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Development of a Modified Lymphocyte Transformation Test for Diagnosing Drug Induced Liver Injury Associated with an Adaptive Immune Response

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Abstract

Drug induced liver injury (DILI) is a growing problem. Diagnostic methods to differentiate DILI caused by an adaptive immune response from liver injury of other causes or to identify the responsible drug in patients receiving multiple drugs, herbals, and/or dietary supplements (polypharmacy) have not yet been established. The lymphocyte transformation test (LTT) has been proposed as a diagnostic method to determine if a subject with an apparent hypersensitivity reaction has become sensitized to a specific drug. In this test, peripheral blood mononuclear cells (PBMC) collected from a subject are incubated with drug(s) suspected of causing the reaction. Cell proliferation, measured by the incorporation of [³H]-thymidine into new DNA, is considered evidence of a drug-specific immune response. The objectives of the current studies were to: 1) develop and optimize a modified version of the LTT (mLTT) and 2) investigate the feasibility of using the mLTT for diagnosing DILI associated with an adaptive immune response and identifying the responsible drug. PBMC collected from donors with a history of drug hypersensitivity reactions to specific drugs (manifested as skin rash) were used as positive controls for assay

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Declaration of interest

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optimization. Following optimization, samples collected from 24 subjects enrolled in the U.S. Drug-Induced Liver Injury Network (DILIN) were tested in the mLTT. Using cytokine and granzyme B production as the primary endpoints to demonstrate lymphocyte sensitization to a specific drug, most samples from the DILIN subjects failed to respond. However, robust positive mLTT responses were observed for two of four samples from three DILIN subjects with hepatitis due to isoniazid (INH). We conclude that the mLTT, as performed here on frozen and thawed PBMC, is not a reliable test for diagnosing DILI caused by all drugs, but that it may be useful for confirming the role of the adaptive immune response in DILI ascribed to INH.

Keywords

Allergic reactions; immuno-allergic; drug induced liver injury; drugs; hepatitis; lymphocytes; lymphocyte transformation test

Introduction

Drug induced liver injury (DILI) is a growing problem that is under-recognized and under-reported (Bonkovsky et al. 2012; Chalasani et al. 2008, 2014, 2015). Among reasons for this are the growing numbers of drugs, herbals and dietary supplements that are consumed by billions of persons worldwide and the difficulties encountered in establishing a diagnosis of DILI (Agarwal et al. 2014). Even when the diagnosis is made, based upon a compatible history and time sequence of drug intake and development of liver injury, and exclusion of alternative causes, such as viral hepatitis, alcoholic liver injury, idiopathic auto-immune hepatitis, etc., it may be difficult to determine which of several possible candidate drugs or supplements is the cause of liver injury in the specific individual case. In order to gain better insights into DILI, the National Institutes of Health (NIDDK) established the US Drug Induced Liver Injury Network (DILIN) in 2004 and has funded work of this cooperative network since that time. The major goals of the Network have been to establish a registry and database and sample repository of patients with well-characterized idiosyncratic DILI (Fontana et al. 2009), in whom other possible causes have been reasonably excluded by a formal process of causality assessment (Rochon et al. 2008; Rockey et al. 2010). Among the major findings of this Network has been the realization that most idiosyncratic, non-dose related and unpredictable DILI is due to host immune responses to the causative drugs, herbals, or dietary supplements. Another has been that herbals and dietary supplements are becoming more frequent as causes (Navarro et al. 2014; Seeff et al. 2015).

Work from the Network recently showed that T cells are frequent in liver biopsies from patients with DILI (Foureau et al. 2015), and that risks of DILI development are associated with certain HLA types (Lucena et al. 2011; reviewed in Bonkovsky et al. 2012). Therefore, some cases of idiosyncratic DILI may be mediated by hypersensitivity or allergic reactions to the drug. These types of reactions, often referred to as immuno-allergic or allergic hepatitis, or immuno-allergic DILI, often (but not always) present with features that include laboratory findings of hepatocellular and/or cholestatic injury along with skin rash, facial edema, eosinophilia, fever, and/or lymphadenopathy (Fontana et al. 2010; Bonkovsky et al. 2012). The mechanism for immuno-allergic DILI reactions has been hypothesized to be

mediated by drug- or drug metabolite-specific T-cells (referenced here simply as drug-specific T-cells) (Ju 2005; Tujios and Fontana 2011; Kim et al. 2015). Activation of these drug-specific T-cells may lead to the generation of cytokine-producing and/or cytotoxic T-cells that lead to liver injury. Indeed, liver biopsies from subjects with acute DILI with immuno-allergic features generally show abundant lympho-plasmacytic inflammation (Kleiner et al. 2014) with a predominantly CD8⁺ T-cell portal infiltrate (Foureau et al. 2015). Clinical cases with some of the symptoms similar to immuno-allergic DILI have been reported but it is not known if these reactions are true allergic reactions to the drug (mediated by drug-specific T-cells) or mediated by other mechanisms.

When drug-induced skin rashes are observed, the lymphocyte transformation test (LTT) has been used to provide evidence that the reactions were mediated by an allergic mechanism (Pichler and Tilch 2004; Kano et al. 2007; Lochmatter et al. 2009a b; Zawodniak et al 2010; Naisbitt et al. 2014). The LTT is an *ex vivo* assay in which peripheral blood mononuclear cells (PBMC) from the patient with a skin rash attributed to a drug are incubated with the suspected drug and lymphocyte proliferation is measured. Modifications of the LTT that involve measuring granzyme B and cytokine production as alternatives to [³H]-thymidine incorporation have also been developed for severe skin reactions (Stevens Johnson Syndrome, toxic epidermal necrolysis) (Porebski et al. 2013). Positive LTT responses have been reported in DILI cases using methods similar to that used for drug-induced allergic skin reactions. Several studies have evaluated the percentage of cases of drug-associated hepatitis that have positive LTT responses (Table 1) (Mizoguchi et al. 1975; Warrington et al. 1978; Yoshimura et al. 1994; Maria and Victorino 1997; Murata et al. 2003). In these studies, the percentage of positive LTT responses ranged from 12–56% for various drugs, to as high as 95% for DILI ascribed to isoniazid (INH). It should be noted that the length of time between blood collection and processing the samples to PBMC were not always indicated in these reports. In general, it must be assumed that fresh PBMC were used for the assays, because the freezing of PBMC prior to use in the LTT was not described in these studies.

In the work reported here, the feasibility of using a modified LTT (mLTT), with cytokines and granzyme B production as the primary endpoints to demonstrate lymphocyte sensitization to a specific drug, was investigated in a collaborative study with the Drug Induced Liver Injury Network (DILIN) (Fontana et al. 2009; Rockey et al. 2010; Kleiner et al. 2014; Chalasani et al. 2015) to determine the utility of the LTT for diagnosing DILI associated with an adaptive immune response. These studies involved the use of PBMC samples collected from DILI subjects by DILIN investigators. As part of the Prospective Protocol of DILIN, blood samples were already being collected and sent to a central repository for PBMC isolation and freezing. For this ancillary study, additional blood samples were also collected and PBMCs were prepared for evaluation in the mLTT.

The first objective of this study was to evaluate the performance of the mLTT using PBMC samples from healthy human donors with and without known drug allergies (manifested as skin reactions). The second objective was to evaluate responses of PBMC samples obtained from DILIN subjects in the mLTT. Cytokine and granzyme B production were evaluated to determine whether the mLTT could aid in the diagnosis of DILI reactions associated with an adaptive immune response and identify the responsible drug.

Materials and Methods

The US DILIN has been described previously (Chalasanani et al. 2008, 2015; Fontana et al. 2009; Rochon et al. 2008; Rockey et al. 2010). In brief, it is comprised of several clinical centers (currently 6 in number) that identify and enroll subjects with acute liver injury due to drugs, herbals or dietary supplements. A formal method for assessing causality, based upon expert opinion of experienced hepatologists, is an important part of the US DILIN because of the difficulty of identifying the cause of liver injury and the need to exclude non-drug causes. Subjects may be enrolled at any time within 6 months of the onset of acute liver injury. Demographic and clinical data are collected, and samples of blood and urine are collected and stored in sample repositories. The Duke Clinical Research Institute serves as the Central Data Repository and Coordinating Center for the Network.

Blood collection and PBMC isolation for initial feasibility experiments

Whole blood was collected into heparinized tubes at the Pfizer Occupational Health Office from human donors with a history of allergic drug reactions or donors with no history of allergic drug reaction. Blood samples were obtained after donors had provided informed consent and signed written informed consent forms. Donor recruitment and blood collection procedures followed protocols approved by the Pfizer Institutional Review Board.

PBMC were isolated using Lymphoprep™ (STEMCELL Technologies, Vancouver, British Columbia) according to the manufacturer's instructions. Following cell isolations, counts per milliliter of whole blood were determined by an Advia™ 120 Hematology System (Siemens Healthcare Diagnostics Inc., Deerfield, IL) and converted into total PBMC per milliliter of whole blood. Twenty million cells were cryogenically frozen in 1 ml freezing media (10% DMSO [Sigma, St. Louis, MO] + 90% filtered heat-inactivated fetal bovine serum [FBS; Invitrogen, Grand Island, NY]) and stored in a liquid nitrogen tank until used for analysis in the mLTT.

Modified Lymphocyte Transformation Test

In brief, 5×10^5 PBMC in 200 μ l medium (RPMI [Invitrogen] supplemented with 2–4% human AB serum, 2 mM L-glutamine [Invitrogen], 25 ng/ml human transferrin [Sigma], and 50 μ g/ml gentamicin [Sigma]) were added to individual wells of a 96-well U-bottomed cell culture plate (Corning Inc., Corning, NY). Cells were treated with either 5 μ g/ml anti-human CD3 (Clone HIT3a, BD Biosciences, San Jose, CA) as a positive control or with the relevant drug (concentration[s] dependent on threshold for cytotoxic effects; data not shown). In some cases, cells were also treated with an irrelevant drug that was not associated with reported allergic response (metformin) to determine specificity of the mLTT. Drugs (purchased from Sigma) and the ranges of concentrations of drugs tested are indicated in Table 2. For each donor, untreated (non-stimulated) PBMCs served as the negative control. Following addition of the appropriate treatment/compounds, cells were cultured in a 5% CO₂ incubator at 37°C for 3 days. At the end of the incubation period, plates were centrifuged (800 \times g) for 10 min and the supernatants collected and stored at –20°C until cytokine and granzyme B analyses were carried out.

Cytokine measurements

Multiplex cytokine assay kits (Millipore Corp., Billerica, MA and Meso Scale Discovery [MSD], Rockville, MD) were used for the cytokine analysis. Interleukin (IL)-2, IL-5, IL-13 and interferon (IFN)- γ were selected for analysis based on previous work that demonstrated the sensitivity of these cytokines in drug allergy responses (Lochmatter et al. 2009). The multiplex bead assay (Millipore) uses beads internally tagged with fluorescent dyes and coated with specific cytokine antibodies to capture cytokines of interest. The internal fluorescent dyes and surface coating of various antibodies allows detection of multiple cytokines in a single sample. Once the beads captured the cytokines, biotinylated detection antibody was added for signal amplification, followed by incubation with Streptavidin-PE (phycoerythrin) conjugate as a fluorescent reporter molecule. After the incubation, each bead passes through two lasers: the first one excites the internal fluorescent dyes to identify each analyte, and the second laser excites the reporter molecule to quantify signals from each analyte. All measures were performed in a Bio-Rad[®] Bio-Plex System using Bio-Plex Manager[™] software (Bio-Rad, Hercules, CA) to analyze the data outputs. The level of sensitivity of the kit was 3.2 pg cytokine/ml.

The MSD cytokine assay plates are pre-coated with capture antibodies spotted at the bottom of the wells. Calibrators or samples are incubated in the multi-spot plate and each cytokine binds its corresponding capture antibody. Cytokine levels are then measured using a cytokine specific detection antibody with MSD SULFO-TAG[™]. All measures were performed in a MSD 6000 system using Discovery Workbench[™] (MSD) software to analyze the data outputs. The level of sensitivity of the kit was 2.4 pg cytokine/ml.

Both multiplex assays were performed according to the manufacturer protocols. Data are presented for each cytokine in pg/ml or as stimulation index (SI) determined by dividing cytokine production of the stimulated PBMC by that of the non-stimulated PBMC.

Granzyme B measurements

A commercially available ELISA kit was used to quantify granzyme B (Cell Sciences, Canton, MA). The assay was performed as per the manufacturer protocols. Data are presented in pg/ml or as a SI determined by dividing granzyme B production of the stimulated PBMC by granzyme B production of the non-stimulated PBMC. The level of sensitivity of the kit was 20 pg granzyme B/ml.

DILIN Sample Testing

PBMC (isolated as described above) obtained from DILIN subjects (Chalasani et al. 2008, 2015; Fontana et al. 2009) were tested in the mLTT and cytokine (IL-2, IL-5, IL-13, IFN γ) and granzyme B concentrations were determined as described above for the respective endpoints. To increase the likelihood of success in identifying a positive response in the mLTT, subjects that experienced DILI reactions with drugs often associated with drug allergy and/or subjects that displayed clinical features such as rash, fever, and/or eosinophilia were prioritized for testing (Table 3); this resulted in a total of 33 samples from 24 DILIN subjects being tested.

Results

mLTT Assay Performance (Cytokines and Granzyme B)

Due to the lack of availability of positive control samples for DILI associated with an adaptive response, PBMC collected from 10 healthy donors that had previously experienced an allergic reaction (manifested as a skin reaction) to a specified drug(s) (considered positive controls) were used to evaluate the performance of the mLTT. Samples collected from 11 healthy donors with no reported allergic reactions to drugs (considered negative controls) were also tested in the assay. Responses in the mLTT for positive and negative control donor PBMC samples are reported in Table 4. A positive mLTT response was defined as an SI ≥ 2 for ≥ 3 of five analytes (e.g., IL-2, IL-5, IL-13, IFN γ , granzyme B) at one or more drug concentrations tested.

Samples from most positive control donors had a positive mLTT response when tested with their relevant respective drugs (that is, the drug that was associated with a previous allergic reaction). However, the positive mLTT response was not consistently reproducible in subsequent experiments using PBMC samples from one positive control donor (Donor D110). Repeat experiments were not performed with samples from the other positive control donors due to inadequate numbers of PBMC. Negative mLTT responses were observed for samples from positive control donors tested with the irrelevant drug, metformin. Positive mLTT responses were observed for samples from some negative control donors. Specifically, PBMC samples from 3 of the 10 negative control donors responded to one or more of the drugs tested.

DILIN sample testing in the mLTT

A total of 33 PBMC samples from 24 DILIN subjects were tested in the mLTT, which included baseline (defined as the first PBMC sample collected following the DILI reaction) and 6-mo follow-up samples from nine DILIN subjects (Table 3). Samples from most subjects had negative mLTT responses based on levels of cytokines and granzyme B (data not shown). For those few samples that did produce a positive mLTT response (six samples from five subjects; indicated in Table 3 with shading and in Table 5), the positive response was not reproducible in repeat experiments, with the exception of one sample from a subject with INH-related DILI (Subject ID 87). For this subject, a positive mLTT response was observed for the 6-month follow-up sample at the highest INH concentration tested in two independent experiments; the baseline sample for this subject (Subject ID 8) did not produce a positive response in the mLTT. A baseline sample from a second subject (Subject ID 23) was negative in the mLTT when PBMC were tested with SMX. However, in a subsequent experiment, a robust positive cytokine and granzyme B response was observed when the PBMC sample from this subject was tested in the mLTT with INH and INA (but not SMX or Trim). Unfortunately, no additional PBMC samples were available from this subject to confirm the positive mLTT result with INH or INA.

Discussion

DILI is a diagnosis of exclusion and it is both under-recognized and under-reported (Bonkovsky et al. 2012). Even when the diagnosis is made, it is often difficult to ascertain which of several possible causative drugs or herbals/dietary supplements are the cause of the liver injury. This is a growing problem in the current era of polypharmacy for most patients, especially those in older age groups who are more susceptible to DILI (Bonkovsky et al. 2012; DeLemos et al. 2016) and in whom chronic and ongoing DILI are more likely (Fontana et al. 2015). Thus, there is growing need for a simple and reliable biomarker, both to identify those with DILI and to predict likely clinical outcomes. In the latter regard, a combination of levels of four cytokines and albumin in the serum was recently found to have high accuracy for predicting prognosis of acute DILI (Steuerwald et al. 2013).

The overall objective of this study was to optimize the mLTT in order to determine if this *in vitro* test could be used to identify DILI reactions associated with an adaptive immune response. In reports from other investigators, the traditional LTT (used as a diagnostic tool for allergic drug reactions) has been shown to result in positive responses in subjects with DILI and, in Japan, the assay is used rather widely to support the diagnosis of DILI and to implicate the putative responsible drugs (Watanabe and Shibuya 2004).

In contrast, however, data from the current study suggest that the mLTT, using cytokines and granzyme B production to determine lymphocyte sensitization to a specific drug, is not useful or reliable for diagnosing DILI associated with an adaptive immune response under the conditions tested. Although a positive control sample from a subject with known DILI associated with an adaptive immune response was not available to assess the performance of the mLTT, the cytokine and granzyme B data generated with the available drug allergy positive control donors were not reproducible, nor were robust responses observed.

Even though robust, reproducible responses were not observed in the mLTT with samples from positive control donors with a history of drug allergies (manifested as skin reactions), samples from selected DILIN subjects were tested in the assay. In order to allow for the greatest probability of success in identifying subjects with DILI reactions associated with an adaptive immune response, samples from DILIN subjects that had clinical features associated with drug allergy (e.g., fever, rash, and/or eosinophilia) (Castell and Castell 2006; Bonkovsky et al. 2012) and/or whose liver injury was associated with a drug known to cause drug allergy, were selected for testing in the mLTT. Similar to what was observed for samples from the drug allergy positive control donors, the majority of samples tested in the mLTT from the DILIN subjects produced non-robust cytokine and granzyme B responses. Samples from only one DILIN subject showed reproducible responses in cytokines and granzyme B in the mLTT. In this particular case (Subject ID 87) where the responsible drug was INH, the positive mLTT response was only observed in the 6-month follow-up sample, and not the baseline sample, and the response was observed following incubation of PBMC with both INH and its metabolite INA. Positive LTT responses have been previously reported for cases of INH-induced hepatotoxicity (Warrington et al. 1978, 1982), and a role for the immune system in contributing to INH-induced liver injury has been described (Metushi et al. 2011). It is worth noting that for the 3 DILIN subjects with INH associated

liver injury, samples from all three subjects had a positive mLTT response in at least one experiment. These results are consistent with the 95% positive LTT response rate observed in INH-induced hepatitis cases reported by Warrington et al. (1978).

Because in prior studies positive LTT responses have been observed in subjects with what appeared to be DILI associated with an adaptive immune response, the lack of consistently and/or strongly positive results in the mLTT described herein was unexpected. Several factors may have contributed to the lack of positive responses in the DILIN subject samples tested in the mLTT. One such factor is the unavoidable delay that occurred in isolating the PBMCs from the whole blood samples that were collected from the DILIN subjects. For this study, whole blood samples were collected at DILIN sites across the United States and then shipped by overnight air to a central processing center where PBMCs were isolated and frozen. The delay in PBMC isolation from whole blood samples may have significantly decreased antigen-specific T-cell responses. In addition, timing of the collection of PBMCs relative to the onset of clinical symptoms of an allergic reaction has also been shown to be important in detecting responses in the LTT (Kano et al. 2007). This may be particularly important in the case of DILI, where drug-specific T-cells may not be in circulation (at least in large numbers) at the time of blood collection and PBMC isolation, especially when these were long after the acute reaction. The concentration of drug tested has also been shown to be important; dose-dependent responses are not always observed in the LTT, which is why it is recommended to test a range of drug concentrations in the assay.

For this study, due to the limited numbers of PBMC available per subject, it was not possible to test a wide range of concentrations, perhaps, reducing the likelihood of detecting a positive response in the assay. Then, too, it may not have been the parent molecule that initiated the DILI response; in some (or all subjects), testing drug metabolites and/or drug-protein conjugates may be necessary to elicit a response in the *ex vivo* LTT. However, with the exception of INA (the metabolite of INH), only the parent molecules were tested in these studies due to the limited number of PBMC available for each DILIN subject. Finally, another possibility that cannot be excluded for why positive responses in the mLTT were not observed for most DILIN subjects is that the DILI reaction in these particular subjects may not have been driven by an adaptive immune mechanism.

Conclusions

Samples from all of the DILIN subjects were negative or not reproducible in the mLTT, with the exception of one subject with INH-associated liver injury. Therefore, it could be concluded that lack of responses from PBMC collected from cases with phenotypes of immuno-allergic DILI could be due to one or a combination of the following reasons: (1) DILI reactions were not mediated by drug-specific T-cells, (2) the mLTT as used in these studies was not sensitive enough, (3) responsiveness of drug-specific T-cells decreased with the 24-hr period between shipment and PBMC preparation, and/or (4) drug metabolites/drug-protein conjugates were not tested for most samples. Given the limitations of the assay, we concluded the mLTT, as performed here, is not a robust test for diagnosing DILI associated with an adaptive response caused by all drugs. However, the mLTT may be useful for diagnosing or confirming DILI ascribed to INH.

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Table 1Positive Lymphocyte Transformation Test Responses in Patients with Drug-induced Liver Injury^a

Reference	Cases	Percent Positive Responses	Endpoint	Comments
Mizoguchi et al. 1975	70	12.8%	[³ H]-uridine uptake	Systemic features included skin eruption (60%), fever (69%), itching (73%), and eosinophilia (50%)
Warrington et al. 1978	20	95%	[³ H]-thymidine uptake	All patients had INH-associated liver injury
Yoshimura et al. 1994	113	41.3%	MTT assay	Systemic features included itching (53%), eruption (35%), and fever (32%)
Maria and Victorino 1997	95	26% (w/o PG inhibitor) 56% (w/PG inhibitor)	[³ H]-thymidine uptake	Systemic features included fever (39%), rash (29%) and eosinophilia (53%)
Murata et al. 2003	17	52.9%	IFN γ production by CD8 ⁺ cells	

^aThese studies did not indicate the amount of time between blood collection and peripheral blood mononuclear cells (PBMC) processing. It is assumed that fresh PBMC were used for these assays since the freezing of PBMC prior to use in the lymphocyte transformation test was not described.

INH-isoniazid; PG-prostaglandin; MTT-3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; w/o-without.

Table 2

Drugs Tested in the Modified Lymphocyte Transformation Test

Name	Abbreviation	Concentration(s) ($\mu\text{g/ml}$) ^a
Allopurinol	ALL	0.1, 1, 10, 100
Amoxicillin	AMX	50, 100, 200, 500
Carbamazepine	CBZ	10, 50
Clavulanic Acid	CA	10, 50
Isoniazid	INH	10, 100, 200
Isonicotinic Acid	INA	10, 100, 200
Levofloxacin	Not used	50, 100
Metformin	MET	100
Minocycline	Not used	0.01, 0.1, 1, 10, 50
Moxifloxacin	Not used	100
Nitrofurantoin	Not used	50, 100
Phenytoin	PHE	10, 50, 100
Sulfamethoxazole	SMX	50, 100, 200
Trimethoprim	TP	10, 25, 50
Valproic Acid	VA	1, 10, 50, 100

^aNot all concentrations tested in samples from all donors.

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Table 3

Summary of DILIN Subjects Tested in the Modified Lymphocyte Transformation Test^a

Subject Baseline ID	Subject 6-mo ID	Pattern of Liver injury	Drug	Days from drug start to onset	Clinical Systemic Symptoms
5	119	Cholestatic	Amoxicillin w/CA	36	Rash
7		Mixed	Amoxicillin	7	Rash
8	87	Hepatocellular	Isoniazid	50	None
9		Hepatocellular	Valproic Acid	6964	None
16		Hepatocellular	Nitrofurantoin	11	Rash, Eosinophilia
18		Cholestatic	Minocycline	386	Eosinophilia
22		Mixed	Isoniazid	171	None
23		Cholestatic	Sulfamethoxazole w/Trimethoprim, Isoniazid	20/131	None
35	128	Cholestatic	Amoxicillin w/CA	16	None
36		Hepatocellular	Sulfamethoxazole w/Trimethoprim	14	Fever
38	135	Cholestatic	Sulfamethoxazole w/Trimethoprim	31	Fever
41	131	Hepatocellular	Allopurinol	50	Fever, Rash
43		Hepatocellular	Amoxicillin w/CA	17	Eosinophilia
58		Mixed	Sulfamethoxazole W/Trimethoprim	29	Rash
61	137	Hepatocellular	Amoxicillin w/CA	33	Eosinophilia
63		Cholestatic	Amoxicillin w/CA	40	None
72		Hepatocellular	Sulfamethoxazole w/Trimethoprim	11	Fever, Rash
78	148	Mixed	Levofloxacin	10	None
81	149	Mixed	Amoxicillin w/CA	14	Fever
86		Hepatocellular	Amoxicillin	45	Eosinophilia
96		Mixed	Amoxicillin	16	Rash, Eosinophilia
98		Cholestatic	Nitrofurantoin	6	None
99	159	Hepatocellular	Amoxicillin w/CA	14	Rash
123		Mixed	Allopurinol	37	Fever, Rash, Eosinophilia

^a Shading indicates a positive cytokine/granzyme B response in at least one experiment.

Table 4Sensitivity and Specificity of the Modified Lymphocyte Transformation Test^a

	Donor Identification	Drug(s) Tested	mLTT Response ^b
Positive Control Donors	D110	SMX, MET	+/- (SMX)
	PID11	SMX	+ (SMX)
	PID31	AMX	+ (AMX)
	PID492	AMX	+ (AMX)
	D33	AMX, MET	-
	PAT32	AMX, PHE, SMX, MET	+ (AMX, PHE, SMX)
	PAT47	CBZ, MET	+ (CBZ)
	PAT535	SMX, MET	+ (SMX)
	PAT553	CBZ, PHE, MET	+ (CBZ, PHE)
	PAT577	SMX, Trim, MET	+ (SMX, Trim)
Negative Control Donors	D138	AMX, SMX	-
	D206	AMX, SMX	-
	D6	MET, AMX, CA, ALL, SMX, Trim	+ (MET, AMX)
	D10	MET, AMX, CA, ALL, SMX, Trim	-
	D229	MET, AMX, CA, ALL, SMX, Trim	+ (CA)
	HD1	AMX, SMX, PHE, MET	-
	HD2	SMX, Trim, MET	+ (SMX, Trim)
	HD3	SMX, MET	-
	HD4	CBZ, PHE, MET	-
	HD5	CBZ, MET	-

^aPBMC from each subject were incubated with drug for 72 hr.

^bPositive response defined as a stimulation index ≥ 2 for ≥ 3 of five analytes (IL-2, IL-5, IL-13, IFN γ , granzyme B) at one or more drug concentrations tested.

Table 5

Summary of Cytokine and Granzyme b Responses (pg/ml) for DILIN Samples Tested in the Modified Lymphocyte Transformation Test^a

Subject ID	Sample	Experiment 1										Experiment 2				
		IFN γ	IL-13	IL-2	IL-5	Granzyme B	mLTT result ^b	IFN γ	IL-13	IL-2	IL-5	Granzyme B	mLTT result ^b			
9 (Baseline)	Media	4.0	9.3	6.4	< 3.2	< 7.8		5.0	6.7	16.6	< 3.2	22.0				
	anti-CD3	190.5	2055.0	< 3.2	1796.4	>1000	+	1351.4	3511.2	< 3.2	1199.0	>1000	+			
	VA (100 μ g/ml)	117.7	1923.5	< 3.2	288.9	>1000	+	< 3.2	14.2	9.6	< 3.2	< 15.6				
	VA (10 or 50 μ g/ml) ^c	< 3.2	3.4	6.4	< 3.2	< 7.8		< 3.2	5.4	9.1	< 3.2	< 15.6				
	VA (1 or 10 μ g/ml) ^c	< 3.2	< 3.2	3.3	< 3.2	< 7.8		< 3.2	< 3.2	< 3.2	< 3.2	< 15.6				
22 (Baseline)	Media	< 2.4	< 2.4	< 2.4	< 2.4	< 20		< 2.4	< 2.4	< 2.4	< 2.4	< 20				
	anti-CD3	264.5	< 2.4	10.0	< 2.4	>1000	+	904.8	13.2	7.5	5.7	>1000	+			
	INA (200 μ g/ml)	< 2.4	< 2.4	< 2.4	< 2.4	< 20		< 2.4	< 2.4	< 2.4	< 2.4	< 20				
	INA (100 μ g/ml)	29.9	< 2.4	14.1	< 2.4	46.6	+	< 2.4	< 2.4	< 2.4	< 2.4	< 20				
	INA (10 μ g/ml)	< 2.4	< 2.4	< 2.4	< 2.4	< 20		ND	ND	ND	ND	ND				
	INH (200 μ g/ml)	< 2.4	< 2.4	< 2.4	< 2.4	< 20		< 2.4	< 2.4	< 2.4	< 2.4	< 20				
	INH (100 μ g/ml)	< 2.4	< 2.4	< 2.4	< 2.4	< 20		4.7	< 2.4	2.7	< 2.4	< 20				
	INH (10 μ g/ml)	< 2.4	< 2.4	3.7	< 2.4	< 20		ND	ND	ND	ND	ND				
MET (100 μ g/ml)	ND	ND	ND	ND	ND		< 2.4	< 2.4	< 2.4	< 2.4	< 20					
23 ^d (Baseline)	Media	< 3.2	< 3.2	< 3.2	< 3.2	42.6		< 2.4	< 2.4	< 2.4	< 2.4	< 20				
	anti-CD3	3918.4	3972.8	< 3.2	380.4	>1000	+	684.1	7.0	17.4	< 2.4	448.9	+			
	INA (200 μ g/ml)	ND	ND	ND	ND	ND		2807.8	77.3	36.1	52.1	739.2	+			
	INA (100 μ g/ml)	ND	ND	ND	ND	ND		3719.3	82.2	36.2	54.8	>1000	+			
	INH (200 μ g/ml)	ND	ND	ND	ND	ND		4357.0	145.8	36.8	130.6	>1000	+			
	INH (100 μ g/ml)	ND	ND	ND	ND	ND		9372.4	151.5	39.1	173.7	749.3	+			
	INH (10 μ g/ml)	ND	ND	ND	ND	ND		65.3	< 2.4	< 2.4	< 2.4	34.4				
	SMX (200 μ g/ml)	< 3.2	< 3.2	< 3.2	< 3.2	34.5		< 2.4	< 2.4	< 2.4	< 2.4	29.8				
	Trim (50 μ g/ml)	< 3.2	< 3.2	< 3.2	< 3.2	23.4		ND	ND	ND	ND	ND				
	Media	< 2.4	8.4	< 2.4	< 2.4	< 20		10.2	21.5	3.4	8.6	103.2				
anti-CD3	3942.3	800.8	61.2	566.2	>1000	+	9989.9	492.6	23.6	343.1	>1000	+				

Subject ID	Sample	Experiment 1						Experiment 2					
		IFN γ	IL-13	IL-2	IL-5	Granzyme B	mLTT result ^b	IFN γ	IL-13	IL-2	IL-5	Granzyme B	mLTT result ^b
135 (6-mo Subject 38)	SMX (200 μ g/ml)	12.6	27.5	26.2	9.1	<20	+	6.6	14.3	5.6	6.8	167.6	
	SMX (100 μ g/ml)	6.0	22.3	13.7	7.0	<20	+	ND	ND	ND	ND	ND	
	TMP (50 μ g/ml)	3.1	10.8	6.6	4.0	<20		4.9	9.1	<2.4	<2.4	540.6	
	MET (100 μ g/ml)	6.5	5.9	8.2	<2.4	<20		6.8	12.2	<2.4	3.9	172.6	
Media	<2.4	13.1	<2.4	3.7	<20		12.6	19.6	<2.4	4.2	<20		
anti-CD3	3710.3	307.3	95.6	221.2	>1000	+	>10000	220.3	53.0	195.7	>1000	+	
SMX (200 μ g/ml)	4.7	14.3	8.8	5.2	<20		5.4	13.9	2.5	3.7	642.8		
SMX (100 μ g/ml)	5.5	12.6	5.9	<2.4	<20		ND	ND	ND	ND	ND		
TMP (50 μ g/ml)	10.2	12.7	10.3	7.3	<20	+	3.8	11.8	<2.4	3.6	118.1		
MET (100 μ g/ml)	2.7	9.0	4.3	2.8	<20		3.6	10.6	<2.4	3.4	80.8		
Media	<2.4	<2.4	<2.4	<2.4	<20		<2.4	<2.4	7.1	<2.4	673.3		
anti-CD3	9000.0	20.9	25.2	3.0	>1000	+	>10000	47.6	64.3	11.5	>1000	+	
INA (200 μ g/ml)	213.5	3.0	7.5	<2.4	146.1	+	1933.3	17.2	8.8	7.1	786.4	+	
INA (100 μ g/ml)	>10000	26.5	12.7	7.8	>1000	+	ND	ND	ND	ND	ND		
INA (10 μ g/ml)	29.3	<2.4	4.4	<2.4	39.7		ND	ND	ND	ND	ND		
INH (200 μ g/ml)	330.0	2.9	5.8	<2.4	271.2	+	2274.7	11.5	2.8	4.9	641.3	+	
INH (100 μ g/ml)	16.0	<2.4	3.6	<2.4	35.9		ND	ND	ND	ND	ND		
INH (10 μ g/ml)	150.2	<2.4	3.0	<2.4	228.8		ND	ND	ND	ND	ND		
MET (100 μ g/ml)	ND	ND	ND	ND	ND		588.7	12.7	<2.4	4.6	926.9		

^aShading indicates an SI ≥ 2 .

^bPositive response defined as ≥ 2 stimulation index (SI) for ≥ 3 of five analytes (IL-2, IL-5, IL-13, IFN γ , granzyme B).

^cVA tested at concentrations of 10, 50, and 100 μ g/ml in Experiment 1 and 1, 10, and 100 μ g/ml in Experiment 2.

^dResponses for Subject 23 in Experiment 1 were from a 6-d incubation;

SMX = sulfamethoxazole; Trim = trimethoprim; INA = isonicotinic acid; INH = isoniazid; MET = metformin; VA = valproic acid; ND = not done.