**In-vitro rapid diagnostic tests for severe drug hypersensitivity reactions in children**

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**Abbreviations/Acronyms:** drug hypersensitivity reactions (DHR), lymphocyte proliferation assay (LPA), interferon (IFN), interleukin (IL), drug–induced exanthems (DIE), erythema multiforme (EM), drug reaction with eosinophilia and systemic symptoms (DRESS), Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), Enzyme-linked immunosorbent spot (ELIspot)

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**Abstract**

**Background**

Previous reports have demonstrated the utility of T-cell proliferation and cytokine release assays as in-vitro diagnostic tests for drug causation in drug hypersensitivity reactions (DHR). However, data from paediatric populations is scarce by comparison to adults.

**Objective**

To compare the lymphocyte proliferation assay (LPA) against combination cytokine assays in the paediatric population and their potential use in the acute and post-recovery phases.

**Methods**

A total of 18 in-vitro tests were undertaken ex-vivo comparing drug-specific proliferation and cytokine release (interferon (IFN)-γ and interleukin (IL)-4). The study included 16 patients, 7 children with DHR in the acute phase, 7 post-recovery and 2 children tested during both acute and post-recovery phases.

**Results**

The sensitivity of LPA was better during the acute stage of DHR in children. Cytokine assays showed a higher frequency of positive drug-specific responses as compared to LPA in both acute (LPA 77.8%; IFN-γ 88.9%; IL-4 100%) and post recovery phase (LPA 33.3%; IFN-γ 66.7%; IL-4 66.7%). Combination cytokine assays (IFN-γ and IL-4) produced a higher positive drug-specific responses in identifying culprit drugs compared to LPA in both acute and post-recovery phases.

**Conclusions**

In-vitro drug-induced T cell proliferation and cytokine release assays are useful for identification of the causative drug in children with DHR. Cytokine assays (IFN-γ and IL-4) were better than LPA, but when combined, they offer even greater utility in the diagnosis of acute and post-recovery DHR. Cytokine detection is rapid and does not involve radioactivity. These novel in-vitro assays offer a significant advancement in our management of DHR in children.

**Introduction**

Drug hypersensitivity reactions (DHR) are common and important concerns for healthcare providers, especially in the paediatric setting.1 There is an over-diagnosis of DHR in children with a parent-reported prevalence of around 10%, and they are 1.5 times less likely to be confirmed as compared to adults.2 Whilst Ig-E mediated drug allergy is usually easily recognised and treated, delayed type (T-cell mediated) hypersensitivity is often hard to diagnose.3 Such T cell mediated reactions may range from mild to severe or life threatening and include mild maculopapular exanthem (MPE), drug reaction with eosinophilia and systemic symptoms (DRESS), and toxic epidermal necrolysis (TEN).3 These may cause be severe life-threatening reactions or cause discomfort, parental anxiety and recurrent visits to healthcare providers.4 In children, skin manifestations are often the most common presentation of these potentially severe systemic reactions.3

The principles of the diagnostic workup for DHR in children and adults are similar because the immunological mechanisms involved are similar.5 However, in reality, protocols for the clinical investigation of drug allergy are usually different in adults and children.5 Intradermal tests are painful while oral provocation tests can pose practical difficulties and are poorly tolerated in children.5 Furthermore, these in vivo tests must only be undertaken after the clinical problem has resolved and for delayed-type hypersensitivity carry a risk of untreatable life-threatening reactions.1 Therefore, the use of in-vitro tests, to identify drug-specific T cells in peripheral blood of patients with DHR-induced skin eruptions may have specific advantages in the paediatric population.

We previously described a cohort of 43 tested individuals which included 9 paediatric cases.7 Despite the small numbers, the paediatric cases showed similar results to the rest of the cohort. However, many consensus statements have highlighted the significant lack of published data specifically addressing in-vitro diagnostics in the management of DHR in children.8,9 We therefore set out to extend the case number to report the analysis of our tested paediatric population.

We aimed to compare the use of LPA versus IFN-γ and IL-4 drug enzyme-linked immunosorbent spot (ELISpot) assays in our cohort of tested paediatric patients. We also addressed the role of the assays in the acute and post-recovery phases when tested against different types of cutaneous DHR.

**Methods**

Patients

In this study, we retrospectively reviewed our cohort of paediatric patients who underwent testing for DHR by our department in University Hospital Southampton NHS Foundation Trust. Children (age 0-18) were diagnosed with DHR on clinical grounds by consultant dermatologists or paediatric allergists experienced in the recognition of these reactions. After a detailed analysis of the medications ingested, and the time course of initiation, up to five drugs were identified as possible culprits by the physician to be discontinued, and the patient was tested to these drugs with in-vitro assays. All cases resolved on cessation of the possible culprit drugs, confirming DHR. A total of 18 in-vitro tests (all 18 tested for LPA and IFN-γ, 13 tested for IL-4) were undertaken in children with DHR, including 7 children tested only in the acute phase, 7 children only in post-recovery and 2 children tested during both acute and post-recovery stages. Clinical reaction patterns were characterised in all cases (Table 1): MPE (n=7), DRESS (n=5), or SJS⁄TEN (n=4). All children with SJS ⁄TEN had confirmatory histology. All acute cases and those with previous skin DHR had demonstrated the classical skin rashes associated with T cell mediated hypersensitivity reactions. Acute DHR testing was defined as testing within 0 to 30 days from rash onset. Post-recovery testing was undertaken 84-1145 days from the rash onset. All testing was undertaken on fresh (not frozen) samples isolated from peripheral blood.

Enzyme-linked immunosorbent spot (ELISPOT) cytokine detection assay

The ELISpot assays were undertaken as described previously.7 Briefly, ex vivo PBMCs were tested at 2.5x 105 cells per well in RPMI 1640 supplemented with 100 IU mL-1 penicillin and 100 lg mL-1 streptomycin (Gibco, Paisley, U.K.), 1% sodium pyruvate (Gibco) plus 10% heat inactivated human serum (Sigma, Poole, U.K.). PBMC were incubated with nil (medium), staphylococcal enterotoxins B (SEB; positive control) or drug in a series of concentrations based on reported physiological plasma concentrations. The plates were incubated overnight at 37 oC 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 enumerated using an automated ELISpot reader (AID, Germany). Positive responses were recorded as those responses greater than the mean of all the background samples plus 2 x SD (standard deviation) of the background. Triplicate averaged test maximal values from the dose series were used for comparisons.7 An example of the IFN-γ ELISPOT from a child with positive response to a culprit drug (Teicoplanin) is shown in Fig 1.

Lymphocyte proliferation assay

The LPA was undertaken as described previously.7,10 Briefly, PBMCs (2.5 x 106 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 hours 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. SI > 2 was considered positive.7

Statistical analysis

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

**Results**

A total of 16 children with DHR were investigated. The mean age of children in our study was 11.6 years (median 13) (± SD 4.5). 56.3% (n =9) were males and 43.8% (n =7) were females (Table 1).

All cases (n=16) were tested to multiple drugs: (38.9% patients to 2 drugs, 16.7% patients to 3 drugs, 27.8% patients to 4 drugs and 16.7% patients to 5 drugs) (Table 1). Of the 59 drugs tested, antibiotics are the most group known to cause DHR (59.3%, n= 35), followed by anticonvulsants (18.6%, n= 11) and antifungals (8.5%, n=5).

Overall, IL-4 identified the culprit drug most frequently (84.6%, 11/13) compared to IFN-γ (77.8%, 14/18) and LPA (55.6%, 10/18). Combination of IFN-γ and IL-4 identified a culprit drug in 92.3% (12/13) of all cases (Fig 2). Only 2 children were tested positive to more than one possible culprit drug when combining LPA with cytokine (IFN-γ or IL-4) detection assay.

To assess the utility of in-vitro assays during the acute phase of DHR, we evaluated the test outcome in 9 cases with DHR within 30 days from rash onset (median 6 days, IQR 4-10) (Table 1). In line with previous reports, the median circulating frequency of drug specific T cells in acute cases identified by ELISpot was 0.39 x10-4 % IFN- γ (IQR 0.26- 2.14 x10-4); 0.47 x10-4 IL-4 % (IQR 0.17- 2.15 x10-4). Causative drugs as identified by positive assays in the acute phase, were most frequently detected by the IL-4 (100%, 7/7) versus IFN-γ (88.9%, 8/9) and LPA (77.8%, 7/9) (Fig 2). A positive drug response was identified by either IFN-γ or IL-4 in 100% (8/8) of cases. The combination of all three assays identified a causative drug in all (100%, 8/8) of all cases (Fig 2).

Of the 9 cases diagnosed with DHR tested after resolution of the acute phase (median 185 days, IQR 128-480) (Table 1), 6 showed positive responses in the IFN-γ (66.7%, 6/9), IL-4 (66.7%, 4/6) and LPA (33.3%, 3/9) (Fig 2). The median circulating frequency of drug specific T cells in post-recovery cases identified by ELISpot was 0.26 x10-4 % IFN- γ (IQR 0.15- 1.17 x10-4); 0.02 x10-4 % IL-4 (IQR 0.01- 0.03 x10-4). The combination of all three assays identified a causative drug in 83.3% (5/6) of cases (Fig 2). In this study, incorporation of the LPA did not enhance detection rate above that of the combined cytokine screen. In the two children tested both during the acute phase and after recovery, 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 phase (Fig 3), and for the IL-4 this reached statistical significance (p= 0.004).

Although the numbers were small for subgroup analysis, the sensitivity of LPA appeared to be better during the acute stage of DHR in children: 83.3% (5/6) for MPE, 50% (1/2) for DRESS, 100% (1/1) for SJS/ TEN in the acute phase versus 33.3% (1/3) for MPE, DRESS and SJS/ TEN in the post-recovery phase (Table 2). Cytokine assays were more useful in the detection of T-lymphocyte function in cases with DRESS in the acute phase: 100% (2/2) for IFN-γ and IL-4, versus 50% (1/2) for LPA. Both LPA and cytokine assays showed similar positive responses when tested for MPE (83.3% (5/6) for LPA versus 83.3% (5/6) for IFN-γ and 100% (4/4) for IL-4) and SJS/ TEN (100% (1/1) for LPA, IFN-γ and IL-4). In the post recovery phase, cytokine assays showed higher positive drug-specific responses than LPA for MPE and SJS/ TEN: 100% (3/3) for IFN-γ and IL-4 versus 33% (1/3) for LPA in MPE, and 100% (3/3) for IFN-γ and 0% (0/2) for IL-4 versus 33% (1/3) for LPA in SJS/ TEN. Combination cytokine assays (IFN-γ and IL-4) produced an overall higher positive drug-specific responses compared to LPA in both acute and post recovery phases. (Table 2).

**Discussion**

Research focussing on children has been the subject of directives from the United States Food and Drug Administration (FDA) and the European Medicines Authority (EMA) which specifically mandate paediatric research.8,9,11 Here we report a series of 16 cases of DHR in children and we are not aware of any other reports specifically addressing the role of in-vitro testing in this age group.5 Testing for DHR causality is important because, whilst the avoidance of possible culprit drugs is important, the use of alternative non-first line drugs may be less effective and unlicensed for infants and children.9,12 Therefore especially in cases of severe DHR, diagnostic testing is important. Currently, the clinical assessment of the exposure timeline is critical, but because challenge testing involves significant risk other approaches are required.4,5 Patch tests show some utility in the investigation of T cell mediated reactions but are very dependent on drug factors,13 less sensitive in some phenotypes (e.g. SJS and TEN) and often not practical in children.5 Furthermore, intradermal and challenge tests are difficult in children and not recommended in severe reaction patterns.1,5 Therefore an in-vitro diagnostic test for DHR would be of great benefit to the paediatric population.

Activation of drug-specific T cells is generally thought to play a central role in mediating adverse cutaneous DHR, such as DRESS, SJS and TEN.1 Previous work has shown that the capacity to generate both T-helper-1 (Th1) and T-helper-2 (Th2) responses are altered in childhood.6 In addition, paediatric immunological responses have shown differences to adults including reduced in-vitro T cell proliferation, induction of anergy and a diminished capacity to produce IFN-γ, all of which are important for when considering the validity of in-vitro T cell assays.6 Therefore, in the context of the in-vitro assays employed by our group and others for the investigation of delayed-DHR it is important to examine the paediatric cohort in detail.7,14 Despite these legitimate concerns, we report that in-vitro diagnostic testing still has its merit in the paediatric population.

Our current cohort of paediatric patients (n=16) allows us to make a valid analysis of the usefulness of the in-vitro diagnostic testing in paediatric population, using previously documented and validated methodology. In this study in contrast to what we predicted, we found the LPA to be positive in 77.8% of acute DHR and 33.3% during post recovery, which is actually higher than for adults. This suggests that LPA in children may be more sensitive during the acute phase of DHR.7 For greater sensitivity, a combination of ELISpot assays (IFN-γ and IL-4) and LPA identified culprit drugs in 100% of cases during the acute reaction and 83.3% after recovery. Cytokine assays showed a higher frequency of positive drug-specific responses as compared to LPA in both acute (100% for IL-4, 88.9% for IFN- γ, 77.8% for LPA) and the post recovery phase (66.7% for IL-4 and IFN- γ, 33% for LPA). In contrast to LPA, measures of drug specific cytokine production by T cells, were not reduced in the paediatric population, and therefore show significant advantages in diagnostic testing, also because they do not rely on radioisotopes and can be completed in less than 24 hours.6

Kano et al. suggested that LPA should be performed within 1 week after the onset of skin rashes in patients with MPE and SJS or TEN; and 5 to 8 weeks after in patients with DRESS.17 In our cohort of children, although we did not adopt the same time points for analysis, our data in children were in broad agreement with this principle: in the acute phase (up to 30 days) LPA showed positive responses in MPE (83.3%), DRESS (50%) and SJS or TEN (100%). However, cytokine ELISpot assays were superior in the detection of all types of cutaneous DHR in the acute settings and showed higher sensitivity positive drug-specific responses for MPE and SJS or TEN in the post recovery phase when compared to LPA (Table 2).

A limitation of the study was the relatively small sample size. Nevertheless, previous larger cohorts in the adult population have shown similar results and this report supported the use of these novel assays for investigation of children with DHR in clinical practice.7 While the LPA would be of limited use alone in the acute management of a patient with possible drug hypersensitivity, its greater specificity supports the value of this assay in combination with cytokine assays, particularly in diagnostic doubt where multiple medications are stopped during the course of the DHR.1,7 Avoidance of the culprit and cross-reactive drugs is the treatment of choice for DHR.1,3 In children, this may be more difficult to achieve because the choice of alternative drugs in infants or children is more limited and due to age there are practical difficulties of ‘lifelong avoidance’ compared to that of adults.5,12 *In-vitro* tests may replace drug provocation challenges in some situations and in others, reduce the risk of accidental induction of a severe DHR during a challenge test.7 Furthermore, the simultaneous assessment of T cell responses to multiple potential harmful drugs, and the potential to test cross-reactivity responses offers a unique advantage.7,15 Confirmation of the test result with drug challenge is ethically not viable for positive results, but we support the approach whereby in selected individuals, test negative drugs can be prescribed if clinical need exists. In two of our cohort we undertook this approach without complication. However, because immunological assays will never show 100% sensitivity and specificity, multidisciplinary teams with experienced clinicians and scientists familiar with the assays will be important for their future use.7 We advocate further studies in larger cohorts of paediatric patients to characterise the precise sensitivity and specificity of these assays.

Our study showed that both drug-induced T cell proliferation and cytokine release assays were useful for identification of culprit drug in DHR in children. To our knowledge, this is the first study to examine the use of in-vitro diagnostic test for drug causation in DHR in the paediatric population. The sensitivity of LPA was better during the acute stage of DHR in children, which is in agreement with previous work in adults. In-vitro assays of drug-specific cytokine production (IFN-γ and IL-4) were as good as LPA, but when combined, they offer greater sensitivity in the diagnosis of acute and post recovery DHR. Cytokine detection is rapid and does not involve radioactivity, which would be more suited to routine clinical use, particularly in children. These novel in-vitro assays appear to be promising and may provide a significant advancement in our management of DHR in children.

(2645 words)

**Tables**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Patient ID | Age (years) | Sex | Rash to test (days) | Acute ⁄recovery | Phenotype | No. of drugs tested |
|
| 1a | 15 | F | 2 | Acute | MPE | 5 |
| 1b | 16 | F | 480 | Post recovery | MPE | 4 |
| 2 | 2 | M | 14 | Acute | MPE | 4 |
| 3 | 15 | M | 4 | Acute | MPE | 2 |
| 4a | 13 | M | 3 | Acute | MPE | 5 |
| 4b | 13 | M | 98 | Post recovery | MPE | 5 |
| 5 | 13 | F | 5 | Acute | MPE | 2 |
| 6 | 16 | M | 6 | Acute | DRESS | 4 |
| 7 | 8 | M | 84 | Post recovery | MPE | 3 |
| 8 | 13 | F | 17 | Acute | DRESS | 2 |
| 9 | 12 | F | 12 | Acute | SJS/TEN | 4 |
| 10 | 13 | M | 255 | Post recovery | SJS | 2 |
| 11 | 15 | M | 574 | Post recovery | SJS | 2 |
| 12 | 18 | M | 158 | Post recovery | DRESS | 3 |
| 13 | 9 | M | 185 | Post recovery | SJS | 2 |
| 14 | 9 | M | 1145 | Post recovery | DRESS | 4 |
| 15 | 3 | F | 16 | Acute | MPE | 3 |
| 16 | 6 | F | 132 | Post recovery | DRESS | 2 |

Table 1. Child characteristics.

MPE, maculopapular exanthem; DRESS, drug reaction with eosinophilia and systemic symptoms; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis. a, b, same child tested in (a) acute and (b) post-recovery phases.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Types of cutaneous DHR | LPA, % | IFN-γ, % | IL-4, % | IFN-γ & IL-4, % |
| Acute | Post recovery | Acute | Post recovery | Acute | Post recovery | Acute | Post recovery |
| MPE | 83 | 33 | 83 | 100 | 100 | 100 | 90 | 100 |
| DRESS | 50 | 33 | 100 | 0 | 100 | 100 | 100 | 25 |
| SJS/TEN | 100 | 33 | 100 | 100 | 100 | 0 | 100 | 60 |

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

MPE, maculopapular exanthem; DRESS, drug reaction with eosinophilia and systemic symptoms; IFN-γ, interferon-γ; IL, interleukin; LPA, lymphocyte proliferation assay; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; DHR, drug hypersensitivity reactions.

**Figures**

|  |  |
| --- | --- |
| Control |   |
| Teicoplanin |  |

Figure 1.

Example of an ex-vivo IFN-γ ELISpot. 2.5 x105 PBMC from a 2 year old boy (Patient ID ‘2’ in Table 1) who had suffered acute maculopapular exanthem, were incubated with media alone (control) against Teicoplanin (0.03 mM), in triplicate for 16 hours, on a pre-coated ELISpot plate after development.

Figure 2.

Bar chart showing percentage of cases with positive assay results to causative drugs in acute and post recovery phase in children.

|  |  |  |
| --- | --- | --- |
| a) |  |  |
| b) |  |  |
| c) |  |  |

Figure 3. Comparison between drug-specific T-cell responses from acute hypersensitivity reactions and post recovery.

Patients with acute drug hypersensitivity reaction (AcHR; black symbols) or post-recovery drug hypersensitivity reaction (PrHR; open symbols) were tested for drug specific proliferation (a, AcHR n = 9, PrHR n = 9) or enzyme linked immunosorbent spot (ELISpot) assay for IFN-γ (b; AcHR n =9 , PrHR n = 9) or IL-4 (c, AcHR n = 7, PrHR n = 6). Each data point represents a single drug (maximum response measured) for each child tested. LPA (a): Dotted lines denote positive response (SI>2). SI, stimulation index; SFU, spot-forming units

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