ORIGINAL ARTICLE

Cytotoxic-based assays in delayed drug hypersensitivity reactions induced by antiepileptic drugs

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KEY WORDS

delayed hypersensitivity, drug allergy, granulysin, granzyme B, in vitro tests

ABSTRACT

INTRODUCTION Cytotoxic mechanisms are present in the majority of delayed drug hypersensitivity reactions, but are not used as a diagnostic tool.

OBJECTIVES The aim of the study was to compare cytotoxic-based assays with a proliferation assay and drug patch tests in patients with maculopapular eruptions induced by antiepileptic drugs.

PATIENTS AND METHODS Peripheral blood mononuclear cells of 23 patients and 24 controls exposed to the drugs were cultured under defined conditions. A drug-specific response was assessed by measuring granzyme B (GrB) release with an enzyme-linked immunospot assay, intracellular expression of granulysin (Grl) in $CD3^{-}NKp46^{+}$ cells with flow cytometry, perforin concentrations in cell culture supernatants with an enzyme-linked immunosorbent assay, and using the lymphocyte proliferation test. Patch tests with culprit drugs were done in all patients.

RESULTS Lymphocyte proliferation, GrB release, and Grl expression were significantly higher in patients than in controls, while perforin concentrations were not elevated. The sensitivities were 30.4%, 55%, 39.1%, and 17.4% for proliferation, GrB, Grl, and perforin-based assays, respectively. A significantly higher rate of positive results was observed when assays were done within 2 years after a drug-induced reaction. The specificities of all assays remained in the range of 95.8% to 100%. The results of patch tests were positive only in 3 patients (sensitivity, 14.3%) and negative in all controls.

CONCLUSIONS In vitro assays based on the detection of Grl, and in particular of GrB, are superior to routine diagnostic tests in patients with hypersensitivity to antiepileptic drugs. They can detect a low-level response that might be overlooked by standard techniques. In the remission phase, drug-specific cells are more easily detectable directly in the circulation than in the skin.

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INTRODUCTION Drug hypersensitivity reactions (DHRs) represent a significant public health problem,[1] as they affect about 7% of the general population.[2] They have extremely heterogeneous manifestations with exanthemas being the most common.[3] DHRs usually require the withdrawal of a culprit drug along with changes in subsequent therapy. Drug causality assessment based on the history of DHR alone is not satisfactory. It may result in unnecessary avoidance of widely used drugs and limit therapeutic options to less effective or more expensive alternative drugs. On the other hand, drug provocation tests, objectively demonstrating drug culpability, are of limited help in delayed DHRs, including exanthemas, because they are not generally accepted by patients, ethically controversial, and may require a full treatment course again, which is no longer a diagnostic procedure but, in fact, a new treatment.[4]

Therefore, to determine the causative drug in delayed reactions, the existing practice is based on skin tests and the lymphocyte transformation test (LTT), which is the most common in vitro assay that allows to identify a drug-specific response. The use of other in vitro assays is still limited to scientific research and has not become a standard in clinical practice as yet. They exploit a variety

TABLE 1 Demographic, anthropometric, and clinical features of the study subjects

Parameter	Patients n = 23	Controls n = 24	*P* value
age, y	34 (18–66)	38 (20–72)	0.21
male/female, n	12/11	8/16	0.31
BMI, kg/m^2	23 (19.5–30.7)	22.3 (20.4–27.1)	0.30
clinical diagnosis			
epilepsy	21	24	0.45
neuralgia	1	0	0.98
depression	1	0	0.98
tested drugs			
carbamazepine	12	16	0.47
oxcarbamazepine	2	4	0.70
lamotrigine	8	2	0.06
phenytoin	1	2	0.97

Data are presented as mean (range) or number of patients.

Abbreviations: BMI, body mass index

of mechanisms involved in drug hypersensitivity, including cytotoxicity, which is present in nearly all delayed DHRs. Until now only a few reports have suggested that assays measuring drug-induced cytotoxicity may be a useful approach to diagnose drug hypersensitivity,[5,6] especially that induced by antiepileptic drugs (AEDs).[7,8]

In the current study, we investigated whether cytotoxic in vitro tests can be translated into a useful diagnostic tool in DHRs and whether they can be superior to the commonly used LTT and skin patch tests. Since there is a high need to tailor the diagnostic procedure to specific manifestations and drugs,[2,9] we focused on patients with a frequent DHR, namely, maculopapular eruptions induced by AEDs. These drugs, beside antibiotics and nonsteroidal anti-inflammatory drugs, are one of the main causes of DHRs. Their primary therapeutic indication is epilepsy, but AEDs are also often used for other psychiatric and neurological conditions, such as neuropathic pain, migraine, bipolar disorder, and depression.[10] Therefore, AEDs are widely prescribed and there is a growing trend in their use.[11]

PATIENTS AND METHODS

Patients and control donors The study included 23 patients with maculopapular exanthema (MPE) and 24 control donors exposed to the tested drugs for at least 12 months without symptoms of any DHRs. In the patient group, there was 1 individual sensitized to animal dander and 2 individuals suffering from contact allergy to cosmetics. Chronic concomitant disorders were diagnosed in 6 patients: arterial hypertension (3 patients), hypercholesterolemia (1 patient), back pain (1 patient), and seborrheic dermatitis (1 patient). They were treated with angiotensin-converting enzyme inhibitors, calcium channel blockers, β-blockers, nonsteroidal anti-inflammatory drugs, and statins. These drugs were taken continuously including a remission period after DHRs. In the control group, there was 1 individual sensitized to animal dander. Chronic concomitant disorders were diagnosed in 4 controls: arterial hypertension (1 individual), hypercholesterolemia (1 individual), and back pain (2 individuals). They were treated with angiotensin-converting enzyme inhibitors, statins, and nonsteroidal anti-inflammatory drugs, respectively.

Clinical characteristics of donors along with a statistical assessment of variable distribution are summarized in TABLE 1. There were more women in the control group than in the patient group; additionally, in the patient group, more individuals were tested with lamotrigine, although these differences did not reach statistical significance. Before the occurrence of hypersensitivity symptoms, patients were not exposed to new drugs other than AEDs. Tests were performed during clinical remission, always more than 1 month after an acute DHR. AED-induced MPE was defined as rash without systemic symptoms that required the withdrawal of the antiepileptic drug within 3 months from the initiation of treatment.[12] Other causes such as autoimmune disorders or viral and bacterial infections were excluded.[13] The study was approved by a local ethics committee. Written informed consent was obtained from all the tested individuals.

Cell culture Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by density gradient centrifugation as described previously[7] and cryopreserved at a temperature of –80°C until testing. After thawing, PBMCs were directly cultured in duplicate at 2×10^5 cells/well for the LTT or 5×10^5 cells/well for other assays in 96-well U-bottom plates in the presence of culprit drugs, positive control, and culture medium as negative control. Culture medium was prepared as previously described.[7] All drugs (Sigma, Basel, Switzerland) for

in vitro stimulations were used at nontoxic concentrations of 20 and 100 μg/ml.[14] Tetanus toxoid (Serum Statens Institute, Copenhagen, Denmark) at a final concentration of 5 μg/ml served as the positive control.

Lymphocyte transformation test The standard LTT was performed as described by Pichler and Tilch.[14] Briefly, PBMCs were incubated for 6 days; then, 3H-thymidine was added, and, after overnight incubation, the cells were harvested for scintillation counting with a Top-Count counter (Perkin Elmer, Waltham, Massachusetts, United States). The result was expressed as the stimulation index, which was calculated as counts per minute (cpm) recorded in stimulated wells divided by cpm in unstimulated ones. A stimulation index exceeding 2 was considered positive.

Granzyme B detection After 48 hours of incubation, PBMCs were transferred into 96-well filtration plates (Millipore, Volketswill, Switzerland), washed, blocked, and coated with capture antihuman granzyme B (GrB) monoclonal antibody. After additional 20 hours of culture, an enzyme-linked immunospot (ELISpot) assay was developed according to the manufacturer's protocol (Mabtech, Nacka Strand, Sweden). Following the removal of PBMCs, GrB secretion was visualized with tetramethylbenzidine (Mabtech). Spots were analyzed automatically with a Bioreader 3000 CL/PRO (BIO-SYS, Karben, Germany).

Granulysin detection in natural killer cells PBMCs were incubated as described above for 72 hours. To enhance the detectability of Grl, brefeldin A (Sigma, Basel, Switzerland) was added to each well (10 μg/ml) for the last 6 hours. Following stimulation, PBMCs were surface-stained with directly conjugated antibodies: anti-CD3 (PeCy5.5, Biolegend, San Diego, California, United States) and anti-NKp46 (PE, BD Biosciences, San Jose, California, United States). Cells were fixed and permeabilized using the BD Cytofix/Cytoperm as per the manufacturer's instructions (BD Biosciences) for intracellular staining and next stained with Alexa Fluor 488-labelled anti-Grl antibody (BD Biosciences) on ice, in the dark, for 30 minutes. Subsequent to 2 washing steps and resuspending in "Cell-Wash" (BD Bioscience), the upregulation of granulysin (Grl) in $CD3^{-}NKp46^{+}$ cells was analyzed on a FACSCanto (BD Biosciences) flow cytometer as described before.[7]

Perforin detection After 72 hours of incubation, perforin in cell-free supernatants was quantified by an enzyme-linked immunosorbent assay (ELISA) using a commercially available human perforin ELISA kit (Diaclone, Besancon, France) and following the manufacturer's protocol. The absorbance values were measured with a Synergy4 reader (BioTek, Winooski, Vermont, United States), and perforin concentrations were calculated from the standard curve. The detection range was from 40 to 2000 pg/ml. The optimal conditions for the above in vitro assays were determined previously.[7,14,15]

Drug patch tests Drug patch tests were performed and read according to the European Society of Contact Dermatitis guidelines. Briefly, the tests were applied for 2 days on the patients' upper backs using IQ-Ultra chambers (Chemotechnique Diagnostics, Vellinge, Sweden). Readings were conducted at days 2 and 4. Commercialized forms of the tested AEDs (carbamazepine, oxcarbazepine, lamotrigine, and phenytoin) were diluted to 30% in petrolatum for patch testing.[16]

Data analysis The higher mean value of replicates obtained in any of the 2 drug concentrations was considered as the outcome of the assay. Subsequently, the final results were expressed as Δ values: the outcome in stimulated conditions (tested drugs) minus the outcome in unstimulated conditions (negative control). Positive results were recorded as Δ values greater than the mean Δ value plus 2 standard deviations measured in control subjects. Differences in proportions between the groups were compared with the χ^2 test with Yates correction or Fisher exact test where necessary. Nonparametric analyses (2-tailed Mann–Whitney test and Spearman correlation) were used in the study, as appropriate, for nonnormally distributed variables (GraphPad Software, Inc., La Jolla, California, United States). A P value of less than 0.05 was considered statistically significant.

RESULTS The results of in vitro tests are presented in FIGURE 1. Both the ELISpot assay (FIGURE 1A) and cytometric analysis (FIGURE 1B) showed a significantly higher expression of GrB and Grl in the patient group than in controls. Culprit drugs induced also an increase in the proliferation of PBMCs in the LTT in the patient group, but not in healthy donors (FIGURE 1C). Perforin concentrations in the supernatants of PBMC cultures from patients did not differ significantly from those in control samples (FIGURE 1D). The LTT showed a significant positive correlation both with the GrB-ELISpot assay and with the Grl-NK assay. In addition, there was a correlation between the expression of GrB in the ELISpot assay and Grl in NK cells (FIGURE 2), while the perforin-ELISA test did not correlate with any other assay ($P < 0.05$).

The cut-offs defined in the methods section allowed us to identify a positive or negative drug response and to attribute sensitivity and specificity to particular assays. The highest sensitivity (55%) was achieved by the GrB-ELISpot assay, followed by the Grl expression in natural killer (NK) cells and the LTT (sensitivity, 39.1% and 30.4%, respectively). The perforin-ELISA test reached a sensitivity of 17.4%. The specificities of all assays remained in the range from 95.8% to 100%

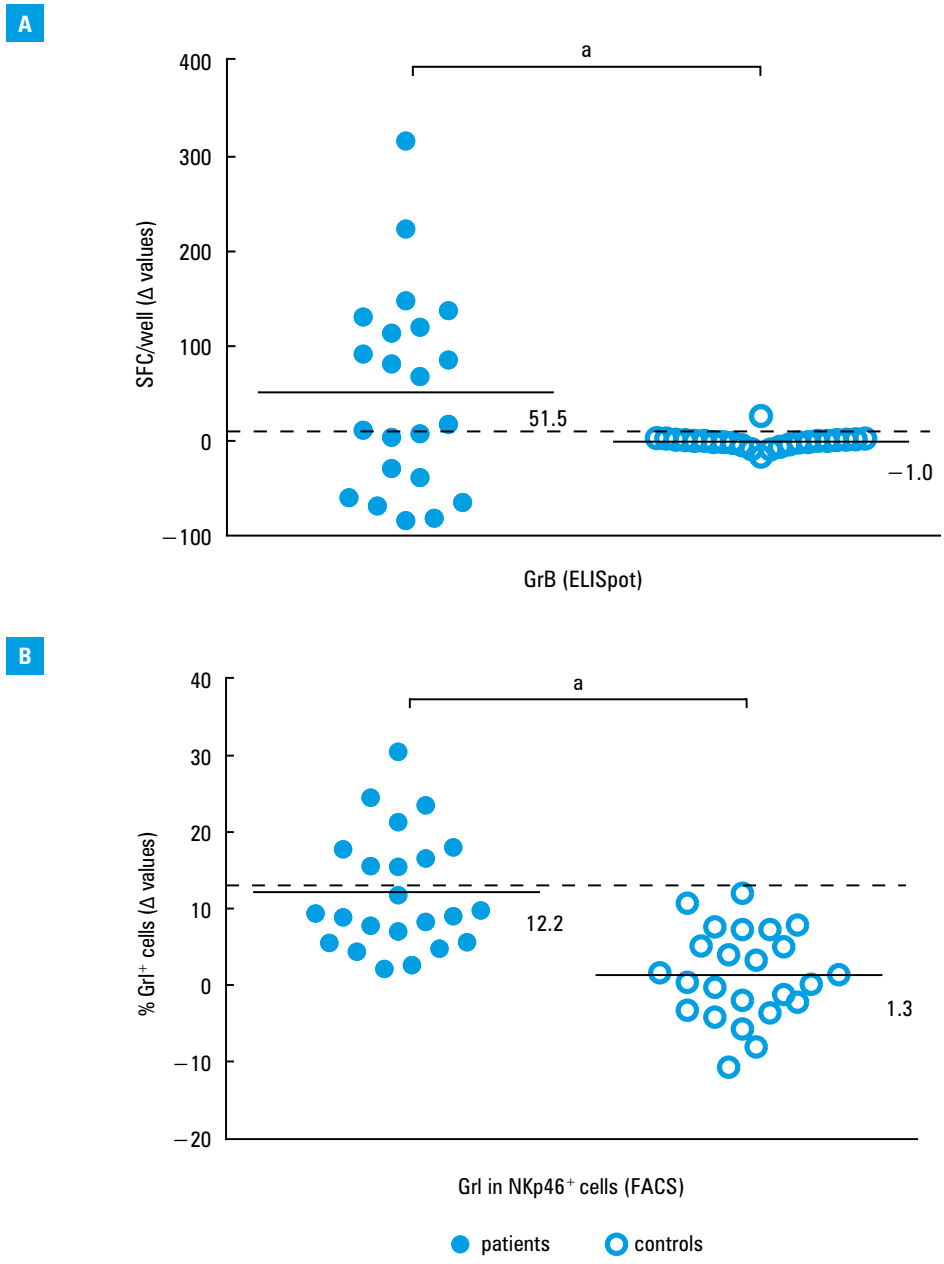

FIGURE 1 Comparison of drug-specific cell responses in patients and controls; **A** – spot-forming cells releasing granzyme B; **B** – upregulation of granulysin in NKp46$^+$ cells

Each data point represents the maximal recorded Δ value (the difference between the response in the presence of tested drug minus background). Dotted horizontal lines represent the cut-off values. Solid lines indicate the group mean with corresponding mean values; **a** $P < 0.05$

Abbreviations: GrB, granzyme B; Grl, granulysin; FACS, fluorescence-activated cell sorting (flow cytometry); NS, nonsignificant; SFC, spot-forming cells; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot assay; LTT, lymphocyte transformation test; SFC, spot-forming cells; SI, stimulation index

(TABLE 2). TABLE 2 also summarizes the results of patch tests with culprit drugs and time intervals between drug-induced symptoms and the tests in individual patients. Drug patch tests were positive in 3 patients and negative in all controls.

In order to assess how the time interval between DHRs and the execution of tests affects the results of the assays, we compared the results of the patients tested within a shorter time than the median time interval (48 months) with the results of the remaining patients. In all assays (FIGURE 3A–C) except for the perforin-ELISA test (FIGURE 3D), we observed a higher number of positive responses and significantly higher read-out values in the group tested over a shorter time span.

The frequency of positive responses to carbamazepine and its analogue, oxcarbamazepine (LTT, 28.6%, 4/14; GrB-ELISpot, 64.3%, 9/14; Grl-NK, 42.9%, 6/14; and perforin-ELISA, 21.4%, 3/14) vs lamotrigine (LTT, 37.5%, 3/8; GrB-ELISpot, 37.5%, 3/8; Grl-NK, 37.5%, 3/8; and perforin-ELISA, 12.5%, 1/8), which were the most

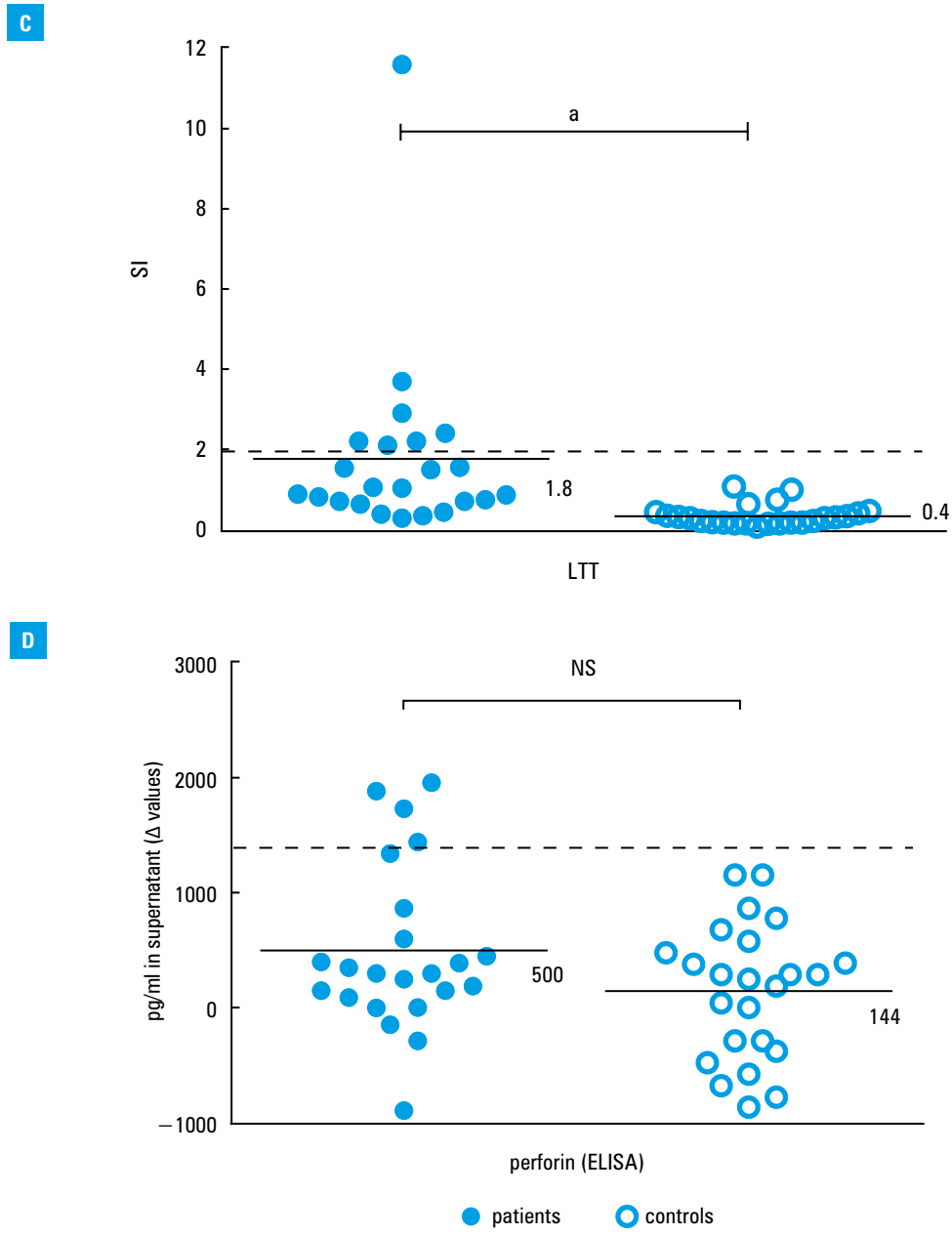

FIGURE 1 Comparison of drug-specific cell responses in patients and controls; **C** – lymphocyte proliferation; **D** – concentration of perforin in cell supernatant

Each data point represents the maximal recorded Δ value (the difference between the response in the presence of tested drug minus background). Dotted horizontal lines represent the cut-off values. Solid lines indicate the group mean with corresponding mean values; **a** $P < 0.05$

Abbreviations: see **FIGURE 1 AB**

often tested drugs, showed no statistical difference between these drugs.

DISCUSSION Previous studies suggested mediators of cytotoxicity as possible targets in causal diagnosis of DHRs. Grl, the key molecule responsible for keratinocytes death in Stevens–Johnson syndrome,[17] was found in various DHRs. Schlapbach et al[6] described drug-specific activation and recruitment of NK cells expressing Grl in MPE, fixed drug eruption, and acute generalized exanthematous pustulosis. They showed that in drug-allergic patients, Grl was expressed mostly by NK cells at significantly higher levels than in controls. Other mediators such as perforin and GrB were found to be expressed by T cells in the epidermis and at the dermoepidermal junction zone of maculopapular drug eruptions[18] and to mediate drug-specific cytotoxicity in T-cell lines and clones derived from patients with different delayed DHRs.[19] This is consistent with another study demonstrating that drug-specific cytotoxic mechanisms can be detected in PBMCs of patients with drug-induced symptoms including maculopapular, bullous, and delayed systemic reactions.[5] In the current study, we attempted to translate

FIGURE 2 Correlations between in vitro assays
A – lymphocyte transformation test vs granulysin in NKp46$^+$ cells; **B** – lymphocyte transformation test vs granzyme B;
C – granulysin in NKp46$^+$ cells (FACS) vs granzyme B
Abbreviations: see **FIGURE 1**

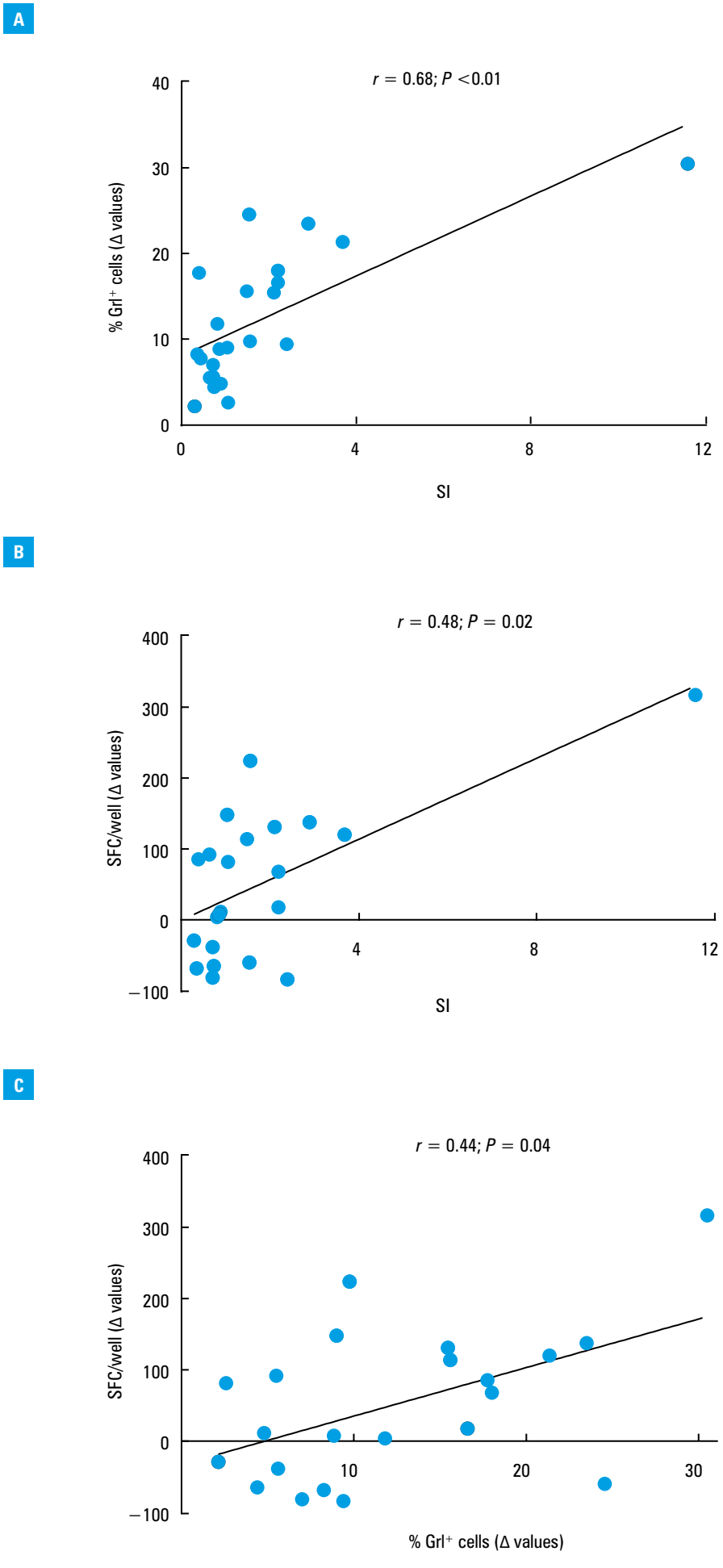

TABLE 2 Summary of tests performed in individual patients; results of in vitro assays and patch tests together with corresponding culprit drugs and time interval between drug-induced symptoms and the execution of tests

Patient	Culprit drug	Time interval, mo	PT	LTT, SI	Perforin, pg/ml (ELISA)	% Grl$^+$ NKp46$^+$ cells	GrB, SCF/well (ELISpot)
1	LTG	135	nd	1.5	188.4	24.6[a]	–60
2	CBZ	39	pos	1.5	865.3	15.6[a]	114[a]
3	CBZ	15	neg	2.9[a]	1957.6[a]	23.5[a]	137.5[a]
4	OXC	76	neg	0.4	388.4	17.8[a]	86[a]
5	LTG	76	neg	0.8	1730.7[a]	11.8	4
6	CBZ	16	neg	2.2[a]	1442.2[a]	18[a]	68[a]
7	CBZ	4	neg	1.6	–288.4	9.8	224[a]
8	CBZ	76	neg	0.9	88.5	4.8	11.5
9	PHT	71	neg	0.7	398.1	4.4	–65
10	CBZ	7	neg.	0.7	–894.2	7	–82
11	LTG	9	nd	2.1[a]	298.1	15.5[a]	131[a]
12	LTG	17	neg	1.0	298.1	9	148[a]
13	CBZ	232	neg	1.1	0.0	2.6	82[a]
14	CBZ	76	neg.	0.3	1885.6[a]	2.2	–29
15	CBZ	1	neg	2.2[a]	1341.3	16.6[a]	18[a]
16	LTG	1	pos	11.6[a]	–1.9	30.5[a]	317[a]
17	CBZ	117	neg	0.4	149.0	7.8	nd
18	LTG	165	neg	0.4	598.1	8.2	–69
19	CBZ	1	pos	3.7[a]	348.1	21.4[a]	120[a]
20	OXC	95	neg	0.9	248.1	8.9	8
21	LTG	59	neg	2.4[a]	149.0	9.4	–84
22	CBZ	48	neg	0.6	–149.0	5.5	92[a]
23	LTG	4	neg	0.7	447.1	5.6	–39
sensitivity, %			14.3	30.4	17.4	39.1	55
specificity, %			100	100	100	100	95.8

Results are expressed as delta values: drug-stimulated condition minus background.

a positive results

Abbreviations: CBZ, carbamazepine; LTG, lamotrigine; nd, not done; neg, negative; OXC, oxcarbamazepine; PHT, phenytoin; pos, positive; PT, patch tests; others, see **FIGURE 1**

preliminary experimental evidence on cytotoxic function of drug-specific T cells into in vitro read-out systems useful in bedside medicine. So far, investigators have often studied drug-allergic patients with different DHRs due to different offending drugs.[5,15,18,20] When an in vitro assay is used in daily practice, there is a serious risk that its results may be distorted by differences in symptoms and culprit drugs between a currently tested individual and patients tested in previous studies. To overcome such limitations, our study group consisted of patients with a specific manifestation provoked by specific drugs, namely AED-induced MPE. Such an approach is in line with the recent expert recommendations[2] and may be considered the strength of this survey.

Although the LTT is the most widely used in vitro test for the diagnosis of delayed DHRs, it has a number of disadvantages, including a long-lasting protocol and the use of radioisotopes. Studies assessing alternative in vitro assays are often based on the enrollment of LTT-positive patients.[5,15,21] In this study, we performed 4 in vitro tests: LTT, GrB-ELISpot assay, Grl-NK assay, and perforin-ELISA at the same time, to be able to compare them directly. In all assays, apart from the perforin-ELISA, we found significantly higher responses to tested drugs in the patient group than in controls (**FIGURE 1**). Furthermore, GrB release by PBMCs and Grl expression in NK cells reached higher sensitivities as diagnostic endpoints than the stimulation index in the standard LTT. To explain these findings, one may speculate that the response of drug-specific cells relies mainly on the release of cytotoxic cytokines and only to a lesser extent—on proliferation. However, we also demonstrated correlations between the LTT, GrB-ELISpot assay, and Grl-NK assay (**FIGURE 2**). All patients with positive results of the LTT, except 1 (patient #21), also had positive results in both the GrB-ELISpot and Grl-NK assays. The next 7 patients (#1, 2, 4, 7, 12, 13, 22) had positive results only in GrB or Grl-detecting tests (or both). Moreover, the longest time spans between acute DHR symptoms and positive response reached 232 months in the GrB-ELISpot (patient #13) and 135 months in Grl-NK (patient #1) in comparison with 59

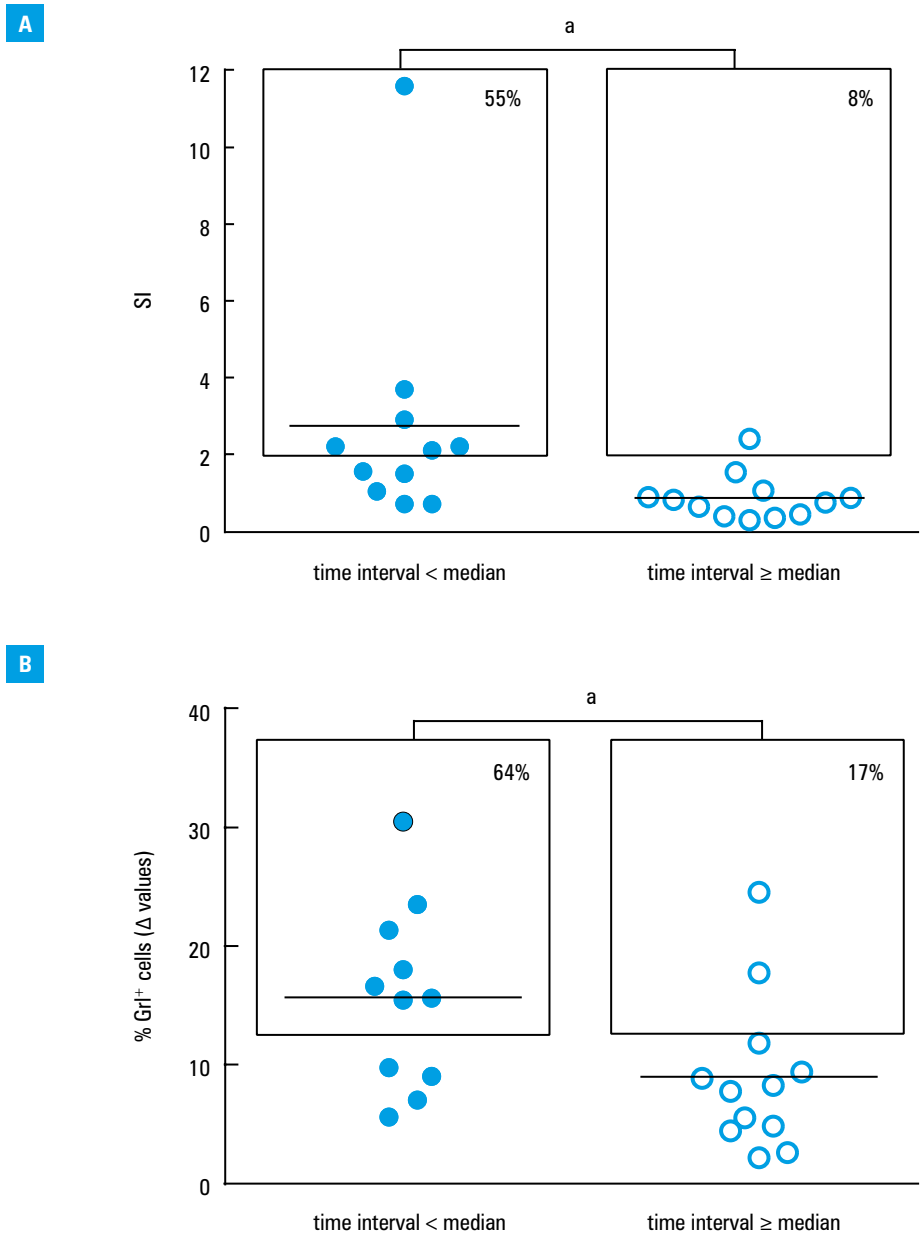

FIGURE 3 Drug-specific cell response in relation to the time point of evaluation. Results of patients tested in a shorter time than the median time interval between drug-induced reaction and the execution of tests (blue circles) were compared with the results of the remaining patients (open circles) in consecutive assays: **A** – lymphocyte transformation test; **B** – granulysin in NKp46^{+} cells.

Boxes contain positive responses data points. Percentages indicate positive results recorded within the groups tested. Horizontal lines represent mean values; **a** $P < 0.05$

Abbreviations: see **FIGURES 1** and **2**

months for the LTT, patient #21. Therefore, it shows that the LTT, GrB-ELISpot, and Grl-NK assays identify similar patients reactive to a culprit drug, but GrB and Grl-based tests are able to detect a weaker drug-specific response and maintain diagnostic responsiveness for a longer time.

In comparison with other assays, the GrB--ELISpot clearly demonstrated the highest sensitivity (55%) along with still a reasonably high specificity (95.8%). Actually, the detection of cytokines with this method is highly sensitive, which was described elsewhere.[19,21] The ELISpot assay allows to detect GrB secreted by single cells present in low frequencies before it is degraded by proteases or absorbed by receptors. In patients not exposed to the culprit drug for many years, only few T cells can persist as resting memory cells releasing cytokines under drug-specific stimulation.[21] For these patients, the ELISpot assay is an especially useful diagnostic tool. The Grl-NK assay also showed potential utility as a sensitive and specific read-out system for the detection of drug-specific response. The Grl expression in different PBMC populations has already been investigated, mostly in patients with bullous skin manifestations of drug-induced reactions. In our

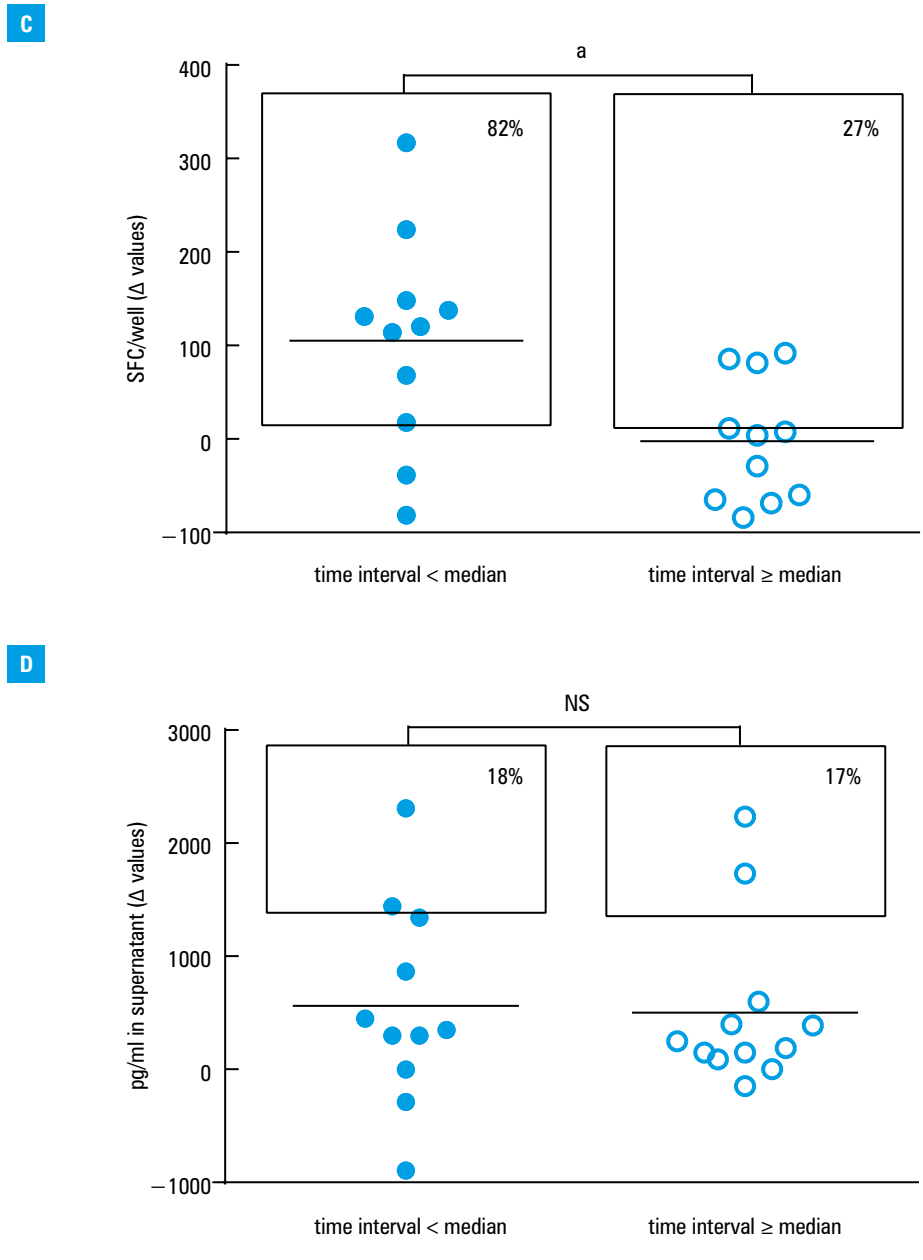

FIGURE 3 Drug-specific cell response in relation to the time point of evaluation. Results of patients tested in a shorter time than the median time interval between drug-induced reaction and the execution of tests (blue circles) were compared with the results of the remaining patients (open circles) in consecutive assays: **C** – granzyme B (enzyme-linked immunospot); **D** – perforin (enzyme-linked immunosorbent assay). Boxes contain positive responses data points. Percentages indicate positive results recorded within the groups tested. Horizontal lines represent mean values.

Boxes contain positive responses data points. Percentages indicate positive results recorded within the groups tested. Horizontal lines represent mean values; **a** $P < 0.05$

Abbreviations: see **FIGURES 1** and **2**

previous study on Stevens–Johnson syndrome, we found a significant increase in the Grl expression in both NK cells and $CD4^+$ cells stimulated by culprit drugs, but considering the results of patients hypersensitive to AEDs, NK cells appeared to be a better diagnostic endpoint.[7] This is consistent with observations that NK cells are the main source of Grl, both in vivo and in vitro, in various DHRs, including MPE.[6] Moreover, the degree of Grl upregulation in NK cells is much higher than in $CD4^+$ cells. Thus, the detection of a significant difference between stimulated and baseline conditions is easier in NK cells, as suggested by other authors.[8] The perforin-ELISA, another investigated assay, did not show sufficient sensitivity and produced the results inconsistent with the results of other in vitro tests. It may be related to the mode of action of perforin, which polymerizes on the activation of effector cells, thus forming cylindrical pores in the target-cell

membrane and enabling other mediators to enter the cell.[22] Therefore, when perforin undergoes transmembrane insertion to target cells during cell culture, it may be no longer present in the supernatant in substantial concentrations. Finally, the analysis of in vitro results showed that the LTT, GrB-ELISpot assay, and Grl-NK assay are reliable across a range of tested drugs, as the differences in the percentage of positive responses to ox/carbamazepine vs lamotrigine were insignificant.

The success rate of drug patch tests is highly variable, ranging from 7% to 87%,[16,23-29] depending on a time span from a hypersensitivity reaction, its clinical manifestation, or differences in the drugs themselves. Investigators often focus exclusively on drug skin tests or in vitro diagnosis, but from a practical point of view, it would be the most interesting to know whether an investigated assay is superior to skin tests, or vice versa. Our main finding was the low rate of positive skin tests (14.3%, 3/21) in comparison with in vitro assays. Probably the most important reason for this was a long interval between a DHR and the execution of tests in the majority of examined patients. However, there was also a patient who demonstrated positive patch test results after a time interval as long as 39 months. This corresponds with the reports on patients remaining sensitized to culprit drugs for many years and demonstrating strongly positive patch test results for many years after DHRs.[23,26,30] We did not perform drug patch tests in 2 individuals from the patient group: 1 patient was pregnant and the other lived far from the study center and could not present for test readings. Such a real-life example illustrates some advantages of an in vitro diagnostic approach.

Although some drug tests may yield negative results after 6 to 12 months,[2] others are still positive owing to long persistence of memory T cells.[14,31,32] Nevertheless, one may expect the higher rates of positive test results within 4 weeks to 6 months after resolution and a decrease in sensitivity of tests over time, which was also observed in our study. Patients tested within a shorter time after a DHR showed a significantly higher number of positive results in the LTT, GrB-ELISpot assay, and Grl-NK assay. We believe that a substantial time span between a drug-induced reaction and the execution of tests (1–232 months) allowed us to achieve a better distinction between investigated assays in terms of sensitivity. Our data are in line with the previous findings that it is possible to detect persistent drug-specific T cells over a long time with a sensitive assay.[21] However, there is still a number of patients with negative results in all tests (7/23, 30.4%). Considering that it is impossible to predict how long specific hypersensitivity will persist in an individual patient, patients could generally lose their reactivity to the culprit drug over time. There is also some evidence that specific T cells may respond to a drug in a dose-dependent manner in delayed DHRs.[33] Most of our patients were included in the study by doctors well aware of DHR, who immediately withdrew culprit drugs when the preliminary symptoms of hypersensitivity occurred. These patients were exposed to culprit drugs for a relatively short time and it may be the reason that some of them did not develop a strong immune response. Therefore, this response might be more difficult to reveal in our tests or even undetectable in some cases. On the other hand, for instance patient #16, initially misdiagnosed and receiving a full therapeutic dose of lamotrigine for 7 days despite drug-induced exanthema, demonstrated highly positive results in the LTT, GrB-ELISpot assay, and Grl-NK assay.

It is necessary to consider the limitations of the study, which was performed exclusively in patients with a defined clinical picture, namely, MPEs due to AEDs. Thus, extrapolation of the results to other clinical manifestations or drugs would be an approximation. Besides, a challenging dilemma is the interpretation of a weakly positive or borderline result of in vitro assays in an individual patient. In such case, a detailed clinical context should be analyzed, since an etiological diagnosis in DHRs is often nothing else but the assessment of the probability of a causative relationship between hypersensitive symptoms and the suspected drug.[34,35] Therefore, in practice, the final conclusion on drug causality derives from a sum of clinical data including experience with the drug and its typical adverse reactions as well as the results of in vivo and laboratory tests.

In the present study, we demonstrated that PBMCs produce GrB, Grl, and, to a lesser extent, perforin under stimulation with a culprit drug. These findings can be translated into diagnostic practice, since in vitro diagnostic methods based on cytotoxic effector cell function, that is, the Grl-NK assay, and in particular the GrB-ELISpot assay, offer an essential advantage over the routine LTT (not only a shorter assay time and nonradioactive approach, but also greater sensitivity). In addition, we proved that Grl-NK and GrB-ELISpot assays have considerably higher positive detection rates than drug patch tests, also in patients in long-lasting remission of hypersensitive symptoms. Therefore, the assays can be used as a promising alternative to drug patch tests - the current most common diagnostic approach. Therefore, drug-specific cells appeared to be more easily detectable directly in the circulation than in the skin. Finally, the percentage of positive results of in vitro assays strongly decreases over time, but the GrB-ELISpot and Grl-NK assays described here allow to detect drug-specific T cells in instances where low-level responses might be overlooked by standard diagnostic techniques. Further studies are needed to develop novel approaches to diagnose delayed allergic reactions and monitor adverse drug reactions.[36,37] Future research directions could follow a drug-specific and symptom-specific approach in

the evaluation of diagnostic methods in DHRs. It seems that the development of assays based on effector mechanisms of reactions is a promising strategy.

Contribution statement GP conceived the idea for the study and study design, participated in the enrollment of subjects into the study and in the execution of patch tests, conducted all in vitro assays, reviewed pertinent raw data, performed the statistical analysis, wrote the original draft of the manuscript, and approved the final version of the manuscript. EC participated in the interpretation of data and read and approved the final version of the manuscript. MB participated in the enrollment of subjects into the study and in the execution of patch tests, as well as read and approved the final manuscript.

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ARTYKUŁ ORYGINALNY

Testy oparte na mechanizmach cytotoksycznych w opóźnionych polekowych reakcjach nadwrażliwości indukowanych lekami przeciwpadaczkowymi

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SŁOWA KLUCZOWE

alergia na leki, granulizyna, granzym B, nadwrażliwość opóźniona, testy *in vitro*

STRESZCZENIE

WPROWADZENIE Mechanizmy cytotoksyczne występują w większości opóźnionych polekowych reakcji nadwrażliwości, ale nie są wykorzystywane jako narzędzie diagnostyczne.

CELE Celem badania było porównanie testów opartych na mechanizmach cytotoksycznych z testem proliferacji i testami płatkowymi u pacjentów z osutkami plamisto-grudkowymi wywołanymi lekami przeciwpadaczkowymi.

PACJENCI I METODY Hodowli w określonych warunkach poddano komórki jednojądrzaste krwi obwodowej 23 pacjentów i 24 osób z grupy kontrolnej poddanych działaniu badanych leków. Swoistą odpowiedź po stymulacji lekiem obserwowano, oznaczając granzym B (GrB) testem ELISpot (*enzyme-linked immunospot assay*), wewnątrzkomórkową ekspresję granulizyny (Grl) w komórkach $CD3^{-}NKp46^{+}$ za pomocą cytometrii przepływowej, stężenie perforyny w nadsączu komórkowym testem ELISA oraz wykonując test proliferacji limfocytów. U wszystkich badanych przeprowadzono testy płatkowe z lekami przyczynowymi.

WYNIKI Proliferacja limfocytów, wydzielanie GrB i ekspresja Grl były znamiennie wyższe u chorych w porównaniu z grupą kontrolną, natomiast stężenie perforyny nie było większe. Czułość testów opartych na oznaczeniu proliferacji, GrB, Grl i perforyny wyniosła odpowiednio 30,4%, 55%, 39,1% i 17,4%. Znamiennie częściej dodatnie wyniki obserwowano, gdy testy były wykonane do dwóch lat po reakcji polekowej. Swoistość wszystkich testów pozostawała w przedziale 95,8–100%. Testy płatkowe były dodatnie tylko u trzech chorych (czułość 14,3%) i ujemne u wszystkich z grupy kontrolnej.

WNIOSKI Badania *in vitro* oparte na detekcji Grl, a szczególnie GrB, są bardziej przydatne niż testy rutynowo stosowane w diagnostyce nadwrażliwości na leki przeciwpadaczkowe. Można dzięki nim wykryć słabą odpowiedź, której mogłyby nie ujawnić standardowe techniki. W fazie remisji komórki lekowo-swoiste łatwiej stwierdzić w krążeniu niż w skórze chorego.

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