

Original article

CD69 upregulation on T cells as an *in vitro* marker for delayed-type drug hypersensitivity

Background: T cells play a key role in delayed-type drug hypersensitivity reactions. Their reactivity can be assessed by their proliferation in response to the drug in the lymphocyte transformation test (LTT). However, the LTT imposes limitations in terms of practicability, and an alternative method that is easier to implement than the LTT would be desirable.

Methods: Four months to 12 years after acute drug hypersensitivity reactions, CD69 upregulation on T cells of 15 patients and five healthy controls was analyzed by flow cytometry.

Results: All 15 LTT-positive patients showed a significant increase of CD69 expression on T cells after 48 h of drug-stimulation exclusively with the drugs incriminated in drug-hypersensitivities. A stimulation index of 2 as cut-off value allowed discrimination between nonreactive and reactive T cells in LTT and CD69 upregulation. T cells (0.5–3%) showed CD69 up-regulation. The reactive cell population consisted of a minority of truly drug reactive T cells secreting cytokines and a higher number of bystander T cells activated by IL-2 and possibly other cytokines.

Conclusions: CD69 upregulation was observed after 2 days in all patients with a positive LTT after 6 days, thus appearing to be a promising tool to identify drug-reactive T cells in the peripheral blood of patients with drug-hypersensitivity reactions.

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The diagnosis of hypersensitivity reactions to drugs remains a major problem in clinical practice as skin tests often do not result in positive reactions even in patients with well-documented histories of drug-allergic reactions. Provocation tests bear the risk of severe reactions and are contraindicated in the most severe forms of drug hypersensitivity reactions like Steven Johnsons syndrome or toxic epidermal necrolysis and do not differentiate between allergic and pseudo-allergic reactions.

Current *in vitro* tests predominantly address immediate-type drug-allergic reactions, assessing e.g. drug-reactive IgE antibodies (CAP-assays), or the drug specific activation of basophils (Flow-CAST or Basotest) (1, 2).

As many drug-hypersensitivity reactions are not mediated by drug-specific IgE antibodies but by drug-reactive T cells, these tests often fail at identifying the culprit drug. Several studies have shown that drug-reacting T cells exist in the affected tissue and peripheral blood of patients suffering from all four types of allergic reactions (3–12), that they have a high frequency in the peripheral blood of patients in remission, and are present even 12 year after the acute reaction (13), implying that drug-reactive T cells might be detectable by *in vitro* tests using T-cell activation, cytokine production or proliferation as parameters (14).

The lymphocyte transformation test (LTT) is the only test to detect a sensitization of T cells to drugs in the peripheral blood of drug-allergic patients, assaying the proliferation of *in vitro* (11, 15). Its specificity has been demonstrated by generating drug-reactive T-cell lines and T-cell clones from peripheral blood mononuclear cells (PBMC) cultures isolated from drug-allergic patients stimulated with drugs like β -lactams, lidocaine, mepivacaine, sulfamethoxazole (SMX), celecoxib, lamotrigine, carbamazepine and *p*-phenylendiamine, none of which induced a response in nonsensitized individuals (4, 6, 16–21). The LTT is often considered more of a research than a diagnostic tool because it is

Abbreviations: AGEP, acute generalized exanthematous pustulosis; AMX, amoxicillin; CBZ, carbamazepine; CFSE, 5, 6-carboxyfluorescein diacetate succinimidyl ester; CFX, cefuroxime; CLA, clavalanic acid; CM, culture medium; CPM, counts per minute; DRESS, drug rash with eosinophilia and systemic symptoms; LTT, lymphocyte transformation test; MPE, maculopapular exanthema; MXFX, moxifloxacin; NMFI, normalized mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHE, phenytoin; SI, stimulation index; SJS, Stevens Johnson syndrome; SMX, sulfamethoxazole; SP, sulapyridine; TEN, toxic epidermal necrolysis syndrome; TT, tetanus toxoid; VCM, vancomycin.

cumbersome, requires sterile cell culture, involves ^3H -thymidine incorporation, is of long duration (6–7 days) and has a sensitivity of at best 60–70% (6, 11, 12, 15, 22–25). Considering the high incidence of delayed drug hypersensitivity reactions, there is an urgent need to develop a new *in vitro* test that can be performed more easily. Such a test should be at least as sensitive and specific as the LTT, discriminate between drug reactive and nonreactive T cells, be more rapid, and involve neither prolonged cell culture nor radioactivity.

The surface molecule CD69 has been widely used as marker of T-cell activation both *in vitro* and *in vivo* (26), but has not been employed for drug hypersensitivity diagnosis so far. In this pilot study, we evaluated its usefulness on T cells of 15 patients with T-cell-mediated drug allergic reactions. All patients were positive in the LTT. Our analyses indicate that CD69 is detectable after 48 h by flow cytometry in all 15 patients tested, as it is upregulated on up to 3% of all CD4^+ T cells. Its upregulation is specific and a stimulation index of 2 appears to discriminate well between allergic and nonallergic patients. Therefore, CD69 measurement is a promising tool to detect drug-reactive T cells in the peripheral blood of drug allergic patients and deserves further validation in a prospective study.

Methods

Donor characteristics

Table 1 summarizes the patients' clinical characteristics. We did not perform provocation tests for ethical reasons as no standardized

protocol exists for delayed hypersensitivity reactions. Patients were analyzed in clinical remission and were healthy at the time of analysis. Five healthy blood donors with no history of drug hypersensitivity served as negative controls. All gave informed consent to this study, which was approved by the ethical committee.

Cell preparation and culture

Peripheral mononuclear cells were isolated and cultured as described before (13).

Drugs and antigens

Drugs were used at nontoxic concentrations and stock solutions prepared directly before use. Drugs were used as follows: 100, 200, 500 $\mu\text{g}/\text{ml}$ amoxicillin (AMX), 100, 200, 500 $\mu\text{g}/\text{ml}$ ampicillin (AMP), 1, 10, 100 $\mu\text{g}/\text{ml}$ vancomycin (VCM), 1, 10, 100 $\mu\text{g}/\text{ml}$ carbamazepine (CBZ), 1, 10, 100 $\mu\text{g}/\text{ml}$ sulfapyridine (SP), 1, 10, 100 $\mu\text{g}/\text{ml}$ cefuroxime (CFX; all from Sigma-Aldrich, St Louis, MO, USA), 50, 100, 200 $\mu\text{g}/\text{ml}$ sulfamethoxazole (SMX; Roche Pharma, Basel, Switzerland), 10, 50, 100 $\mu\text{g}/\text{ml}$ phenytoin (PHE; Siegfried Holding AG, Zofingen, Switzerland), 1, 10, 50 $\mu\text{g}/\text{ml}$ clavulanic acid (CLA; Lek, Ljubljana, Slovenja) and 1, 10, 100 $\mu\text{g}/\text{ml}$ moxifloxacin (MXFX; Bayer AG, Leverkusen, Germany).

Patch tests

Patch test was performed according to (27, 28).

Lymphocyte transformation test

Lymphocyte transformation tests were performed as described before (13, 15). Stimulation indices (SI) were calculated as counts per minutes (cpm) in culture medium (CM) with antigen divided by cpm in CM without antigen (13), and interpreted as described (15).

Table 1. Patient's characteristics

Patient	Age/sex	Culprit drug	Disease	LTT	Patch test	Duration*
AS	35 years/f	AMX	MPE	Positive†	–/–‡	14 months
BK	35 years/m	AMX	MPE	Positive	–/–	8 months
LDM	57 years/f	AMX	MPE	Positive	–/–	5 months
HH	47 years/m	AMX	SJS	Positive	++/+++	9 months
SF	26 years/f	AMX	MPE	Positive	++/++	2 months
ES	82 years/f	AMX, CLA	E.L.E.§, fever, thrombocytosis	Positive	n.d./n.d.¶	5 months
WZ	64 years/m	AMX, PHE, SMX	DRESS, MPE, erythrodermia	Positive	–/–	19 months
UNO	45 years/m	SMX	MPE, malaise	Positive	++/++	12 years
HB	71 years/m	SMX	SJS	Positive	–/–	2 months
AA	48 years/m	SP	DRESS	Positive	–/–	13 months
EK	19 years/m	CFX	MPE, fever	Positive	–/–	8 months
GR	80 years/f	MXFX	MPE	Positive	+/+	10 months
VG	31 years/f	VCM	AGEP	Positive	+++/n.d.	14 months
CS	35 years/f	CBZ	DRESS	positive	–/–	14 months
GO	45 years/f	CBZ	AGEP, erythrodermia	positive	+++/n.d.	2 months

*Time interval between acute allergy and testing.

† $\text{SI}_{\text{LTT}} > 2$.

‡–, no reaction; +, exanthema and sparse induration; ++, exanthema with well-defined induration; +++, exanthema, induration and vesicles after 48 h/72 h respectively.

§Elevated liver enzymes.

¶Not determined.

CFSE proliferation assay

5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE) proliferation assays were performed as described (13).

Flow cytometry analysis of CD69 upregulation

Freshly isolated PBMC (2×10^5) were cultured in triplicates in 96-well U-bottomed tissue culture plates in the presence of indicated drug concentrations as described, of TT (10 μ g/ml), or of IL-2 (Proleukin, Roche Pharma, Basel, Switzerland). CD69 expression on CD4⁺/CD8⁺ T cells was measured by flow cytometry with anti-human monoclonal antibodies (mAb) (PE-CD69, APC-CD3, FITC-CD8, PerCP-CD4 and PE-IgG1 as isotype control; BD Biosciences, San Jose, CA, USA). Fluorescence was determined with a FACSCanto™ Flow Cytometer (BD Biosciences). Data were expressed as normalized mean fluorescence intensity (NMFI), which was calculated by multiplying the percentage with the mean fluorescence intensity of CD69⁺. SI were calculated as NMFI in CM with antigen divided by NMFI in CM without antigen.

Intracellular staining

Peripheral blood mononuclear cells were stimulated with drugs or antigens as indicated for 24 h with unstimulated PBMC incubated in parallel as a negative control. Monensin (Sigma-Aldrich corporation) was added at 6 μ g/ml for the last 8 h of incubation. Cells were washed twice in ice-cold phosphate buffered saline (PBS) containing 0.5% BSA and 0.1% sodium azide and stained for 20 min with anti-human mAb (PE-CD4 mAb, PerCP-CD3 mAb and FITC-CD69 mAb. Cells were fixed and permeabilized with Cytotfix/Cytoperm solution for 20 min (BD Biosciences), and resuspended in Perm/Wash solution (BD Biosciences) containing APC conjugated anti-IFN- γ /IL-5 mAb and incubated at 4°C for 30 min in the dark. As isotype controls, FITC- and APC-conjugated IgG1 and IgG2a were used. All antibodies were obtained from BD Biosciences. After washing twice with Perm/Wash, cells were resuspended in PBS containing 0.5% BSA and 0.1% sodium azide, and analyzed as described.

IL-2 detection in cell-culture supernatants

T cells (2×10^5) were stimulated in 200 μ l of CM for 48 h in round-bottom 96-well plates with drugs as indicated, 10 μ g/ml of tetanus toxoid as a positive control antigen, or CM alone as control. A cytokine ELISA was used for detection of secreted IL-2 (BD

Biosciences). Duplicate samples were measured according to the manufacturer's protocols. The detection limit of the assay was 1 pg/ml for IL-2.

IL-2 receptor blockade

Peripheral blood mononuclear cells from blood donors were either preincubated for 1 h at 37°C with blocking antibodies against recombinant human interleukin-2 receptor alpha (rh IL-2 R α , 1 μ g/ml) and beta (rh IL-2 R β , 10 μ g/ml; R&D Systems, Minneapolis, MN, USA) or with their corresponding mouse IgG1 and goat IgG isotype controls, and stimulated with drugs, TT as positive control, or CM alone as negative control.

Results

Characterization of patients

Table 1 summarizes the clinical characteristics of 15 patients. All patients showed a positive *in vitro* reactivity in the LTT as defined by Pichler and Tilch (15), which were the inclusion criteria. Six of 15 patients showed positive patch test reactivity to the drugs. Results were grouped into β -lactams (AMX, CLA), sulfanilamides (SMX, SP), antiepileptics (PHE, CBZ), and other antibiotics including quinolones (CFX, MXFX) and vancomycin (VCM). Drug hypersensitivity reactions had occurred 2 months–12 years before testing (Table 1). Five patients have previously been described with regard to precursor frequency of drug-reactive T cells (13). Five healthy nonallergic individuals served as negative controls.

CD69 upregulation

A typical example of CD69 upregulation on CD4⁺ T cells of a SP-allergic patient is shown in Fig. 1A. Upon exposure to SP and TT, 1.9% and 1.1% of CD4⁺ cells showed CD69 expression, respectively, while only 0.1% were positive upon incubation with CM. Time kinetics of the mean CD69 upregulation on CD4⁺ T cells from five drug-allergic patients (patients UNO, WZ, CS, HH and VG) after 18, 36 and 72 h of stimulation with the culprit

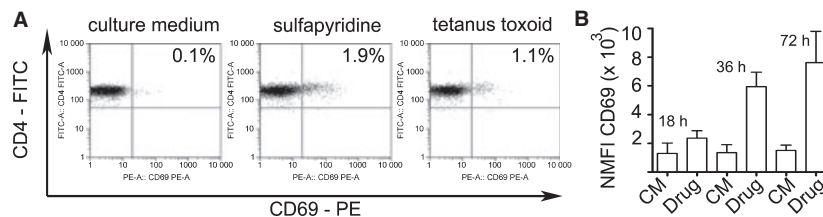


Figure 1. Flow cytometric analysis and time kinetics of CD69 upregulation. (A) CD69 upregulation on CD4 T cells from a sulfapyridine (SP)-allergic patient 48 h after culture medium (negative control), SP (drug), or tetanus toxoid (positive control) stimulation. (B) Time kinetics of CD69 upregulation on T cells shown as normalized mean fluorescence intensity (NMFI) after coincubation of peripheral blood mononuclear cells from allergic patients with different drugs (drug) or CM for 18, 36 or 72 h. Shown is the mean $\pm$ SE of five drug-allergic patients.

drug (Table 1) are shown in Fig. 1B. CD69 upregulation occurred on both CD4⁺ and CD8⁺ T cells (not shown). No upregulation of CD69 on CD4⁺ and CD8⁺ T cells was observed after 6 and 12 h of antigen-stimulation (not shown). Incubation period (36 or 72 h) increased the frequency of activated cells substantially while 18 h was the minimal time to detect any increase. No CD69 upregulation occurred in the control cultures (CM) without drug. For practical reasons, we chose to incubate the cells for 48 h subsequently.

Comparison of the LTT with CD69 upregulation

Next, we analyzed the same 15 drug-allergic, LTT-positive patients for CD69 upregulation. Both assays were performed with three different concentrations of the drugs (low, middle, high, see Methods), which had previously been found not to be toxic (13). The results were grouped into the four drug classes as above. As a positive control, cells of all blood donors were stimulated with TT, which induced CD69 upregulation in all patients (data not shown). Cells in CM without antigen/drug served as negative control.

Figure 2A shows the proliferation of PBMC from 15 patients. Some drugs elicited a dramatic proliferation *in vitro* as described earlier (15, 19, 29), which was dose dependent, as in most instances the middle and highest doses gave the highest values. Concomitant with the LTT and using the same concentrations, CD69 upregulation was evaluated on CD4⁺ and CD8⁺ T cells after 48 h. An enhanced CD69 upregulation was observed with all three concentrations on CD4⁺ (Fig. 2B) as well as on CD8⁺ T cells (data not shown).

All drugs to which the 15 patients had a confirmed allergy were then tested in LTT with PBMC from five healthy individuals to identify any unspecific proliferative effects. None of the drugs induced a proliferation in these nonallergic individuals (Fig. 3A), apart from vancomycin, which elicited a moderate proliferative response at the two higher concentrations. No proliferation was seen in the allergic patients to drugs they were not allergic to (data not shown). The T cells of the five nonallergic controls showed CD69 upregulation neither on CD4⁺ (Fig. 3B) nor CD8⁺ T cells (Fig. 3C) after co incubation with three different concentrations of all drugs tested. Clavulanic acid was the only exception, inducing a consistent increase of CD69 on CD4⁺ T but not on CD8⁺ T cells.

Stimulation indices of LTT and CD69

To normalize for heterogeneous background proliferation, LTT results are often expressed as SI, which is proliferation in the presence of drug divided by proliferation in the absence of drug. A $SI_{LTT} > 2$ has habitually been interpreted as a sign of sensitization (15). The data in Fig. 4 (x-axis) confirm the $SI_{LTT} > 2$ as cut-off value,

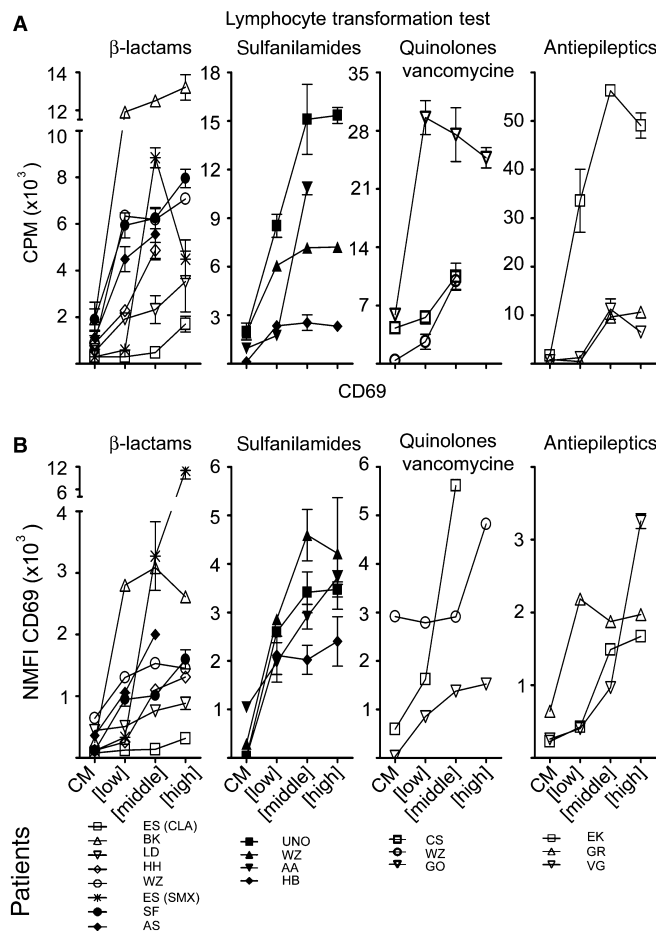


Figure 2. Drug-specific proliferation (LTT) and CD69 upregulation after incubation of peripheral blood mononuclear cells isolated from 15 drug-allergic patients with different drugs at three different concentrations (low, middle, high). Culture medium alone served as negative control. (A) LTT Proliferation (counts per minute, cpm) and (B) normalized mean fluorescence intensity (NMFI) of CD4⁺ CD69⁺ T cells after incubation for 48 h with different drugs.

as the PBMC of all healthy donors had a $SI_{LTT} < 2$ to any drug tested (Fig. 4A,C), while drug allergic patients showed a $SI_{LTT} > 2$ (Fig. 4B,D). Calculating the SI based on CD69 upregulation on CD4 T cells (y-axis), no $SI_{CD69} > 2$ was observed in PBMC isolated from healthy individuals stimulated with the different drugs with the exception of high and middle concentrations of clavulanic acid (Fig. 4A). All 15 patients analyzed showed a $SI_{CD69} > 2$ to the eliciting drug except for some stimulations with low concentrations (Fig. 4B), indicating that a $SI_{CD69} > 2$ is an appropriate cut-off value for CD69 upregulation as well. Similar SI values were found for CD69 upregulation on CD8⁺ T cells (Fig. 4C,D), although no upregulation of CD69 on CD8⁺ T cells was observed after stimulation of PBMC from healthy individuals with clavulanic acid (Fig. 4C).

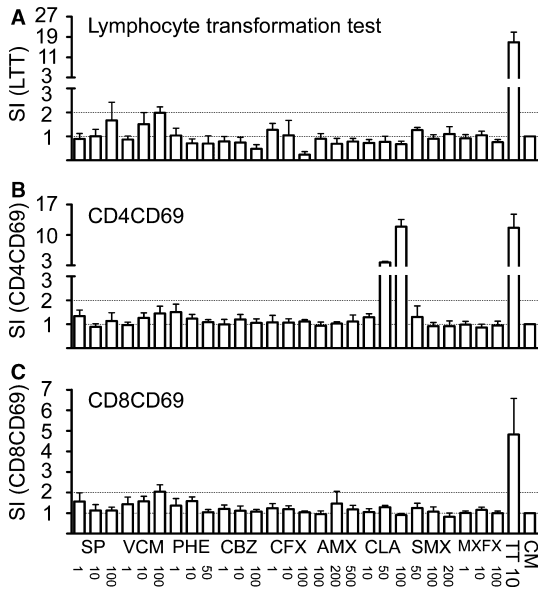


Figure 3. Proliferation and CD69 upregulation after drug stimulation of peripheral blood mononuclear cells isolated from five healthy individuals. Mean of stimulation indices (SI) of (A) lymphocyte transformation test and CD69 upregulation on (B) CD4⁺ and (C) CD8⁺ T cells after drug-stimulation with three different concentrations (µg/ml).

Intracellular cytokine staining

There is a striking difference between the frequency of drug-reacting T cells based on the analysis using the ELISPOT/CSFE methodology as recently reported (13),

and the percentage of CD69⁺ T cells after drug stimulation as shown here. CD69 upregulation on T cells was observed in up to 0.5–3% of drug- or TT-stimulated CD4⁺ T cells, hence about 20–50 fold more frequently. While this observation rendered the detection of CD69 easy and practicable, it also raised doubts concerning the specificity of this assay. To define the specific reactivity of CD69⁺ T cells more precisely, we simultaneously stained drug-exposed PBMC from three different drug-hypersensitive patients for intracellular cytokines and CD69. We chose IFN-γ for patients UNO and WZ, and IL-5 for patient CS, as these were the predominant cytokines according to previous ELISPOT data (13). As shown in Fig. 5A by the filled portion of the bars, maximally 5% of the drug-stimulated CD4⁺ T cells that became CD69 positive after 24 h produced these cytokines. Thus, only a minority of CD69⁺ T cells is cytokine producers upon drug stimulation for 24 h, whereas the majority of T cells expressed CD69 but was not synthesizing cytokines. Of note, the frequency of cytokine-producing CD69⁺ T cells (*c.* 1 : 1000) is close to the frequency of drug reactive T cells as previously determined by ELISPOT and CSFE analysis (13).

IL-2 dependent upregulation of CD69

To better understand the upregulation of CD69 on T cells that were apparently neither able to proliferate nor to produce cytokines, we evaluated the effect of IL-2 or IL-2 receptor (IL-2R) blockade. As shown previously (30), addition of IL-2 to cell cultures for 48 h without antigen (TT or drug) in healthy controls led to CD69 upregulation on CD4⁺ and CD8⁺ cells (Fig. 5B). IL-2 receptor

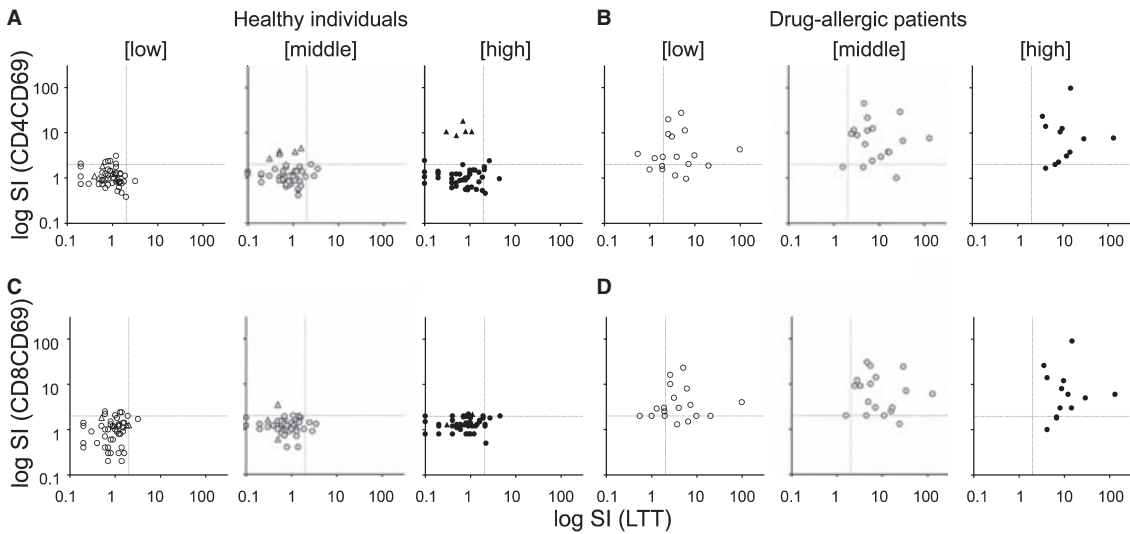


Figure 4. Comparison of individual stimulation indices of lymphocyte transformation test (LTT) and CD69 upregulation on CD4⁺ (A,B) and CD8⁺ (C,D) T cells. (A) LTT and CD69 measurements of CD4⁺ cells of five healthy individuals or (B) from 15 drug-allergic patients in response to low, middle and high concentrations of drugs. (C) LTT and CD69 measurements of CD8⁺ cells of five healthy individuals or (D) from 15 drug-allergic patients in response to low, middle and high concentrations of drugs. ▲: clavulanic acid.

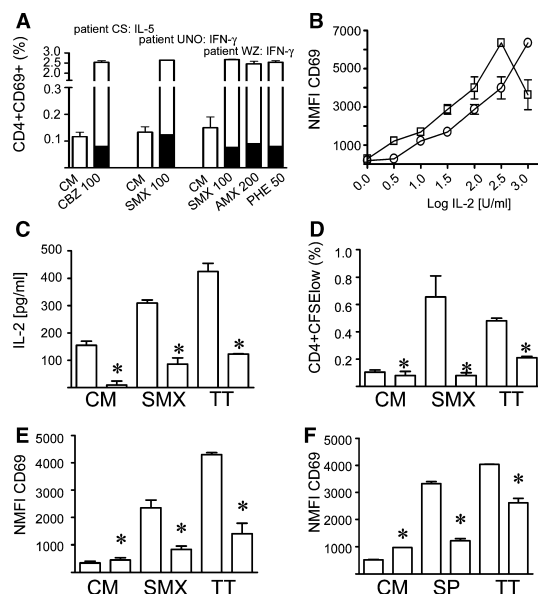


Figure 5. Intracellular cytokine staining of T cells after drug-stimulation and upregulation of CD69 by IL-2. (A) Intracellular IL-5/IFN- γ producing CD4⁺ CD69⁺ T cells after activation (shown by filled portion of the bar). (B) Flow cytometric measurement of CD69 upregulation (NMFI) after 48 h incubation with different concentrations of IL-2 in two healthy donors. (C) IL-2 production of drug or TT stimulated peripheral blood mononuclear cells (PBMC) from the SMX-allergic patient UNO after 48 h. (D) Percentage of proliferating PBMC (CFSE low) from the SMX-allergic patient UNO after 48 h of drug- or TT-stimulation as measured by the CFSE proliferation assay. CD69 upregulation on CD4 T cells after 48 h stimulation with drug or TT for patient (E) UNO and (F) patient AA. In C-F, cells were cultivated with blocking antibodies to the IL-2 R α /IL-2 R β chain (*) or isotype controls.

blockade with antibodies against the α - and β -subunits of the IL-2 receptor resulted in a strongly diminished IL-2 production (Fig. 5C) and proliferation (Fig. 5D) after stimulation with SMX or TT in PBMC isolated from a SMX hypersensitive patient. Likewise, it reduced CD69 expression on CD4⁺ T cells induced by SMX (Fig. 5E) or SP (Fig. 5F), suggesting that antigen-induced IL-2 secretion by antigen-specific cells enhanced CD69 upregulation.

Discussion

In vitro tests have the advantages of being safe by avoiding new or recall sensitizations or other adverse drug effects and of providing insight into the underlying pathomechanisms. While the LTT is used to diagnose T-cell-mediated drug hypersensitivity *in vitro* (15), it imposes technical problems (14, 31). Here, we show that the measurement of CD69 upregulation on T cells is a promising alternative.

Compared with other early T-cell activation markers like CD71 or CD25, CD69 seemed the most suitable, as it was upregulated quickly (within 18 h) and showed the biggest difference to baseline values (data not shown). Moreover, CD69 upregulation was easily detectable by flow cytometric measurements, as between 0.5% and 3% of the CD4⁺ T cells reacted. CD69 upregulation was specific for drug and TT stimulation and not an inherent property of cells derived from patients, as it occurred neither in nonstimulated cultures nor in drug-stimulated PBMC from nonallergic donors and drug-allergic subjects reacted only to the culprit but not to any other of the tested drugs.

As CD69 upregulation was observed already after 18 h, the high frequency of activated T cells cannot be explained by a rapid expansion of few drug reactive T cells. Further characterization of CD69⁺ T cells revealed that only a small proportion of CD69⁺ T cells (up to 5%) reacted to drug stimulation with cytokine production. When IL-2 binding to the IL-2R was blocked, proliferation and IL-2 secretion were partially abrogated and CD69 upregulation strongly reduced. Most likely, other cytokines are involved in this upregulation of CD69 as well, as IL-2R blockade had only a partial effect. Thus, the surprisingly high frequency of CD69⁺ T cells after drug-incubation is due to two distinct populations of T cells: (i) few drug-reactive T cells, which are most likely identical to the proliferating and/or cytokine-secreting T cells and (ii) many drug-activated ‘bystander’ T cells, which reacted to cytokines secreted by the truly drug-specific T cells, in particular to IL-2. As this inherent amplification occurs only if drug-reactive T cells are present, it does not affect the specificity of the test, but substantially increases its sensitivity. Why only a proportion of T cells reacted and what the phenotype of these CD69⁺/cytokine-reactive T cells might remain unclear.

In the LTT, a cut-off value of $SI_{LTT} > 2$ has been determined empirically to determine if a patient’s cells are drug reactive (15). In analogy and based on the 15 patients and five healthy donors analyzed, an $SI_{CD69} > 2$ seems an equally appropriate cut-off value.

That drugs may also stimulate CD69 upregulation without prior sensitization was revealed by clavulanic acid, which increased CD69 expression on all CD4⁺ but not on CD8⁺ T cells, but failed to induce proliferation. Conversely, vancomycin increased the proliferation of nonallergic cells but did not affect CD69 expression. These examples illustrate that every drug used in either assay should first be evaluated carefully in nonallergic individuals before one concludes from any measurements to a sensitization.

Collectively, our data show that CD69 upregulation is comparable with the LTT. CD69 upregulation offers some advantages over the LTT, however, as it (i) provides results after 48 h instead of 7 days, (ii) does not require 3H-thymidine incorporation and (iii) seems particularly suitable for routine diagnosis thanks to the high number

of reactive cells. On the other hand, the CD69 test cannot overcome the inherent deficiencies of drug-specific *in vitro* T-cell stimulation assays, as it is only positive if the correct antigen and sufficient memory T cells are present. Like the LTT, it may be false negative if the immune response was mainly directed to a metabolite not available *in vitro*, if T cells were not activated strongly enough, if no previous memory T-cell response had been generated, or if the response was mainly due to cytotoxic but not proliferating T cells. Nevertheless, the data presented

here warrant a prospective study with a larger number of suspected drug allergics, which will hopefully provide data on the sensitivity and specificity of this test for drug hypersensitivity diagnosis.

Acknowledgments

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