

A pilot study evaluating biomarker development for drug-induced chronic eczematous eruptions of aging individuals

Erika Mae Summers¹, Nicholas Ray Blickenstaff¹, Garrett Curtis Coman¹, Thomas Bernd Martins², Harry Raymond Hill^{1,2}, Richard Dennis Sontheimer¹

¹Department of Dermatology, University of Utah School of Medicine, Salt Lake City, UT, USA; ²ARUP Laboratories, Salt Lake City, UT, USA

Contributions: (I) Conception and design: EM Summers, TB Martins, HR Hill, RD Sontheimer; (II) Administrative support: EM Summers, GC Coman, NR Blickenstaff; (III) Provision of study materials or patients: EM Summers, TB Martins, HR Hill, RD Sontheimer; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Erika M. Summers, MD. Department of Dermatology, University of Utah School of Medicine, 30 North 1900 East #4A 330, Salt Lake City, UT 84132, USA. Email: erika.summers@hsc.utah.edu.

Background: Identifying the drug(s) responsible for drug-induced chronic eczematous eruptions of aging individuals (CEEAA) is a clinical challenge in patients on multiple medications. Reliable *in vitro* testing methods and biomarkers are needed to identify the causative agent and allow simultaneous assessment of T-cell responses to multiple drugs being taken concurrently. This study examined the feasibility of using *in vitro*, drug-specific T cell activation responses as a biomarker for drug-induced CEEAA.

Methods: This was a single center, proof-of-concept pilot study at the University of Utah Hospital, Salt Lake City, Utah. Eight aging study subjects having a history suggestive of chronic eczematous drug eruptions suspected to have resulted from calcium channel blocker (CCB) and/or hydrochlorothiazide (HCTZ) hypersensitivity plus three matched aging control subjects were identified. Drug patch testing for CCB and/or HCTZ, *in vitro* drug antigen-induced lymphocyte proliferation assays, and multianalyte-determined cytokine release assays were performed before and after HCTZ and/or CCB incubation.

Results: All study and control subject blood samples tested failed to demonstrate detectable enhanced lymphocyte proliferation or cytokine release to *in vitro* CCBs or HCTZ challenge when tested with a fairly wide range of drug concentrations. Additionally, none of the enrolled patients developed a positive patch test to CCBs and/or HCTZ.

Conclusions: This pilot study aimed to correlate *in vitro* drug-induced T lymphocyte transformation and cytokine production with the presence of drug-induced CEEAA. Failure to identify T cell proliferative responses to CCB drug antigens in our *in vitro* studies could have, in part, resulted from a pharmacologic inhibiting effect of CCB on T cell activation.

Keywords: Chronic eczematous eruptions in aging individuals; delayed hypersensitivity reactions (DHR); calcium channel blockers (CCBs); hydrochlorothiazide (HCTZ); lymphocyte proliferation assay; cytokine release assay; T cell proliferative response; drug reaction; eczematous drug eruptions

Submitted May 24, 2017. Accepted for publication Jun 05, 2017.

doi: 10.21037/atm.2017.07.16

View this article at: <http://dx.doi.org/10.21037/atm.2017.07.16>

Introduction

Chronic eczematous eruptions of aging individuals (CEEAA) (i.e., ≥ 50 years) has been a neglected area of clinical research in dermatology. CEEAA can result from aging-

onset atopic dermatitis, widespread contact dermatitis, cutaneous T cell lymphoma, prodromal/non-bullous pemphigoid, autoeczematization reaction/ID reaction and eczematous drug eruptions. Such eczematous drug eruptions are thought to result from T cell-mediated

delayed hypersensitivity reactions (DHR). Drug-induced DHR have been shown to account for approximately 15% of all adverse drug reactions and are of major medical significance (1).

Several aspects of drug-induced CEEA present considerable challenges to clinicians. Aging individuals are typically taking multiple prescription medications, over-the-counter medications, vitamins, and supplements on a daily basis. Thus, when a drug eruption develops in this setting, there is always uncertainty as to its specific cause. In addition, drug-induced CEEA can develop in patients who have been taking the triggering medication for years. Due to this potential delay in development of CEEA, patients and their healthcare providers often do not recognize a causal relationship between taking a medication and the development of a drug-induced CEEA.

In recent work, we confirmed the prior observations by Joly *et al.* indicating that CEEA can result from DHR to calcium channel blocker (CCB) medications. Our studies also indicated that hydrochlorothiazide (HCTZ) can trigger CEEA (2,3).

Aging individuals being treated with CCB and/or thiazide diuretics are frequently taking other medication classes as well. Identification of the drug(s) responsible for a cutaneous adverse reaction in these patients following onset of the drug eruption poses a significant clinical challenge. The current gold standard for DHR is drug withdrawal and re-challenge, however this method is inefficient for multiple medications and carries the risk of severe clinical reactions resulting from drug withdrawal in patients. Management of drug-induced CEEA would be greatly facilitated by a biomarker for drug class-specific CEEA to identify the offending medication causing the eczematous eruption. We chose to perform a pilot study to examine the feasibility of using *in vitro*, drug-specific T cell proliferative responses as a biomarker for drug-induced CEEA. We had hoped to use the results of this pilot study to recruit funding for a larger systematic study of this important clinical problem.

Methods

Ethical approval was obtained from the Institutional Review Board at the University of Utah for a single center, proof of concept pilot study. The initial design of this pilot study included ten study subjects and ten matched controls. Biopsy and billing records were searched in order to recruit study subjects and controls.

Study subjects

Inclusion criteria for study subjects were: (I) age 50 or older; (II) a symmetrical eczematous eruption; (III) the eruption evolved continuously or recurrently during a period of at least 3 months before the study period; (IV) affected individuals had taken either a CCB and/or HCTZ for at least 2 months preceding the onset of the eruption; (V) skin biopsy for routine hematoxylin-eosin examination showed presence of spongiotic dermatitis with eosinophils in the inflammatory infiltrate and/or the presence of overlapping elements of other inflammatory histologic patterns; (VI) skin biopsy from direct immunofluorescence (if available) was negative for bullous pemphigoid. Exclusion criteria for study subjects were: (I) a history of atopic dermatitis; (II) leukopenia; (III) systemic immunosuppressive therapy within the 4 weeks prior to initiation of the study; (IV) congenital or acquired immunodeficiency disorders.

Controls

Control subjects were gender and age-matched (± 5 years) to a respective study subject. Inclusion criteria for control subjects also included current intake of a CCB or HCTZ, with medication class matched to a respective study subject. Exclusion criteria included the same as those for the case subjects, as well as manifestation of any type of eczematous eruption.

Protocol

Eight study subjects having a history suggestive of chronic eczematous drug eruption suspected to have resulted from CCB and/or HCTZ hypersensitivity plus three control subjects were initially identified. All participants provided their written consent to participate in the study. Following enrollment, patients underwent venipuncture and drug patch testing for either CCB and/or HCTZ.

Purified formulations of HCTZ and the CCB medications were obtained from Sigma Aldrich®. Drug concentrations of 1%, 10%, and 30% in petrolatum were compounded and applied to the medial upper arms of the participants. Patch test readings were performed on day 2 and either day 4 or 5.

In vitro drug antigen-induced lymphocyte proliferation assays and multiplexed-determined cytokine/marker release assays for interferon (IFN)- γ , interleukin (IL)-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-17,

tumor necrosis factor (TNF- α), cytokine receptor IL-2r, and soluble CD40 ligand (sCD40L) were performed on HCTZ and/or CCB-stimulated and unstimulated cells after five days of incubation at 37 °C with 5% CO₂. Purified formulations of HCTZ and amlodipine besylate were obtained from Sigma Aldrich®, St. Louis, MO and were used for cell challenge. Inhibition studies were performed on control subjects, in which drugs were titrated in two-fold dilutions in cell culture media from 500 to 15.6 ng/mL, adding 100 μ L of each dilution per well based on previous studies (4,5). For the study subjects, amlodipine besylate was titrated in cell culture media from 31.25 to 1,000 ng/mL, and HCTZ was titrated from 718 to 50,000 ng/mL. Phytohemagglutinin (PHA) was included as a positive stimulation control for the lymphocyte proliferation assays. Inhibition of lymphocyte proliferation and cytokine release by the drugs themselves was determined by spiking control and patient cells with PHA alone, or PHA plus the drugs. Participants were also screened for BP230 and 180 antibodies by ELISA. Total serum IgE concentrations were determined using ImmunoCAP 1000 instrument (Phadia Uppsala Sweden) (6).

In vitro stimulation assays

Peripheral blood mononuclear cells (PBMC's) were obtained from anticoagulated peripheral blood by Ficoll-Paque density gradient centrifugation. The cells were counted and adjusted to a concentration of 1×10^6 /mL in RPMI 1640 medium containing 10% heat-inactivated pooled normal human serum, antibiotic/antimycotic, and L-glutamine. Cell cultures were set up in 96-well microtiter plates using 100 μ L (1×10^5 PBMC's/well). PBMC's were stimulated with PHA as a positive control, and cells in medium alone as a background/endogenous stimulation control. Cells were cultured for 3 and 5 days at 37 °C with 5% carbon dioxide, after which the plates were either pulsed with titrated thymidine for lymphocyte proliferation studies or the supernatant was harvested and stored at -20 °C until cytokine studies were performed.

Multiplexed cytokine assay

A multiplexed fluorescent microsphere immunoassay system was developed in house and utilized in this study to simultaneously assess the concentrations of 14 cytokines/markers including IFN- γ , IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, p70, IL-13, IL-17, TNF- α , cytokine

receptor IL-2r, and sCD40L in only 100 μ L of tissue culture supernatant and 75 μ L serum sample using optimized protocols for each sample type (7-9).

Results

A total of eight study subjects and three control subjects were enrolled. All study and control subject blood samples tested failed to demonstrate any detectable enhanced *in vitro* lymphocyte proliferation or cytokine release following challenge with CCBs or HCTZ, even when tested with a wide range of drug concentrations. In addition, none of the eight study subjects and three control subjects tested developed a positive patch test to CCBs and/or HCTZ. As this was a pilot study, we chose to discontinue enrolling study and control subjects given the uniformly negative T cell stimulation results. Even if positive results had been obtained with the additional study and control subjects according to initial study design, the pilot study would still have ended with negative results overall.

Inhibition studies were performed to determine the non-specific effects of the included drugs on *in vitro* cell proliferation and cytokine stimulation. The amount of inhibition due to the drug compared to PHA alone is shown in Table 1. HCTZ showed no inhibition up to 50,000 ng/mL, which is 100 times greater than peak physiologic blood concentrations. Amlodipine showed significant inhibition at a concentration of 500 ng/mL. Cytokine responses were also similarly suppressed at these same concentrations (data not shown).

One study patient tested positive for BP 180 antibody, indicating an underlying possible diagnosis of eczematous bullous pemphigoid (Table 2). However, bullous pemphigoid autoantibodies detected by solid phase immunoassay can be seen in clinical settings other than bullous pemphigoid.

Three study subjects had an elevated blood IgE level suggesting the possibility of aging onset atopic dermatitis rather than drug-induced CEEA (Table 2). However, two control subjects also had elevated levels of IgE.

Discussion

Dermatologists are frequently challenged with identifying the drug(s) responsible for adverse cutaneous reactions in patients taking multiple medications. The current gold standard for diagnosing drug eruptions in this setting is drug withdrawal and re-challenge. This method is inefficient for patients on numerous medications and increases risk

Table 1 Drug inhibition of lymphocyte proliferation

Drug	Drug class	Drug concentration (ng/mL)	Inhibition* (%)
Hydrochlorothiazide	Diuretic	≤50,000	0
Amlodipine	CCB	500	99.8
		250	95
		125	16
		≤62.5	0

*, percent inhibition of Drug + phytohemagglutinin (PHA) to PHA alone.

Table 2 IgE levels and BP antibody levels

Patient	Age (years)	Gender	Total IgE (IU/mL)*	BP 180/230 antibodies (units)**
1	78	F	828	0/0
2	77	M	<2	0/0
3	78	F	5	0/0
4	72	M	590	0/0
5	68	M	59	(+) 47/0
6	75	F	10.4	0/0
7	90	M	1,971	0/0
8	65	M	8.99	0/0
Control 1	76	M	292	0/0
Control 2	68	M	253	0/0
Control 3	70	F	65.6	0/0

*, normal ranges for adults (≥18 yrs) are <214 IU/mL; **, IgG BP 180 and 230 antibody reference range: positive if ≥9 units; negative if <9 units.

in patients who may need the discontinued drug(s). Other diagnostic procedures, including patch testing, prick testing, and intracutaneous prick testing have proven to have a low sensitivity for detection of T cell-mediated drug eruptions. Lymphocyte transformation testing (LTT) and *in vitro* drug-induced T cell cytokine production studies have shown promise in the detection of T cell sensitization in a wide variety of drug eruptions (4,10,11). Our experimental study protocol for drug-induced CEEA was designed using the approaches that were employed in these earlier studies.

Our pilot study was designed to test the hypothesis that CEEA occurring in individuals taking CCB or HCTZ have circulating T cells that can be shown to display specific proliferative responses to the drug antigenic structures. We had also hoped to develop a clinically useful *in vitro* assay that could efficiently confirm a drug-specific cause for

CEEA occurring in individuals on multiple medications. Unfortunately, our pilot study was unsuccessful in this regard.

The *in vitro* lymphocyte transformation assays and T cell cytokine release assays employed in this study were the same as those used in the Cellular and Innate Immunology Laboratory of ARUP Laboratories, a national reference laboratory. These assays are routinely standardized with the appropriate positive and negative stimuli. It was unfortunate that none of the eight CEEA study subjects displayed a positive drug patch test. Negative or positive *in vitro* lymphocyte transformation assays and T cell cytokine release assays would have been more informative in a patient with a positive drug patch test.

Although prior studies have shown an association between CEEA and CCB and HCTZ medications, it is plausible that these medications are not truly causative in

our study (2,3). A weakness of our study was that we did not re-challenge study subjects with CCBs or HCTZ to confirm drug causation. One of our study subjects tested positive for bullous pemphigoid autoantibodies, raising the possibility that the chronic eczematous eruption in this setting was not caused by an eczematous drug eruption. However, as previously noted, circulating bullous pemphigoid autoantibodies detected by solid-phase immunoassay are not specific for bullous pemphigoid. Also, it is possible that some of our study subjects may have been suffering from aging-onset atopic dermatitis, chronic contact dermatitis, or autoeczematization/ID reaction rather than eczematous drug eruptions. Several of our study subjects and control subjects did have elevated IgE blood levels. However, none of the study or control subjects had *in vitro* drug-induced positive T-cell proliferative responses/cytokine production or drug patch tests, so the question of aging-onset atopic dermatitis being a confounder in our study is moot.

Additionally, our identification of study participants was based upon skin biopsy for routine hematoxylin-eosin examination showing presence of spongiotic dermatitis with eosinophils in the inflammatory infiltrate and/or the presence of overlapping elements of other inflammatory histologic patterns. Seitz *et al.* recently performed a study in which 91 patients with skin biopsy specimens suggestive of an acute exanthem temporally related to an identifiable medication were allergy tested to the possible offending medication (12). Prick testing, intradermal prick testing, LTT, and provocation testing excluded drug hypersensitivity in 56 of the patients and confirmed non-IgE-mediated drug hypersensitivity in 35 of the patients (positive predictive value of 40.7%). The authors concluded that histopathology is not useful in differentiating drug-mediated exanthems from non-drug-mediated exanthems. Thus, it is plausible that many of the participants we identified by dermatopathologic evaluation of skin biopsy specimens did not truly have a drug-mediated eczematous eruption.

Despite our negative findings, case reports and general clinical experience support the hypothesis that a number of drug classes are capable of inducing eczematous drug eruptions in all age groups. In addition, there is a growing body of evidence supporting the idea that different drug classes, including CCBs and HCTZ, can induce other T cell-mediated inflammatory dermatoses such as subacute cutaneous lupus erythematosus (13-16).

There are other ways to consider the negative results

of our pilot study. Due to their pharmacologic effects on intracellular calcium signaling, at certain concentrations CCB are known to inhibit mitogen-induced T cell activation *in vitro* (17,18). Since *in vitro* mitogen-induced T cell proliferative responses are typically stronger/higher than antigen-induced T cell responses, it would seem safe to assume that drug antigen-specific T cell proliferative responses would also be suppressed at the same concentrations of CCB. Also, given that calcium flux is essential for lymphocyte proliferation and cytokine release, it is unlikely that CEEA from a CCB would be due to a T cell-mediated process.

CCB (and/or their metabolites) at therapeutic dosing levels are not known to be clinically immunosuppressive. That is to say, CCB at therapeutic doses in humans do not result in an observable decrease in resistance to opportunistic infection nor an increase in opportunistic malignancies. Supporting this is the fact that there are no warnings of immunosuppression as an adverse effect in the package inserts of the individual drugs in the CCB class. However, it has been suggested that CCBs used in conjunction with clinically significant immunosuppressives, such as cyclosporine, may provide a mild additive immunosuppressive effect (19,20).

The concentrations of CCB examined in our study subjects ranged from 31.25 to 1,000 ng/mL. Koike *et al.* reported that after oral administration of a therapeutic dose of verapamil in humans, the peak serum concentration was 219.09 ng/mL on average (21). Other pharmacokinetic studies have documented that steady-state blood levels of CCB after therapeutic dosing in humans is in the 100–300 ng/mL range (22–24). Our *in vitro* experimental conditions employed therapeutic concentrations of CCB and demonstrated inhibition of *in vitro* cell proliferation and cytokine responses only at the upper levels of physiological blood concentrations. Thus, our negative results were not entirely due to CCBs pharmacologically blocking *in vitro* T cell responses.

It has been suggested that topical application of pharmacological concentrations of CCB may have a local immunosuppressive effect in the skin (25). However, previous work has suggested that drug patch testing with CCBs is feasible. Girardi *et al.* reported a positive drug patch test to diltiazem in a patient with diltiazem-induced acute generalized exanthematous pustulosis (26). This argues against the possibility that percutaneous penetration of the CCB drug applied topically under occlusion in very

high concentrations (10–30% in petrolatum) in the patch test protocol produced a local immunosuppressive effect on the cutaneous DTH response.

Given that CCBs are extensively metabolized by first pass hepatic metabolism, it is also possible that CCB metabolites are the pathogenic mediators responsible for drug-induced chronic eczematous reactions. In addition, CCB and HCTZ pills/capsules can contain dye chemicals that have been documented to be responsible for drug-induced hypersensitivity clinical phenomena. This hypothesis was not tested in our studies. Future studies in this area might include testing for dye hypersensitivity and utilize hepatic microsomal systems to “pre-metabolize” the parent drug *in vitro* prior to testing for drug antigen-specific T cell memory responses. We would propose that such studies exclude subjects having any evidence of prodromal/non-bullous bullous pemphigoid and aging-onset atopic dermatitis as reflected by elevated blood IgE levels.

Finally, it can be argued that by testing circulating T cells in our pilot study, we were not testing the most skin disease-relevant subpopulation of T cells. It has been reported that the human body contains 10–20 billion resident memory T cells having a skin-homing phenotype (CD45RO/CLA/CCR4) (27). There are 20-fold more T cells with this skin-homing phenotype in the skin than in blood. Skin-homing T cells consist of a mix of helper, cytotoxic, and regulatory subsets. It is quite possible that the subpopulation of memory T cells reactive with CCB and/or HCTZ antigens might be diluted beyond the point of detection with available clinical immunology techniques in peripheral blood. It is also not yet known whether the antigen-specific reactivity of such skin resident T memory cells can be assayed by conventional drug patch testing.

It was our hope that publication of the negative results of this pilot study might be of value in guiding others in the future who decide to try to move this important area of clinical research forward. As a note of caution, care should be taken in selecting the *in vitro* assay concentrations of CCB for T cell stimulating studies. CCB concentrations higher than therapeutic levels in human plasma (100–300 ng/mL) could be expected to have a broad inhibiting effect on T cell proliferation due to their impact on intracellular calcium signaling.

Porebski and colleagues have recently suggested that a single parameter of *in vitro* T cell activation may not be adequate in identifying a drug-induced DHR in human skin (28). In future studies of CCB-induced T cell activation within this context, appropriate care should be taken to examine

in vitro CCB drug antigen concentrations within the *in vivo* CCB therapeutic blood level range.

Acknowledgements

The authors would like to express their appreciation to Dr. Gerald J. Gleich, M.D., University of Utah, Department of Dermatology and Dr. Douglas L. Powell, M.D., University of Utah, Department of Dermatology, for their thoughtful guidance on study methods at the beginning of this study. We also would like to thank Colby Bingham, M.D., University of Missouri, Department of Dermatology, for his assistance in acquisition of data. These individuals did not receive compensation for their help.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: Ethical approval was obtained from the Institutional Review Board at the University of Utah for a single center (No. IRB_00044966), proof of concept pilot study.

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Cite this article as: Summers EM, Blickenstaff NR, Coman GC, Martins TB, Hill HR, Sontheimer RD. A pilot study evaluating biomarker development for drug-induced chronic eczematous eruptions of aging individuals. *Ann Transl Med* 2017;5(20):393. doi: 10.21037/atm.2017.07.16