

In vitro diagnostic assays are effective during the acute phase of delayed-type drug hypersensitivity reactions

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Summary

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Conflicts of interest

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Background Previous reports have suggested that drug-specific lymphocyte proliferation assays (LPA) can be used retrospectively to confirm the culprit drug following delayed-type drug hypersensitivity reactions (DHR). However, only limited evidence supports their use in aiding acute clinical management. The aim of this study was to compare the LPA against combination cytokine assays for potential use in the acute setting.

Methods A total of 43 patients with DHR (19 during the acute reaction, 20 after recovery, four during acute and after recovery) and 14 control subjects without DHR were investigated using *ex vivo* analysis of drug-specific proliferation, and interferon (IFN)- γ and interleukin (IL)-4 production.

Results Healthy controls showed negative drug-specific proliferation and cytokine release in contrast to individuals with a known sensitivity ($P < 0.0001$). The assays demonstrated a test specificity of 95% (LPA), 83% (IFN- γ) and 92% (IL-4). The sensitivity of combined measurement of drug-specific IFN- γ and IL-4 cytokines during acute DHR was better than LPA (82% vs. 50%), but all assays were less sensitive during the recovery phase. The correlation between LPA and IFN- γ assays was strong ($r = 0.7$, $P < 0.0001$), whereas the IL-4 assay did not correlate as well with either of these assays. In contrast to LPA, drug enzyme-linked immunosorbent spot assays showed positive responses in patients concurrently taking immunosuppressive medication.

Conclusions *In vitro* assays of drug-specific IFN- γ and IL-4 production offer potential for use as rapid diagnostic tests. Cytokine detection offers distinct advantages over the LPA, including a shorter assay time, a greater sensitivity and effectiveness in testing immunosuppressed patients.

Delayed-type drug hypersensitivity reactions (DHR) are common and occasionally fatal. As a consequence of the delay in type IV T cell-mediated hypersensitivity, drug culpability is often hard to diagnose. These potentially severe systemic reactions frequently show early involvement of the skin but may manifest with early or late organ dysfunction during the course of the reaction.¹ The most critical intervention in DHR is to stop administration of the culprit medication. However, diagnostic testing is not routinely available, hence most clinicians use an educated guess of disease causality based on known parameters of the medications ingested and time course of the response.² In some acute situations, because of the possibility of alternative diagnoses, the severity of the reaction or the lack of improvement, this approach is not satisfactory and a diagnostic test would significantly improve patient care. Similarly, after recovery, testing may be required

to ascribe causality. Challenge tests are useful for investigation of IgE-mediated drug allergy and have been used for investigation of skin test-negative mild β -lactam-induced exanthems,³ but are generally not recommended in type IV DHR because of the risk of untreatable life-threatening reactions.^{4,5} Past guidelines have suggested the utility of delayed intradermal testing in the diagnosis of type IV DHR.³ However, more recent data from a cohort of drug challenge-positive β -lactam-sensitized individuals (with mild exanthems) showed that delayed intradermal testing was positive in only 9% of confirmed cases.⁶ Furthermore, recent guidelines have highlighted the limitations of intradermal testing and stipulate that it is only appropriate for the milder exanthems because of the possibility of inducing severe reactions.⁷ Although U.K. guidelines suggest that in contrast to intradermal testing, drug patch testing may be utilized in all delayed-type hypersensitivity

reactions,⁷ the test demonstrates a highly variable success rate (positive in 9–59% of cases) due to differences in the drugs themselves (e.g. lipophilicity) and is not useful in the acute setting.^{1,8–10}

Drug-specific T cells have been shown to infiltrate the skin in DHR-induced skin eruptions¹¹ and assays of circulating peripheral blood mononuclear cells (PBMCs) for drug-specific T-cell proliferation [synonyms: lymphocyte transformation test; ³H-thymidine incorporation assay; lymphocyte proliferation assay (LPA)] are the most widely reported *in vitro* tests used to identify drug-specific T cells. LPA testing has been reported to be a useful diagnostic assay.^{12–14} However, drug-specific cytokine production by circulating lymphocytes has also been shown to identify drug-sensitized individuals.^{12,15} Although measures of interferon (IFN)- γ production have been the most widely utilized cytokine assay,^{16–18} the presence of T helper (Th) 2 cells has been documented in drug-induced exanthems, and assays for multiple T-cell cytokines have been suggested to be a more sensitive approach to diagnosis of DHR.^{12,15,19,20} Thus, the specific repertoire of cytokines released by drug-specific T cells represents the functional outcome of sensitization, e.g. cytotoxic T cells/Th1 (IFN- γ , tumour necrosis factor- α) and Th2 [interleukin (IL)-4, IL-5, IL-13]; this is likely to differ in individuals and in different DHR patterns.

In this study we compared DHR testing using the LPA vs. IFN- γ and IL-4 drug enzyme-linked immunosorbent spot (ELISpot) assays. We also addressed the role of the assays in the acute phase and after recovery. Furthermore, in 83% of cases we tested the individuals to multiple relevant medications to establish the frequency of multiple drug hypersensitivities in these patients.

Patients and methods

Patients and controls

The project was approved by the local research ethics committee (NRES07/Q1704/46). Patients were diagnosed with DHR on clinical grounds by consultant dermatologists experienced in the recognition of these reactions. Clinical reaction patterns were characterized in all cases (Table 1), corresponding to diagnostic criteria for drug reaction with eosinophilia and systemic symptoms (DRESS) and Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) as published previously.^{21,22} All patients with SJS/TEN had confirmatory histology and, although not mandatory for the diagnosis, all patients with DRESS had eosinophilia [median $2.45 \times 10^9 \text{ L}^{-1}$; interquartile range (IQR) 1.8–2.7; normal range 0.04–0.8]. A total of 47 *in vitro* tests were undertaken in patients with DHR, including 19 patients tested in the acute phase, 20 post-recovery and four patients tested during both acute and post-recovery stages, recruited through the Department of Dermatology, University Hospital Southampton NHS Foundation Trust. For comparison, 14 nonallergic controls (including four treated with vancomycin, but without

DHR symptoms) were also tested. All acute cases and those with previous skin DHR had demonstrated classical T cell-mediated hypersensitivity reactions in the skin [TEN/SJS/erythema multiforme (EM), DRESS, drug-induced exanthema, fixed drug eruption or eczema]. There was no statistically significant difference in the age and sex distribution of the controls and patients with drug hypersensitivity. Acute DHR testing was defined as testing within 30 days from rash onset (median 9 days, IQR 6–13.5). Postacute testing was undertaken 40–1596 days from the rash onset (median 231 days, IQR 101–815) (Table 1). All testing was undertaken on fresh (not frozen) samples isolated from anticoagulated peripheral blood.

Enzyme-linked immunosorbent spot cytokine detection assay

ELISpot assays were undertaken as per the manufacturer's instructions (Mabtech, Nacka Strand, Sweden). Briefly, *ex vivo* PBMCs were tested at 2.5×10^5 cells per well in RPMI 1640 supplemented with 100 IU mL⁻¹ penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin (Gibco, Paisley, U.K.), 1% sodium pyruvate (Gibco) plus 10% heat inactivated human serum (Sigma, Poole, U.K.). Drugs were tested in a triplicate dose series of concentrations based on reported physiological plasma concentrations. Medium and staphylococcal enterotoxin B (SEB) were utilized as negative and positive controls, respectively. The plates were incubated overnight at 37 °C in 5% CO₂ and were developed with streptavidin–alkaline phosphatase (Mabtech) and an alkaline phosphatase conjugate substrate kit (Invitrogen, Abingdon, U.K.). Spot-forming units per million cells from test and control wells were analysed. Positive responses were recorded as those responses greater than the mean of all the background samples plus $2 \times \text{SD}$ of the background. Triplicate averaged test maximal values from the dose series were used for comparisons.

Lymphocyte proliferation assay

The LPA was undertaken as described previously.^{23,24} Briefly, PBMCs ($2.5 \times 10^6 \text{ mL}^{-1}$) were co-incubated with a dose series of the relevant drug (as above). Negative (medium with drug vehicle) and positive (SEB) controls were used in all assays. ³H-thymidine was added on day 5, and the cells harvested 6 h later for scintillation counting. The stimulation index (SI) was calculated as the fold difference between counts per minute recorded in wells stimulated by drug over the negative control.¹³ As previously, SI > 2 was considered positive.^{12,13}

Statistical analysis

As appropriate for non-normally distributed data, nonparametric analyses were used throughout the study (Mann–Whitney and Spearman correlation; GraphPad Prism Software, La Jolla, CA, U.S.A.). Median responses and IQR are reported.

Results

Lymphocyte proliferation assay vs. enzyme-linked immunosorbent spot assay in allergic patients vs. controls

To examine the specificity of the LPA and the ELISpot assays we assessed the frequency of drug-specific T-cell responses to the four most frequent culprit drugs in our department (amoxicillin, meropenem, carbamazepine, vancomycin) in healthy controls with no history of DHR, including 10 patients never exposed to the drug and four patients actively taking the medication without complication.

Assays for drug-specific T cells from healthy volunteers showed a low false-positive detection rate for LPA (4.89%, specificity 95.11%, median SI 0.84, IQR 2.4–8.8), IFN- γ (17.14%, specificity 82.85%, median frequency 0%, IQR $1.4\text{--}12.3 \times 10^{-3}$) and IL-4 (8%, specificity 92%, median frequency 0%, IQR $1.4\text{--}4.2 \times 10^{-3}$). Combined assays of IFN- γ and IL-4 gave a specificity of 77.27%, and all three assays combined of 73.3%. In contrast, individuals with a known sensitivity to any of the four drugs showed positive responses with the LPA (median SI 3.67, IQR 2.27–4.72, $P < 0.0001$, $n = 12$), IFN- γ (median frequency $4.6 \times 10^{-3}\%$, IQR 1.3×10^{-3} to 1.04×10^{-2} , $P < 0.0001$, $n = 11$) and IL-4 (median frequency $3.2 \times 10^{-3}\%$, IQR 1.4×10^{-3} to 4.2×10^{-3} , $P < 0.0001$, $n = 10$) (Figure 1a–c).

The calculated median circulating drug-specific T-cell frequencies within patients detected positive using IFN- γ and IL-4 assays were in accordance with previous studies reporting a frequency of 4×10^{-4} to $1.3 \times 10^{-2}\%$.^{16,25,26}

In vitro dose dependency of drug-specific T-cell responses

To confirm the sensitivity of the in vitro assays we examined ex vivo T-cell responses to vancomycin over an extensive range of tested concentrations for both IFN- γ (Figure 2a) and IL-4 (Figure 2c) in a vancomycin-sensitized individual who was willing to provide multiple samples for replication. The IFN- γ ELISpot was weakly positive even at very low drug concentrations (6.1×10^{-5} mmol L⁻¹) but showed a significant enhancement in detection at 0.0015 mmol L⁻¹, whereas the IL-4 ELISpot showed a similar step-up in detection at 0.06 mmol L⁻¹. LPA showed a positive response only when tested with vancomycin at 0.25 mmol L⁻¹ (Figure 2a,c). In contrast, both drug-specific proliferation and cytokine responses in nonallergic patients were flat at all drug concentrations (Figure 2b,d).

Detection of drug-specific T cells in patients during the acute phase or after recovery from classical skin drug hypersensitivity reaction

To assess the utility of in vitro assays during the acute phase of DHR, we tested 23 patients with DHR within 30 days

(median 9, IQR 6–13.5) from rash onset (Table 1). On the basis of detailed analysis of the medications prescribed and the timeline of initiation, up to five possible culprit drugs were identified and discontinued, and these medications employed in the in vitro assays. All patients responded to withdrawal of the medication consistent with DHR as the correct diagnosis. Eighty-three per cent of cases ($n = 19$) were tested to multiple medications (median number of tested drugs per sample 3 ± 1.5). As expected, the possible culprit drugs tested included medications widely recognized as frequent causes of DHR, including antibiotics (64.5%) and anticonvulsants (17.7%). Culprit drugs as identified by positive assays in the acute phase were most frequently detected by the IFN- γ ELISpot assay (17/22, 77%) vs. IL-4 (12/18, 67%) and LPA (11/22, 50%) (Figure 3). A positive drug response was identified by either IFN- γ or IL-4 ELISpot in 82% (18/22) of cases confirming that these assays were complementary and not duplicative. The combination of all three assays identified a putative culprit drug in 83% (19/23) of cases.

Of the individuals diagnosed with DHR tested after resolution of the acute phase (Table 1), 24 showed positive responses in the IFN- γ ELISpot (12/24, 50%), IL-4 ELISpot (nine of 18, 50%) and the LPA (six of 24, 25%) (Figure 3). Combining the IFN- γ and IL-4 assays gave positive results in 67% of cases. Combination of the LPA and IFN- γ assay showed positive responses in 54%, whereas combination of all three tests showed positive responses in 67% of cases examined. In three out of the four patients tested both during the acute phase and after recovery (median time interval between the tests 191 days, IQR 79–335) the drug identified by positive in vitro testing in the acute phase was also positive in the delayed test.

Both cytokine assays and LPA showed a higher frequency of positive responses in the acute setting (Figure 3), and for the IFN- γ assay this reached statistical significance ($P = 0.029$). In acute reactions, the median LPA SI for drug-specific proliferating T cells in positive assays was 3.9 ± 10.3 (Figure 3a). Circulating IFN- γ and IL-4 drug-specific T-cell frequency was detected at $4.2 \times 10^{-3}\%$ (IQR $2.4\text{--}10.6 \times 10^{-3}$) and $3.9 \times 10^{-3}\%$ (IQR $1\text{--}8.6 \times 10^{-3}$), respectively, in the acute phase (Figure 3b,c). However, median circulating drug-specific T-cell frequency for patients tested following recovery was lower at $2.7 \times 10^{-3}\%$ (IQR $1.1\text{--}9.7 \times 10^{-3}$) and $0.7 \times 10^{-3}\%$ (IQR $0.4\text{--}4.8 \times 10^{-3}$) for IFN- γ and IL-4, respectively (Figure 3b,c). The median SI for recovery phase drug-specific proliferating T cells was 3.7 (IQR 2.8–5.1) (Figure 3a).

Nine cases were tested to only one medication: four during the acute phase and five during recovery (Table S1; see Supporting information). In this small data subset, 50% of acute cases and 60% of those in recovery tested positive. Of the two acute cases with negative test results, drug-induced cytokine assays were unavailable in one case (for technical reasons), limiting the interpretation of these data. All positives were detected with the drug-induced IFN- γ assay and four out of five (80%) were detected in more than one assay (two of five detected with the LPA and IFN- γ ; three of five detected with IL-4 and IFN- γ).

Table 1 Patient characteristics

Patient ID	Age (years)	Sex	Rash to test (days)	Acute/recovery	Phenotype	No. of drugs tested
1a	15	F	2	Acute	DIE	5
1b	16	F	480	Recovery	DIE	4
2	2	M	14	Acute	DIE	4
3 ^a	15	M	4	Acute	DIE	2
4a	13	M	3	Acute	DIE	5
4b	13	M	98	Recovery	DIE	5
5 ^a	13	F	5	Acute	DIE	2
6a	47	F	13	Acute	DIE	2
6b	47	F	300	Recovery	DIE	1
7 ^a	54	M	29	Acute	DIE	3
8	75	F	16	Acute	DIE	3
9	56	F	1044	Recovery	DIE	4
10	61	M	8	Acute	DIE	2
11	51	M	11	Acute	DIE	3
12	65	M	40	Recovery	DIE	1
13	84	F	9	Acute	DIE	2
14 ^a	63	M	1317	Recovery	DIE	2
15 ^a	63	M	1296	Recovery	DIE	4
16	60	F	141	Recovery	DIE	1
17	35	F	184	Recovery	DIE	3
18	32	F	190	Recovery	DIE	2
19	70	F	312	Recovery	DIE	1
20	56	F	6	Acute	DIE	1
21	48	F	11	Acute	DIE	1
22	20	F	1226	Recovery	DIE	4
23	42	F	93	Recovery	DIE	2
24	82	F	144	Recovery	DIE	2
25	90	F	6	Acute	DIE	1
26 ^a	87	M	48	Recovery	DRESS	2
27 ^a	58	M	18	Acute	DRESS	3
28	16	M	6	Acute	DRESS	4
29	28	F	44	Recovery	DRESS	4
30a ^a	55	M	13	Acute	DRESS	4
30b ^a	55	M	44	Recovery	DRESS	3
31	73	F	396	Recovery	DRESS	3
32	81	M	40	Recovery	EM	2
33	69	M	8	Acute	SJS	1
34	32	F	103	Recovery	SJS	1
35	63	M	11	Acute	SJS	2
36 ^a	13	M	17	Acute	SJS	2
37	66	F	273	Recovery	SJS/TEN	2
38 ^a	59	F	8	Acute	SJS/TEN	4
39 ^a	20	M	19	Acute	SJS/TEN	2
40	65	F	1036	Recovery	TEN	2
41 ^a	21	F	6	Acute	Eczema	4
42	93	F	106	Recovery	Eczema	3
43 ^a	42	F	742	Recovery	FDE	5

DIE, drug-induced exanthems; DRESS, drug reaction with eosinophilia and systemic symptoms; EM, erythema multiforme; FDE, fixed drug eruption; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis. ^aPatient on immunosuppressive medication; a, b, same patient tested in (a) acute and (b) post-recovery phases.

Correlation between drug-specific T-cell proliferation and cytokine production

Of the 47 *in vitro* tests undertaken, 39 (83%) were tested to more than one possible culprit drug. For each drug tested, the

LPA showed a highly significant positive correlation with the IFN- γ ELISpot ($r = 0.71$, $P < 0.0001$) but did not correlate well with the IL-4 assays ($r = 0.05$; not significant) suggesting that they identified different populations of drug-specific T cells (Figure 4a–c). Sensitization to more than one drug was

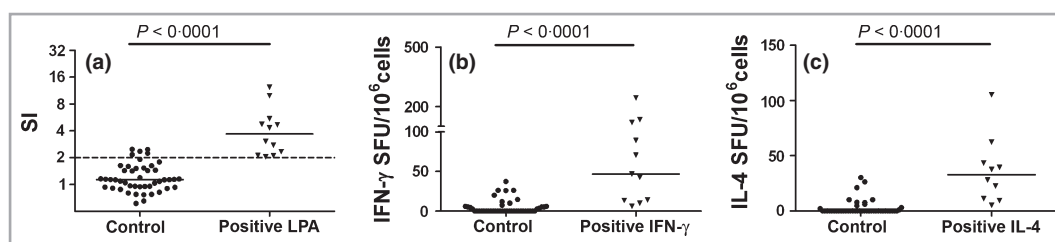


Fig 1. Frequency of positive responses in drug-allergic patients vs. controls. Frequency of positive responses induced by amoxicillin, carbamazepine, meropenem and vancomycin detected in healthy controls ($n = 14$) and drug-allergic patients ($n = 12$) as shown by lymphocyte proliferation assay (LPA) (a) and enzyme-linked immunosorbent spot assay (b, c). Each data point represents a maximum measured response to the drug tested. IFN, interferon; IL, interleukin; SFU, spot-forming units; SI, stimulation index.

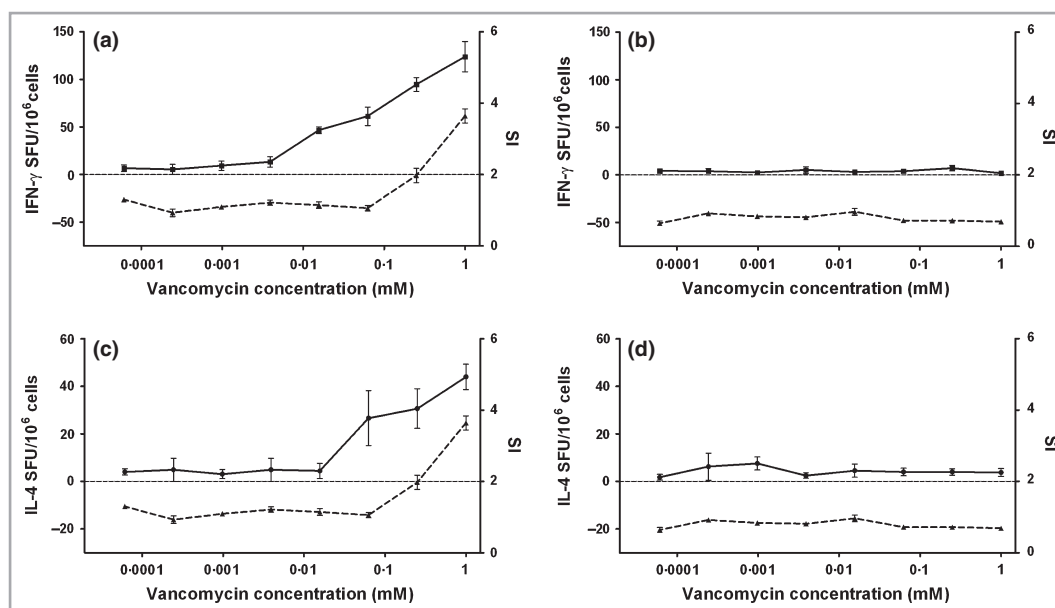


Fig 2. T-cell responses to vancomycin. Ex vivo peripheral blood mononuclear cells from a vancomycin-allergic patient (a, c) vs. four healthy controls (b, d) were tested in a dose range for drug-specific interferon (IFN)- γ (a, b) or interleukin (IL)-4 (c, d) production (left y-axes, solid line) vs. proliferation (right y-axes, dotted line). Horizontal dotted line represents the assay positive cut-off. SFU, spot-forming units; SI, stimulation index.

identified in all three assays: LPA 8% (three of 38); IFN- γ 38% (15/39); IL-4 52% (15/29). For each individual tested to multiple drugs, the same drug was identified (maximal test response) by both the LPA and IFN- γ in 56% (26/46) of cases, LPA and IL-4 in 58% (21/36), LPA and one of the cytokines in 63% (29/46), IFN- γ and IL-4 in 72.2% (26/36) and all three assays in 50% (18/36) of cases. Cytokine assays identified positive drug-specific T cells even when the LPA was negative in 18/29 (62%) cases (Figure 4d–f).

Clinical correlates with detection of drug-specific T cells

EM/SJS/TEN are recognized to be clinically the most severe DHR patterns and positive IFN- γ assay results were more frequent (78%, seven of nine) in these reaction patterns compared with drug-induced exanthems (52%, 14/27). Similarly,

cytokine assays were more useful in detecting T-lymphocyte function in DRESS: 71% (five of seven) and 80% (four of five) for IFN- γ and IL-4, respectively, vs. 14% (one of seven) for LPA (Table 2).

The frequency of positive reactions in skin DHR to antibiotics (LPA 15%, 13/84; IFN- γ 42%, 37/87; IL-4 42%, 29/69) and anticonvulsants (LPA 38%, six of 16; IFN- γ 62%, eight of 13; IL-4 50%, three of six), which are the most often tested in the context of DHR vs. all other medications (LPA 5%, two of 37; IFN- γ 42%, 11/26; IL-4 72%, 13/18), showed no statistical difference, suggesting that the *in vitro* assays are reliable across a range of drugs (Figure 5a–c). However, when testing individuals who were concurrently taking immunosuppressive therapies (prednisolone, dexamethasone and azathioprine, $n = 14$), we found that the LPA tests were uninformative in all but one case, in contrast with the results of cytokine release

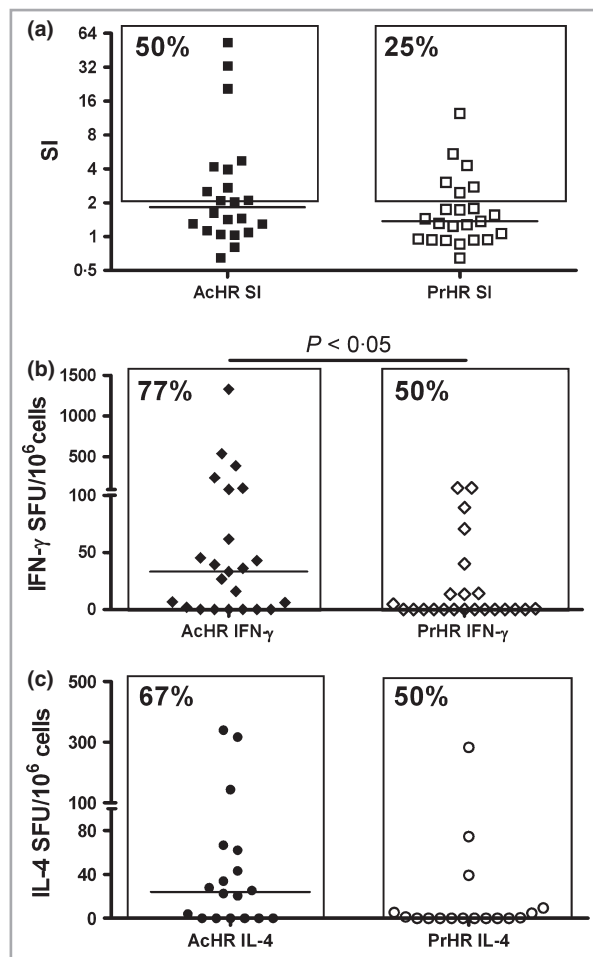


Fig 3. Comparison between drug-specific T-cell responses from acute hypersensitivity reactions or following recovery. Patients with acute drug hypersensitivity reaction (AcHR; black symbols) or previous drug hypersensitivity reaction (PrHR; open symbols) were tested for drug-specific proliferation (a, AcHR $n = 22$, PrHR $n = 24$) or enzyme-linked immunosorbent spot (ELISpot) assay for interferon (IFN)- γ (b, AcHR $n = 22$, PrHR $n = 24$) or interleukin (IL)-4 (c, AcHR $n = 18$, PrHR $n = 18$). Each data point shows the maximum response measured for each patient tested. Percentages indicate positive responses recorded within the group tested. Horizontal bars: median. Boxes contain positive assay data points (see Patients and methods). SFU, spot-forming units; SI, stimulation index. Statistical analysis using Mann-Whitney test.

assays (LPA 10%, four of 40; IFN- γ 92%, 37/40; IL-4 51%, 18/35) (Figure 5d–f). The frequencies of positive responses to all medications tested are indexed in Table 3.

Discussion

Type IV drug hypersensitivities create a diagnostic conundrum. While removal of culprit medication is critical for patient recovery, tests to identify the causative drug are largely unavailable. The LPA has been reported to identify drug-specific T-cell proliferation in 58–100% of cases.^{27–32} It is

considered unethical to rechallenge patients who may potentially develop life-threatening reactions,^{4,5} therefore the sensitivity of the LPA has been estimated at between 60% and 70% by testing well-characterized case series.¹³ We found the LPA to be positive in 50% of acute DHR in our cohort, but a combination of three assays identified 83% during the acute reaction and 67% after recovery. The higher sensitivity of *in vitro* assays compared with patch testing¹ in the recovery phase may reflect that drug-specific long-term memory T cells are more easily detectable in the circulation than in the skin. By comparing sensitized patients vs. drug-exposed but nonallergic individuals or nonexposed individuals we showed the LPA specificity to be 95%, in agreement with others reports examining single drugs.^{27–33} Although it has been widely assumed that the assay may show false-negative reactions < 1 month after the clinical reaction, we have shown here that the LPA may be more sensitive in the acute stage of a DHR. However, for translation into routine diagnostic laboratories this assay has a number of disadvantages including the use of radioisotopes and a 5-day assay protocol.

In contrast to lymphocyte proliferation, measures of drug-specific cytokine production by T cells do not rely on radioisotopes and can be detected in < 24 h. Such assays have been previously reported for DHR investigation and shown positive detection rates in 36–100% of cases.^{12,15–18,25,34} However, in contrast to our work here, these studies largely examined cohorts of β -lactam- or anticonvulsant-sensitized individuals rather than clinically representative samples and did not directly compare with the LPA. Here we report the *in vitro* assay utility by testing for multiple drugs during the acute stage and after recovery as well as the frequency of circulating IFN- γ - and IL-4-producing drug-specific T cells. Assessment of other T-cell populations may be useful, but previous reports have not found measurement of IL-17 to be informative,¹² suggesting that the circulating T cells producing IFN- γ and IL-4 on drug exposure represent the majority of drug-specific lymphocytes.¹² We showed that the LPA significantly correlates with the IFN- γ drug ELISpot ($P < 0.0001$), but not with the IL-4 ELISpot assay. Furthermore, combining IL-4 and IFN- γ increased the detection of positive responses to 82%, which is greater than either alone or that of the LPA (50%) during the acute-reaction phase. The dual cytokine approach was similarly advantageous in the postacute recovery phase. We confirmed that positive IL-4 assays reflected drug-specific Th2 cells and were able to expand drug-specific Th2 lines *in vitro* (data not shown). Thus, identification of drug-specific Th2 cells better reflects the repertoire of drug-specific phenotypes than T-cell proliferation or IFN- γ alone, as suggested previously.³⁵ Furthermore, we showed that cytokine assays were superior in the more severe reaction patterns such as SJS/TEN, which is consistent with previous reports describing low LPA sensitivity in these diseases.³⁶

Although these findings confirm the distinct advantages of the cytokine assays, it is important to note that the specificity is 3–9% lower than the LPA. However, the LPA was not useful for individuals currently treated with immunosuppressant

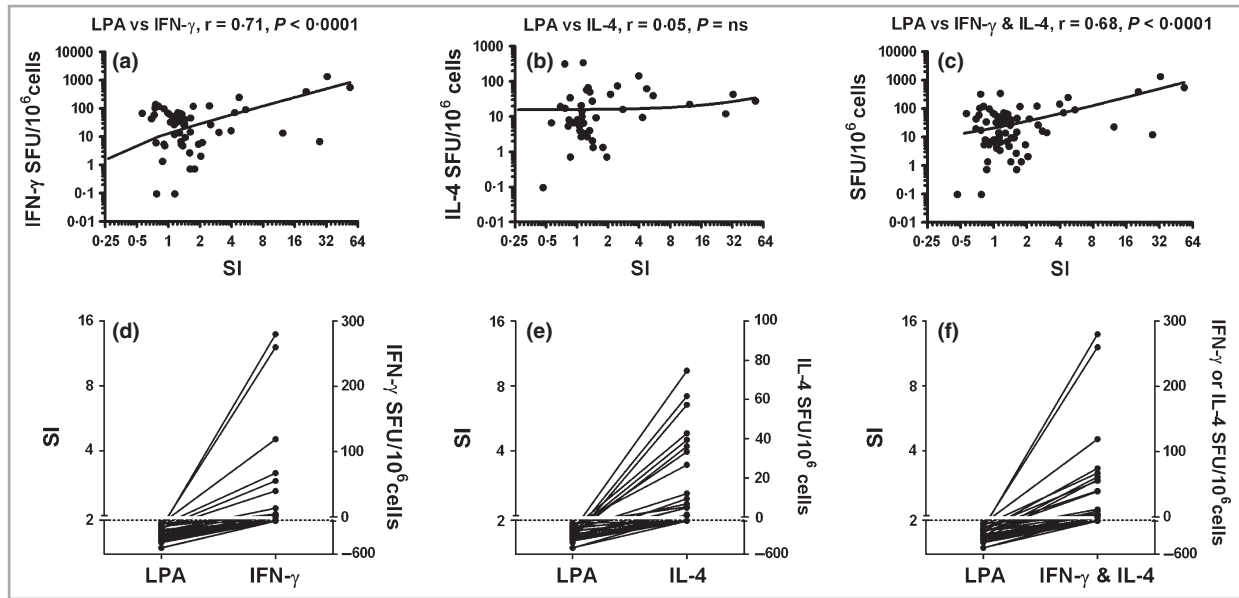


Fig 4. Correlations between detection of drug-specific proliferation and cytokine production assays. Correlation between maximal lymphocyte proliferation assay (LPA) and interferon (IFN)- γ (a), or interleukin (IL)-4 (b), or combined IFN- γ and IL-4 enzyme-linked immunosorbent spot assay (ELISpot) (c). (d–f) Patients with clinically proven drug hypersensitivity reaction with negative LPAs and positive IFN- γ (d) or IL-4 (e), or combined IFN- γ and IL-4 ELISpot (f) assay in peripheral blood mononuclear cells. SFU, spot-forming units; SI, stimulation index.

Table 2 Percentage of positive drug-specific responses in different cutaneous manifestations of drug hypersensitivity

	No. of tests	LPA, %	IFN- γ , %	IL-4, %
DIE	28	33.33	51.85	59.09
DRESS	7	14.29	71.43	80.00
EM/SJS/TEN	9	55.56	77.78	50.00
Eczema	2	50.00	100.00	0.00
FDE	1	100.00	100.00	100.00

DIE, drug-induced exanthems; DRESS, drug reaction with eosinophilia and systemic symptoms; EM, erythema multiforme; FDE, fixed drug eruption; IFN, interferon; IL, interleukin; LPA, lymphocyte proliferation assay; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

medication whereas drug-specific cytokine production was still evident. This may be relevant for DRESS syndrome, where glucocorticoid therapy is widely used early in the onset of the disease and where a diagnostic assay would be particularly useful especially during disease flares, which can arise 1–2 weeks after cessation of the culprit drug. We acknowledge that there exists the possibility of false-positive assays but in our investigation of healthy controls and individuals taking medications without complication, we were able to estimate the specificity of our assays to be 82.9–95.1%. Using the combination of LPA and cytokine assays, we were able to detect immunological sensitization to multiple medications in 8% of LPA tests, and 38% of IFN- γ and 52% of IL-4 ELISpot assays of cases, which is in accordance with previously published data¹⁷ and which raises the possibility that clinical algorithms

may falsely identify a single culprit medication. In our cohort in whom multiple sensitization was detected, only 16% of patients showed positive responses to two drugs of the same class (commonly β -lactam antibiotics), suggesting that cross-reactivity was an unlikely explanation for multiple sensitizations. Further work is warranted to explore the clinical relevance of multiple sensitizations. It seems likely that this may mirror the IgE allergy field, where it is widely accepted that serological sensitization is a valid but imperfect marker for clinical sensitization and that biological sensitization is not necessarily life-long.

For practising clinicians these assays could prove useful tools for investigation of patients with DHR. We suspect that in most cases of DHR caused by an easily identifiable culprit medication (such as a single drug exposure with a typical timeline for DHR development), further investigations with *in vitro* assays are not necessary. However, diagnostic doubt about drug culpability is not uncommon in patients who are on multiple medications. Although overnight drug ELISpots could prove useful in the acute management of severe DHR, the possibility of false-negative results (at least 18% in these data) must be recognized. False-positive responses are undesirable, but unnecessary avoidance of a medication is likely to be of less importance than potential re-exposure to a culprit medication. Challenge testing of individuals with negative drug ELISpots would be required to optimize definitively the assays to minimize false-negative results. However, this is only likely to be ethical for those with mild exanthems, and application of the findings to more severe cases is uncertain.

While the LPA would be of limited use alone in the urgent management of a patient with possible drug allergy, its greater

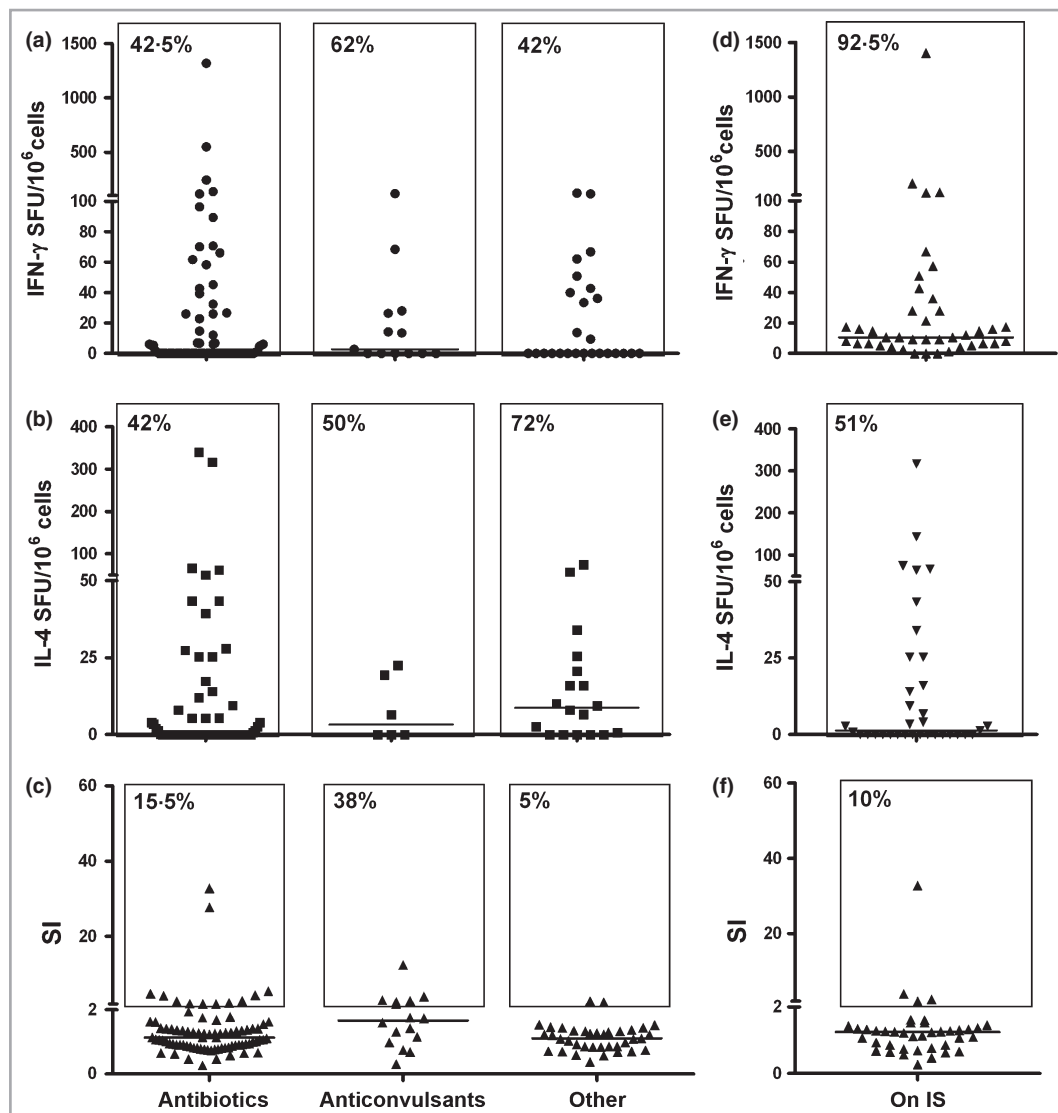


Fig 5. Drug-specific T-cell responses to different drug groups. Drug-specific T-cell responses measured by interferon (IFN)- γ release (a), interleukin (IL)-4 release (b) and lymphocyte proliferation assay (LPA) (c) induced by antibiotics, anticonvulsants and other drugs. Each data point represents maximal recorded response for a single drug tested per patient. Solid lines indicate group median. Gates indicate percentage of positive responses recorded. (d–f) IFN- γ release (d), IL-4 release (e) and LPA (f) measured in patients taking immunosuppressive medications (on IS). SFU, spot-forming units; SI, stimulation index.

specificity supports the value of this assay in combination with cytokine assays. We predict that *in vitro* assays may be of particular value in cases where multiple medications are stopped during the course of the DHR. Currently, all possible culprit medications need to be avoided life-long. *In vitro* testing would be attractive as it may be undertaken during the acute stage and is entirely safe. Furthermore, it is also likely that in the setting of a positive assay, these immunological tests could be utilized to test for potential cross-reactivity (e.g. between penicillins, sulphur-containing drugs and anticonvulsants), but we have not tested this possibility here. These tests remain largely confined as research tools for the moment and we and others are in the process of validating these assays against current standards for use in clinical practice. However, because immu-

nological assays will never show 100% sensitivity and specificity, multidisciplinary teams with experienced clinicians and scientists familiar with the assays will be critical to their future use.

In conclusion, we have tested the utility of *in vitro* assays for delayed-type DHR in a clinically heterogeneous population with expert-diagnosed DHR typical of routine clinical practice and found a combined sensitivity of 77–83% and a specificity of 83–95%. *In vitro* assays show potential diagnostic value in the acute setting as they allow safe testing for multiple drugs. Combined IFN- γ and IL-4 ELISpot assays demonstrate a number of advantages over the LPA, including rapidity of assay, a greater detection rate of drug sensitization and utility during concomitant immunosuppression.

Table 3 Frequencies of positive responses detected for each drug tested

Drug	No. of patients tested	IFN- γ , %	IL-4, %	LPA, %	DIE, n	DRESS, n	EM/SJS/TEN, n	Eczema/FDE, n
Allopurinol	4	0	50 ^a	0	0	4	0	0
Amoxicillin	10	50	0 ^a	10	7	1	2	0
Atenolol	1	0	nd	0	0	1	0	0
Atorvastatin	1	0	100	0	0	0	1	0
Aztreonam	1	0	0	0	1	0	0	0
Bisoprolol fumarate	1	100	nd	0	0	0	0	1
Candesartan	1	0	100	100	1	0	0	0
Carbamazepine	9	62.5 ^a	40 ^a	33	5	0	3	1
Ceftazidime	1	0	0	0	1	0	0	0
Ceftriaxone	1	0	0	0	0	0	0	1
Cefuroxime	5	20	33 ^a	20	4	0	1	0
Cetirizine hydrochloride	1	100	nd	0	0	0	1	0
Ciprofloxacin	1	0	nd	0	1	0	0	0
Clarithromycin	3	67	67	0	1	2	0	0
Clindamycin	2	50	100 ^a	0	2	0	0	0
Co-amoxiclav	1	0	0	0	1	0	0	0
Codeine	3	0	33	0	3	0	0	0
Co-trimoxazole	3	67	67	0	0	3	0	0
Doxycycline	2	0	0 ^a	0	1	0	1	0
Erythromycin	2	0	50	0	1	1	0	0
Esomeprazole	1	0	100	0	0	0	1	0
Flucloxacillin	6	17	40 ^a	17	4	0	1	1
Fluconazole	3	33	100 ^a	33	2	0	1	0
Furosemide	1	0	nd	0	0	1	0	0
Gentamicin	2	0	100 ^a	0	1	1	0	0
Hydroxychloroquine	1	100	100	0	1	0	0	0
Ibuprofen	1	0	0	0	0	1	0	0
Itraconazole	1	100	0	0	1	0	0	0
Levetiracetam	1	0	nd	0	1	0	0	0
Mercury	1	100	0	100	0	0	0	1
Meropenem	8	29 ^a	57 ^a	0	6	1	1	0
Metronidazole	1	0	0	0	0	0	1	0
Omeprazole	1	100	100	0	1	0	0	0
Oseltamivir	1	0	0	0	1	0	0	0
Paracetamol	2	50	50	0	1	0	0	1
Penicillin G	4	25	0 ^a	0	2	1	0	1
Penicillin V	3	0	33	0	2	1	0	0
Phenobarbital	2	100	100	0	2	0	0	0
Phenytoin	3	33	nd	33	2	0	1	0
Premarin ^{®b}	1	100	100	0	0	0	0	1
Ramipril	2	50	100	0	1	0	0	1
Rifampicin	1	0	0	0	0	1	0	0
Senna	1	100	100	100	0	0	0	1
Simvastatin	1	100	nd	0	0	1	0	0
Sodium valproate	2	100	100 ^a	50	2	0	0	0
Sulfasalazine	3	67	0	33	0	0	3	0
Tazocin ^{®b}	6	67	75 ^a	40 ^a	5	1	0	0
Teicoplanin	1	100	100	100	1	0	0	0
Thalidomide	1	100	100	0	0	1	0	0
Tolterodine tartrate	1	100	nd	0	0	0	0	1
Topiramate	1	100	100	100	0	0	1	0
Trimethoprim	2	0 ^a	0 ^a	0	2	0	0	0
Vancomycin	10	100	89 ^a	44 ^a	9	1	0	0

Percentages represent the actual frequency of positive tests. DIE, drug-induced exanthems; DRESS, drug reaction with eosinophilia and systemic symptoms; EM, erythema multiforme; FDE, fixed drug eruption; IFN, interferon; IL, interleukin; LPA, lymphocyte proliferation assay; nd, test not done; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis. ^aIncomplete dataset; ^bWyeth, Maidenhead, U.K.

What's already known about this topic?

- Lymphocyte proliferation assays can aid the identification of the culprit drug in hypersensitivity reactions.

What does this study add?

- The measurement of interferon- γ and interleukin-4 production is more sensitive than lymphocyte proliferation for detection of drug-specific T cells.
- *In vitro* assays can be used in the acute stages of a drug hypersensitivity reaction in a clinical setting.

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Supporting information

Additional Supporting information may be found in the online version of this article.

Table S1. Individuals tested to one medication during acute or recovery phase reactions (overall percentage positive and individual results).

Supplementary Table 1.

Individuals tested to one medication during acute or recovery phase reactions (overall percentage positive) and individual results.

Acute (50%)	
#20	Positive (IFN- γ /IL4)
#21	Negative
#25	Negative *
#33	Positive (LPA/IFN- γ)
Recovery (60%)	
#6	Positive (LPA/IFN- γ /IL4)
#12	Negative
#16	Negative
#19	Positive (IFN- γ /IL4)
#34	Positive (IFN- γ)

* cytokine assays not available