

Flow Cytometric Analysis of Regulatory T Cells During Hyposensitization of Acquired Allergic Contact Dermatitis

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Background: We previously demonstrated that repeated intradermal steroid injections administered at weekly intervals into positive patch-test sites induce hyposensitization and desensitization.

Objective: To examine changes in CD4⁺CD25⁺CD127lo/− regulatory T cells during the attenuation of the patch-test response.

Methods: Ten patients with known allergic contact dermatitis were patch tested weekly for 10 weeks. The patch-test site was injected intradermally with 2 mg triamcinolone. At weeks 1 and 7, a biopsy was performed on the patch-test site in 6 patients, and flow cytometry was performed assessing CD4⁺CD25⁺CD127lo/− regulatory T cells. Secondary outcomes were clinical score, reaction size, erythema, and temperature. Statistical analysis included regression, correlation, and repeated-measures analysis of variance.

Results: The percentage of CD4⁺CD25⁺CD127lo/− regulatory T cells, measured by flow cytometry, increased from week 1 to week 7 by an average of 19.2%. The average grade of patch-test reaction decreased from +++ (vesicular reaction) to ++ (palpable erythema). The mean drop in temperature following treatment was 0.28°C per week. The mean area decreased 8.6 mm²/wk over 10 weeks.

Conclusions: Intradermal steroid injections of weekly patch-test reactions resulted in hyposensitization of the allergic contact dermatitis reaction. CD4⁺CD25⁺CD127lo/− regulatory T cells showed a tendency to increase; however, further studies are needed to determine if this is significant.

The development of allergic contact dermatitis (ACD) is divided into 2 stages, sensitization and elicitation. During sensitization, the allergen is taken up and presented to effector CD8⁺ and CD4⁺ T cells. During elicitation, the sensitized individual is re-exposed to the allergen, and the specific effector T cells induce an inflammatory reaction. Regulatory T cells suppress the expansion and function of effector T cells.¹ Regulatory T cells have been classically divided into 2 populations of cells, namely, natural and inducible regulatory T cells. Natural regulatory T cells are produced in the thymus and express high levels of the interleukin 2 receptor α chain (CD25) and the transcription factor forkhead box P3 (FOXP3).² Inducible regulatory T cells arise from naive T cells in the periphery because of the cytokine environment present and antigenic stimuli

and include the interleukin 10–producing CD4⁺ cells (Tr1) and T_H3 cells.³ Foxp3 is considered the definitive marker of natural regulatory T cells, which are thus defined by their phenotype as CD4⁺CD25⁺Foxp3⁺ regulatory T cells.³

Many studies have highlighted the role that CD4⁺CD25⁺Foxp3⁺ regulatory T cells play in promoting tolerance to allergens in ACD. Gomez de Agüero et al⁴ demonstrated that tolerance induction to 2,4-dinitrothiocyanobenzene in a murine model of contact hypersensitivity relies on Langerhans cells activating CD4⁺CD25⁺Foxp3⁺ regulatory T cells. Luckey et al⁵ induced tolerance to contact allergy in a murine model and discovered that this coincided with increased numbers of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in the draining lymph nodes of mice. The same study demonstrated that depletion of CD4⁺CD25⁺Foxp3⁺ regulatory T cells impaired the development of tolerance and led to sensitization resulting in severe contact dermatitis. These studies indicate that CD4⁺CD25⁺Foxp3⁺ regulatory T cells are crucial for tolerance development, and in their absence sensitization occurs and contact dermatitis develops. Vocanson et al⁶ demonstrated that CD4⁺CD25⁺Foxp3⁺ regulatory T cells suppress hapten-reactive CD8⁺ T cells in vivo and in vitro models of contact hypersensitivity. This suggests that CD4⁺CD25⁺Foxp3⁺ regulatory T cells have the ability to suppress the CD8⁺ T-cell effector response after sensitization. Park et al⁷ found that, in a mouse model of contact hypersensitivity,

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pretreatment with the antioxidant heat shock protein 70 augmented the resolution of contact dermatitis by increasing the number of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. This indicates that CD4⁺CD25⁺Foxp3⁺ regulatory T cells may dampen the inflammatory reaction associated with the elicitation phase of ACD. Deane et al⁸ demonstrated that CD4⁺Foxp3⁺ regulatory T cells bind to intercellular adhesion molecule 1 in the dermal vessels, which attenuates the adhesion of neutrophils and CD8⁺ cells to the vessels, thus decreasing the level of proinflammatory leukocytes that are recruited to the site of challenge in a murine model of contact hypersensitivity. Collectively, these studies have demonstrated that CD4⁺CD25⁺Foxp3⁺ regulatory T cells promote tolerance induction to allergens, suppress the effector response in primed CD8⁺ T cells, decrease proinflammatory cell migration, and augment the resolution of inflammation in ACD, thus indicating that CD4⁺CD25⁺Foxp3⁺ regulatory T cells exert their effects on tolerance induction during both the sensitization and elicitation steps of ACD development.

In view of the fact that Foxp3 is located within the cytoplasm or nucleus in natural regulatory T cells, an alternative cell surface marker has been identified to use as surrogate. Liu et al⁹ demonstrated that CD127 is an excellent marker of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in the human blood, as Foxp3 interacts with the promoter region of CD127, resulting in its reduced expression. Shen et al¹⁰ isolated CD4⁺CD25⁺CD127lo/− T cells from peripheral blood of patients with gastric cancer and demonstrated that 88.1% to 96.1% expressed Foxp3. Since its discovery, CD4⁺CD25⁺CD127lo/− cells have been used to isolate and measure natural regulatory T cells in numerous reports in the literature.^{11–14} Furthermore, the steps involved in Foxp3 staining may reduce the signal of other antibodies used.¹⁵ For these reasons, we used CD127 as a substitute marker for Foxp3, and identified CD4⁺CD25⁺CD127lo/− regulatory T cells as a proxy for CD4⁺CD25⁺Foxp3⁺ regulatory T cells, also known as natural regulatory T cells. For the remainder of this article, natural regulatory T cells will be defined as CD4⁺CD25⁺CD127lo/− regulatory T cells.

In a previous study of 10 patients with ACD, repeated patch tests followed by intralesional steroid injections of 4 mg of triamcinolone acetate were able to desensitize 3 patients and attenuate the reaction in an additional 6 patients.¹⁶ It is hypothesized that the observed attenuation of ACD reactions is due to expansion of natural regulatory T cells in the skin. This study aims to assess the expansion of CD4⁺CD25⁺CD127lo/− regulatory T cells during hyposensitization and desensitization of ACD.

Throughout this article, desensitization will refer to a reversal of a prior state of sensitivity to nonreactivity and hyposensitization to a state of lessened reactivity. In all cases, this refers to the patch-test site and does not refer to a clinical state, although this would be surmised.

ETHICS APPROVAL

Ethics approval for this study was obtained from the University of Saskatchewan Research Ethics Board.

METHODS

Patient Selection

Patients were identified through the patient database in the Division of Dermatology. Inclusion criteria were disease duration of at least 6 months, previously active ACD causing self-reported functional impairment, age older than 18 years, and ability to provide informed consent. Exclusion criteria included patients with active ACD, immunosuppressive therapy, significant psychiatric comorbidity, malignancy, or bleeding disorder.

Ten patients who met the eligibility criteria were recruited and form the cohort for the present study. The significance of the allergens was assessed based on the clinical presentation of contact dermatitis, possible exposures, and the patient's occupational history.

Intervention

At the first appointment, subjects were patch tested to the previously identified specific antigen (Table 1). The patch was removed at 48 hours, and the patch-test sites assessed at 96 hours. A preliminary reading was performed (baseline). Following these measurements, a 4-mm skin punch biopsy was taken from the center of the positive test site in 6 of the 10 patients who consented to the biopsy. On successive weeks, for 10 weeks, the patch test was repeated at a different site on the back, and the patch was assessed at 96 hours. On each occasion, the patch-test site was injected intradermally with 2 mg of triamcinolone acetonide. The skin biopsy for flow cytometry was repeated at 7 weeks in the same 6 of 10 patients who gave a biopsy during week 1.

Assessment Measures

These were the same as were used in our previous study and included clinical assessment, spectrophotometric estimation of the erythema, and infrared assessment of heat generation.

Clinical Assessment

Rating of the patch-test reaction was assessed using the International Contact Dermatitis Research Group scoring system,¹⁷ by the following criteria:

- (a) + denotes reactions with only erythema
- (b) ++ denotes reactions with palpable erythema
- (c) +++ denotes a vesicular reaction

The size of the reaction was measured weekly based on the diameter in the horizontal and vertical axes.

Spectrophotometric Estimate of Erythema

The degree of erythema was measured weekly using the Minolta CR 200 spectrophotometer (Konica Minolta Optics, Inc, Osaka, Japan).¹⁸ The values obtained were a*, which assesses the red-green color axis, and L*, which assesses lightness.

TABLE 1. Contact Allergen, Affected Site, Exposure Source, and Occupation

Patient	Allergen	Presentation	Exposures	Occupation	Work-Related Exposure
1	Formaldehyde 2%	Hand dermatitis	Hand soap, hand sanitizer containing formaldehyde	Nurse	Yes
2	4-Phenylenediamine	Scalp dermatitis	Hair dye containing 4-Phenylenediamine	Electrician	No
3	Nickel	Hand dermatitis	Soaps, coins, keys: nickel release from coins, keys	Sales/management	No
4	Hexahydro-1,3,5-triahydroethyltriazine	Eyelid dermatitis	Cosmetics containing hexahydro-1,3,5-triahydroethyltriazine	Pharmacist	No
5	4-Phenylenediamine	Scalp dermatitis	Hair dye containing 4-phenylenediamine	Nurse	No
6	Formaldehyde 2%	Hand dermatitis	Hand sanitizer containing formaldehyde	Technologist	No
7*	4-Phenylenediamine	Scalp dermatitis	Hair dye containing 4-phenylenediamine	Aesthetician	No
8	Ethyl acrylate	Hand dermatitis	Gel nails containing acrylates	Owner of hair salon	Yes
	Kathon CG	Hand dermatitis	Shampoo, conditioner, hair -styling products containing Kathon CG		Yes
9	Nickel	Axillary dermatitis	Natural antiperspirants, lotions: unknown ingredient list, nickel	Social worker	No
10	Carba mix	Hand dermatitis	Medical gloves containing carba mix	Physician	Yes

Measure of Heat Generation

The measurement of heat generation was assessed using the FLIR i3 infrared camera (FLIR Systems Inc, Boston, Mass).¹⁹ A standard distance of 1 m from the subjects was used for measurement, and ambient room temperature was controlled for.

Flow Cytometry

The 4-mm skin biopsies were processed as previously described to isolate cells.²⁰ Tissue was incubated for 3 hours with 2% collagenase 1A (Sigma, St Louis, Mo) in 37°C and 5% CO₂. The biopsies were mechanically disaggregated using the BD Medimachine (BD, Franklin Lakes, NJ) and filtered to isolated cells within the skin. Sufficient cells for flow cytometry were obtained in 5 of the 6 patients. The cells were stained with CD3, CD4, CD127, CD25, and 7-AAD antibodies (Beckman Coulter, Inc, Fullerton, Calif).¹⁵ Viable regulatory T-cell populations were gated on by isolating the 7-AAD- CD3⁺ CD4⁺ CD25⁺ CD127lo/- population of cells.

Data Analysis

To analyze patterns, trend analysis of least-squares regression fitted to our data with the correlation is depicted in the graphs (Figs. 1 and 2). A linear model fit for each graph was chosen. To investigate changes in temperatures, area, a*, and L* over 10 weeks, a repeated-measures analysis-of-variance procedure with Mauchly's

test of sphericity was used. We assumed that observations within a treatment are independent, and first-week measurements were taken as baseline. Little's Missing Completely at Random test was performed to determine whether the data are missing completely at random. Expected maximization method was used for imputation (to populate the missing values). Analysis was undertaken using SPSS Statistics (version 20; IBM, New York, NY).

RESULTS

Clinical Relevance

The clinical relevance of the allergens, as it related to the patient's presentation of dermatitis, possible exposures, and occupational

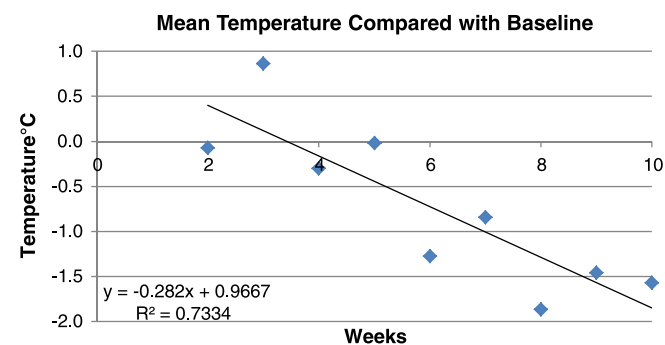


Figure 1. Linear regression analysis indicating a reduction in irradiated heat as compared to baseline for each week of treatment.

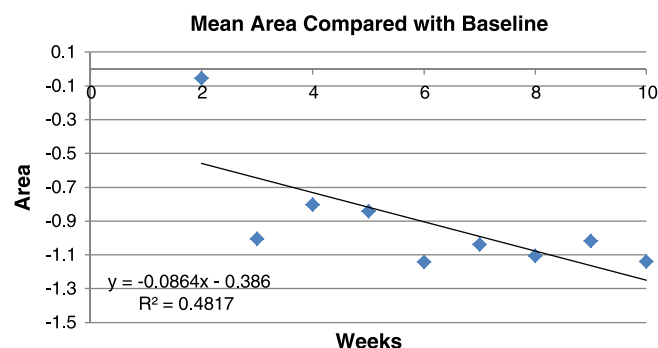


Figure 2. Linear regression analysis of average reaction size (area in mm²) compared to baseline for each week of treatment.

history were assessed (Table 1). Eight of the 10 patients had an obvious relationship between the allergen and exposures to allergen-containing products at the location of the presenting dermatitis. Patients 3 and 9, both nickel allergic, did not have a clear correlation between the allergen, exposures, and the resultant dermatitis. Four of the 10 patients had allergens that were related to occupational exposures. Hand dermatitis was the most common presenting complaint, with 6 of the 10 patients having hand involvement.

Clinical Analysis

The disease-specific antigen for each patient, the duration involved in the study, and the reactions elicited in the initial patch testing(s) and final patch testing are presented in Table 2. Of the 10 patients, 9 were tested to 1 allergen, and 1 patient (patient 7) received patch tests to 2 separate allergens. Eight patients completed the 10-week course, and 2 patients completed only 9 weeks. The reasons for early completion included no change in reaction (patient 9) and loss to follow-up (patient 2). One patient had a decrease in clinical assessment of reaction from +++ to + over 10 weeks. Six patients had a decrease in clinical assessment from +++ to ++. The patients who experienced a reduction of clinical assessment also noted

TABLE 2. Maximum and Final Clinical Assessments of the Patch Test Response

Patient	Allergen	Duration in Study, wk	Original Assessment	Final Assessment
1	Formaldehyde 2%	10	+++	++
2	4-Phenylenediamine	9	+++	++
3	Nickel	10	+++	+
4	Hexahydro-1,3,5-triahydroethylthiazine	10	++	++
5	4-Phenylenediamine	10	+++	+++
6	Formaldehyde 2%	10	+++	++
7*	4-Phenylenediamine	10	+++	++
	Ethyl acrylate	10	+++	++
8	Kathon CG	10	+++	++
9	Nickel	10	+++	++
10	Carba mix	9	++	++

decreased pruritus at the site of the patch-test reaction. Three patients experienced no change in patch-test reaction clinical assessment.

Objective Measures

Reaction Size: Area

Linear regression analysis of average reaction size (area in mm²) indicates that there was a statistically significant ($P < 0.05$) decrease in the area of the patch-test reaction following treatment with triamcinolone compared with baseline with a moderate correlation ($R^2 = 0.48$). On average each week, area decreased by 8.6 mm² (Fig. 2).

Irradiated Heat: Temperature

Linear regression analysis indicates that there was a significant reduction of irradiated heat following triamcinolone injection compared with baseline ($P < 0.05$) with a strong correlation ($R^2 = 0.73$). On average each week, temperature decreased by 0.28°C (Fig. 1).

Color Change: Spectrophotometric Analysis of “a*” (Red-Green Axis)

Linear regression analysis of a*, which reflects the value of the reaction on the red-green color axis, was performed comparing weekly treatments with triamcinolone to baseline values. These values were not significant ($P > 0.05$) with nearly nonexistent correlation ($R^2 = 0.03$). However, on average, a* decreased by 0.05 units each week.

Spectrophotometric Analysis of L* (lightness)

Linear regression analysis of L*, which reflects the lightness of the reaction, was performed comparing weekly treatments with triamcinolone to baseline values. These values were not significant ($P > 0.05$) with a poor correlation ($R^2 = 0.25$). Each week, L* on average increased by 0.22 units.

Flow Cytometry

Punch biopsies were taken from 6 patients at weeks 1 and 7. Of these, 5 harvested enough cells to run flow cytometry. Viable regulatory T cells were selectively gated on, and the percentage of these cells in comparison to the total T-cell population was assessed. Week 1 served as the baseline amount of regulatory T cells present in the patch-test reaction. Week 7 was selected as the second reading based on previous results, indicating that the weekly treatment of triamcinolone was effective at this time point.¹⁶ The percent change of regulatory T cells within the punch biopsy of the patch-test reaction site, as measured by flow cytometry, increased from weeks 1 to 7 by an average of 19.2%. This value was not statistically significant ($P < 0.05$) (Fig. 3).

Subanalysis at Week 8

Results calculated at week 8 show the temperature decreasing by 0.35°C/wk ($R^2 = 0.70$). Area decreased by 12.7 mm²/wk ($R^2 = 0.54$),

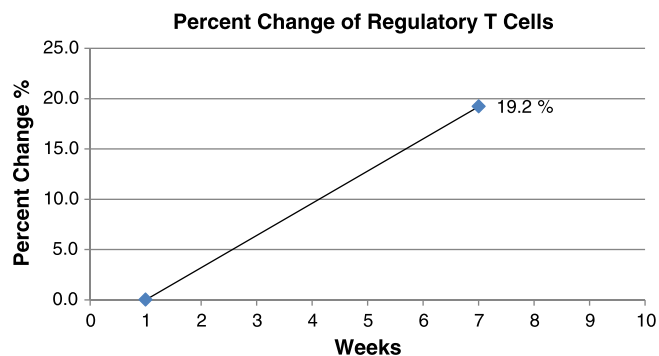


Figure 3. Mean percent change in regulatory T cells—baseline to 7 weeks.

a* by 0.09 unit/wk ($R^2 = 0.040$), and L* increased by 0.33 unit/wk ($R^2 = 0.26$).

DISCUSSION

Allergic contact dermatitis in the population is rising in prevalence worldwide.^{21,22} An estimated 20% of the population suffers from contact allergies.²³ Occupational contact dermatitis is one of the most common workplace diseases reported in many countries.²⁴ This can have far-reaching implications for the careers of those affected, including permanent disability and change of vocation.²⁵ Currently, the mainstay of treatment of ACD is allergen avoidance. Desensitization has been previously attempted by a variety of mechanisms, including oral tolerance induction, repeated cutaneous exposures, and subcutaneous injections of allergen.^{26–28} Hardening has seldom been described.²⁹ Where hardening has developed, the hyposensitivity induced has almost always been associated with either oral ingestion of the antigen and/or perhaps even inhalation.^{30,31} Kligman,³² in an attempt to induce hyposensitization to poison ivy, did indeed show that repeated topical application of PDC (3-n-penta-decylcatechol) could lead to a hyposensitized state. Initially, after 15 to 25 daily patches, the trend was toward greater reactions. Hyposensitization was achieved with increasing dosages of PDC and only after 300 applications and 9 to 11 months. The test reactions at this point were described as having “no more reaction than the original patch tests.”³² Was this then really hyposensitization?

In our previous study, repeated patch-test application followed by intralesional steroid injections showed great promise in being able to desensitize and hyposensitize patients to their contact allergies.¹⁶

This small, open study again showed a decreased reactivity at the repeat patch-test sites. Six of the 10 patients in the study experienced a decrease in the clinical assessment of their patch-test reaction following weekly repeated patch testing and intralesional steroid injections. Irradiated heat, a measure of inflammation, and patch-test reaction size decreased significantly over the course of the study. In this study, in contrast to our previous study, none

achieved negative patch tests. However, it should be noted that the dose of triamcinolone used was reduced from 4 mg in the previous study, to 2 mg in this study, in order to prevent previously noted steroid-induced skin atrophy. In reducing the dose of steroid used, there was also a reduction in the efficacy of the intervention previously observed. This indicates that steroid-induced hyposensitization and desensitization of ACD may be dose dependent. We also noted that on average, reduction in area seems to be occurring by a greater magnitude earlier during treatment. Our results show that area decreases on average by 12.7 mm²/wk ($R^2 = 0.54$) for the first 8 weeks compared with an average reduction of 8.6 mm²/wk ($R^2 = 0.48$) for the 10 weeks. Similarly, temperature is decreasing on average by 0.35°C/wk ($R^2 = 0.70$) during the first 8 weeks and by 0.28°C/wk ($R^2 = 0.73$) during the 10 weeks of treatment. Thus, the effects of triamcinolone treatment seem to be targeting different aspects of inflammation as the duration of treatment progresses.

CD4⁺CD25⁺Foxp3⁺ regulatory T cells have previously been shown to decrease the inflammatory infiltrate and attenuate the effector T cell response in ACD.^{4–8} This leads to the hypothesis that these cells are responsible for the observed decrease in patch-test reaction following repeated patch testing and intralesional steroid injections. CD4⁺CD25⁺CD127lo/− regulatory T cells were identified by flow cytometry as a proxy for CD4⁺CD25⁺Foxp3⁺ regulatory T cells, also known as natural regulatory T cells. The overall percentage of CD4⁺CD25⁺CD127lo/− regulatory T cells, measured by flow cytometry, increased from week 1 to week 7 by an average of 19.2% following repeated triamcinolone injections after patch tests. This trend of increased CD4⁺CD25⁺CD127lo/− regulatory T cell was not statistically significant, which may be due in part to the small sample size ($n = 5$). The trend indicates that CD4⁺CD25⁺CD127lo/− regulatory T cells may play an important role in suppressing the inflammatory reaction in ACD and promoting hyposensitization and desensitization. There are several possible hypotheses to explain the nonsignificant increase in CD4⁺CD25⁺CD127lo/− regulatory T cells despite clinical attenuation of inflammatory responses. For example, Gomez de Agüero et al⁴ observed increased activation of natural regulatory T cells in the draining lymph nodes, by additionally staining for ICOS⁺ cells, but not greater overall numbers. Similarly, the patients in this article could have experienced an increase in activation, but not overall percentage of CD4⁺CD25⁺CD127lo/− regulatory T cells, which would have been missed by our flow cytometry assay. In addition, the assessment of natural regulatory T cells within the skin lesion may not depict the full environment in which these cells exert their effects. Luckey et al⁵ identified changes in the amount of natural regulatory T cells in the draining lymph nodes following tolerance induction in contact hypersensitivity. It could be hypothesized that these cells are exerting their tolerizing effect in the local lymph node, thus altering antigen presentation to effector T cells. In our study, changes in natural regulatory T cells in the local draining lymph node were not assessed, but could be crucial in causing the observed hyposensitization in this article.

Future directions include repeating the experiments with a larger number of participants to determine if the observed trends of increased CD4⁺CD25⁺CD127lo/− regulatory T cells are significant with a larger sample size. In addition, staining for ICOS⁺ cells could be added to the flow cytometric regimen, to determine if there is an increased percentage of activated natural regulatory T cells.

In conclusion, intradermal steroid injections, administered to the positive patch-test site and repeated weekly at new patch-test sites, reduce the patch-test site reaction and are associated with the trend of increased CD4⁺CD25⁺CD127lo/− regulatory T cells at that site.

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