally based on the measurement of proliferation using radioactive thymidine incorporation onto dividing cells. When the aim of the test is the assessment of lymphocyte reactivity towards potentially allergenic small molecules, other readouts are also possible and reasonable with current technologies. The viability test we employed has small variability when an adequate number of parallels are used (Figure 1). Therefore, a modest increase in SI value, as a result of increased cellular survival by the direct (proliferation) or indirect (cytokines, chemokines) effect of responding cells, can be confidently distinguished from the absence of response. Even though mitogen-triggered responses

decline with age, the responses triggered by the tested substances showed no such decline (Figure 2). The likely reason is the distinct mechanism of action: whereas PHA is a lectin that binds cell surface glycans and triggers agglutination and finally mitosis [50,51], drugs mostly act as a bridge between MHC and TCR molecules [52–54].

The use of multiple identical measurements (parallels) is necessary for increasing the precision of the method, but it also raises the question of how to average the observed individual signals. When the SI values calculated from the arithmetic means and the medians of parallel wells give discordant results (Figure 3a), we suggest examining the distribution of signals. A single outlier value can be reasonably excluded from evaluation, while a cluster of two outliers suggests non-random but true biological effects. In this latter case, the use of the mean value of all parallels better reflects biological events.

The clinical diagnostic accuracy of LTTs for drug allergy is very difficult, if possible at all, to establish. Each substance may act via distinct mechanisms, and each individual may respond in different ways to distinct substances, with the timing and exact methodology of the test also influencing accuracy. Accordingly, reported values of diagnostic sensitivity and specificity lie in very large ranges [21,22,41,42]. The analytical (metrological) sensitivity and specificity can be established, however, and may provide a cue regarding the reliability of the measurement method. We designed our assay so that the number of parallel measurements was suitable for distinguishing SI values above the cut-off with high confidence. Our analysis shows that if the analytical specificity is indeed high, 99% of the measurements above the lower cut-off value are predicted to be true cellular responses (Figure 1b). Accordingly, we assume that the diagnostic specificity of the test is also high. However, the diagnostic sensitivity of the test is assumed to be low, simply because an in vitro test cannot reproduce the in vivo effects of a drug. We cannot rule out that the sensitivity of the approach could be reduced if responses dominated by cytotoxic T cells [55] result in cell death of the target cells, which would in turn counterbalance the activation and proliferation of the responding cells. We used different drug concentrations in the test, partially with the goal of finding conditions where cellular activation and survival stood out over cell death. For these reasons, the LTT is not suitable for excluding drug sensitivity, but only for confirming hypersensitivity and comparing the reactivity of related drug substances.

There was a strong female to male bias (560 to 178) in the number of patients tested, which confirms previous reports that women are more likely to develop drug allergies [56,57]. Antibiotics and non-steroid anti-inflammatory drugs were the two most frequently tested groups (Figure 4, Table 2), in agreement with other reports [56,58,59]. Two beta-lactam compounds, amoxicillin and clavulanic acid, were the top two tested substances (Table 2), as these two are frequently used in combination. The observed positive responses to clavulanic acid confirm that this beta-lactamase inhibitor can also induce adverse reactions [60]. Messaad et al. [58] carried out drug provocation tests in patients with histories suggesting drug allergy and observed a 17.6% positivity rate for oral provocation. Our percentages of positivity for the four major drug categories (Table 2, antibiotics, anesthetics, NSAID, and miscellaneous) were 11.4%, 9.7%, 13.5%, and 18.7%, alongside additional 13.3%, 8.3%, 7.3%, and 7.6% equivocal results. Overall, these numbers suggest that this in vitro test can efficiently supplement clinical history and perhaps precede skin testing and provocation testing in the diagnostic work-up.

The analysis of particular substances (Table 2) suggests that the observations are not due to random noise (observed positivity is more frequent), but less than what would be expected as a result of a general stimulatory effect (when the majority of measurements would be positive). These measurements can therefore act as good correlates, but cannot explain the molecular nature of the cellular response and immunological consequences

in the body. These tests can therefore only provide support for the diagnosis of drug hypersensitivity and should not be interpreted without consideration for clinical data.

A critical aspect of the testing of cells is the variability in an individual's responsiveness, which may be modulated by general health, ongoing immunological processes, and the time of latency from last allergic symptoms. Our results highlight that increased background activity is associated with decreased incidence of positive results, pointing towards decreased sensitivity (Figure 3b). These results suggest that the test should ideally be repeated in cases where the background signal was high and the clinical symptoms were strongly suggestive of allergic reactions.

From the theoretical point of view, LTTs are suited for detecting cellular responses of delayed type-hypersensitivity. Nevertheless, IgE-mediated immediate allergic reactions may also be identified by the test, because antigen specific T-helper cells are involved and can be stimulated in vitro. LTT can be combined with other in vitro allergy tests, such as a basophil activation test [13], in order to broaden the range of immunological mechanisms behind drug reactions that can be detected and increase diagnostic sensitivity.

5. Conclusions

The in vitro testing of potentially allergenic drugs and drug components is feasible using viability tests as a readout for LTT. Our results confirm that the method is applicable throughout adulthood and highlight that increased background, potentially caused by ongoing immune responses, can decrease testing sensitivity. The most frequently tested substances (antibiotics, NSAIDs and anesthetics) were comparable regarding the percentage of positive results, confirming that the widespread use of drugs is accompanied by a range of adverse drug reactions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/allergies5010001/s1; Table S1: LTT-results; Table S2: list-of-tested-drugs.

Author Contributions: Conceptualization, A.G. and J.P.; methodology, G.M.; software, K.P.; validation, J.P.; formal analysis, A.G. and K.P.; investigation, A.G.; resources, J.P.; data curation, K.P.; writing—original draft preparation, A.G. and J.P.; writing—review and editing, J.P.; visualization, A.G.; supervision, J.P.; project administration, A.G.; funding acquisition, J.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors are employed by a company that provides allergy testing services.

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