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ORIGINAL ARTICLE

# Can the Cytokine Analysis of the Scales on Alopecic Patch Predict the Response to Diphenylcyclopropenone Treatment in Alopecia Areata Patients?

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**Background:** Contact immune modulating therapy with diphenylcyclopropenone (DPCP) is a topical treatment option for extensive alopecia areata (AA). Because the response to DPCP treatment varies according to the patient, and it takes several months to evaluate the clinical effectiveness of the treatment, it is necessary to identify the factors that can predict the prognosis of the disease while treating with topical DPCP. **Objective:** In this study, cytokine levels in the scales of alopecic patches were investigated to identify whether they could predict response to DPCP during the early treatment period. **Methods:** Scale samples were taken from the alopecic patches in eight AA patients at 1 week, 2 months, and 4 months after DPCP sensitization. The patients were divided into responders and non-responders according to the clinical responses of DPCP treatment. Interferon (IFN)-gamma, interleukin (IL)-2, IL-12 and IL-10 levels of the subjects were compared in several perspectives. **Results:** Cytokine levels after 1 week of DPCP sensitization showed no statistically significant difference between two groups. After 4 months of treatment, IFN-gamma levels were significantly lower in responders than in non-responders. **Conclusion:** The results of this study show IFN-gamma levels in the scales of alopecic patches might possibly reflect the clinical response in AA patients treated with DPCP. However, initial cytokine levels could not predict the treatment response. **(Ann Dermatol 30(2) 150～157, 2018)**

**-Keywords-**
Alopecia areata, Cytokines, Diphenylcyclopropenone

## INTRODUCTION

Alopecia areata (AA) is a common cause of hair loss and afflicts 1% to 2% of the population. If an alopecic patch is small, the lesion may regress spontaneously and generally responds well to treatment. However, alopecia totalis or generalized alopecia may resist treatment and exhibit a poor prognosis[1].

Treatment of AA mainly focuses on immune modulation using, intralesional steroid injection, oral steroid, topical immunotherapy, topical or systemic immunomodulator, or photochemotherapy[2-4]. Diphenylcyclopropenone (DPCP) contact immune modulating therapy is recommended as a topical treatment for extensive AA patients[5-7]. The effect of DPCP is dependent on disease extent and can be evaluated after three months to six months of therapy. DPCP treatment is effective in 39.5% to 83.3% of patients (mean, 70%)[8]. Because it takes several months to determine whether DPCP is clinically effective, considerable patience is required of patients and doctors, and thus, an early predictor of response to DPCP therapy would be useful for resolving this problem. It has been suggested changes in T cell function and cytokine secretion contribute to the pathogenesis of AA. In particular, cytokines associated with T helper 1 cells appear to play an important role[1,9]. Studies on the pe-

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ripheral blood of AA patients have shown interleukin (IL)-2, IL-12, interferon (IFN)-gamma, IL-13 and IL-17A are significantly increased and that transforming growth factor-$\beta$1 are decreased[10,11]. Because changes in cytokines during treatment seem to reflect clinical response to DPCP treatment, it was proposed cytokines be used for the clinical monitoring of the effectiveness of long term DPCP treatment[12]. Adhesive tape stripping of skin scales collection provides a non-invasive means of accessing cytokine levels[13,14], and some studies have been conducted to measure cytokine levels in stratum corneum taken from patients suffering atopic dermatitis or allergic contact dermatitis using adhesive tape (D-Squame®; CuDerm, Dallas, TX, USA)[15-17]. This method has the advantages of rapid, simple, non-invasive sampling as compared with skin biopsy. Although several many reports have been issued on serum cytokine analysis in AA, levels of cytokines in skin scales taken from alopecic patches have not been previously determined.

In this study, we collected skin scales from AA patients being treated with DPCP to investigate the differences of cytokine levels with time, and to compare differences between the cytokine levels of responders and non-responders during the course of DPCP treatment to identify a factor that could predict response to DPCP during the early treatment period.

## MATERIALS AND METHODS

### Subjects

The study was performed on eight AA patients who visited the Department of Dermatology at Inha University Hospital (Incheon, Korea) between January 1, 2013 and May 31, 2015. This study was approved by the institutional review board of Inha University Hospital (IUH-IRB 13-060).

The criteria for inclusion were a diagnosis of AA and scheduled for treatment with DPCP. Patients with the following histories were excluded: Those with a comorbidity, such as, an endocrinal or an immunological disorder, those that had applied a topical hair growing solution or treated with topical or systemic steroids or an immunosuppressive agents within the previous 1 month.

The degree of hair loss was assessed by Severity of Alopecia Tool (SALT) score based on the percentage of scalp surface area involved on the top, back, and each side of the scalp[18,19]. The disease severity was classified by SALT subclasses based on the extent of scalp hair loss ($S_0$=no hair loss, $S_1$=<25% hair loss, $S_2$=25%~49% hair loss, $S_3$=50%~74% hair loss, $S_4$=75%~99% hair loss, $S_5$=100% hair loss)[18].

### Methods

DPCP sensitization was performed by applying 1% DPCP 2~3 times to the inner part of the plastic ring of 2 cm diameter on the alopecic patch, and 1 week later, when the lesion was not sensitized, the sensitizing procedure was repeated. After sensitization, hair growth was induced by applying low concentration of DPCP to the entire alopecic patches every 1 or 2 weeks.

Scales of sensitized alopecic patches were taken in 1 week after DPCP sensitization, and during DPCP immunotherapy, skin scales on alopecic patches were taken at 2 and 4 months. The scales were taken by using the 'sequential tape stripping' method, which involves the application of an adhesive D-Squame® skin sampling disc (22-mm diameter; CuDerm) to a cleaned, dry lesion (lightly wiped out with cotton soaked in saline 15 minutes before applying the adhesive disc). At each visit, scales were sampled 4 times to obtain a sufficient amount for analysis, on the sensitization site or treatment site using 'pressure device' which aids taking scales by providing constant pressure on the tape applied for 5 seconds. Specimens were stored at −80°C until required for analysis.

### Measurement of cytokine levels

To elute cytokines from samples, we used an elution buffer consisting of phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA) containing 0.005% Tween-20. This buffer (1.5 ml) was poured on 4 tape samples obtained from each patient in a 6-well plate and placed on ice for 30 minutes. Plate with D-Squame® tapes soaked in buffer is sonificated by being floated on sonicator for 15 minutes at a temperature of 4°C. After sonification, supernatant (225 $\mu$l) was centrifuged at 15,000×$g$ for 1 minute, and concentrations of IFN-gamma, IL-2, IL-12, and IL-10 were measured using a Milliplex® human cytokine/chemokine kit (Millipore, Darmstadt, Germany). The above measurements of cytokine levels from twenty four scale samples (1 week, 2 months, and 4 months; three samples of each eight patient) were repeated total three times.

### Assessments of therapeutic effects and adverse events

To categorize the eight subjects as responders and non-responders, therapeutic response was evaluated 2 months and 4 months after DPCP sensitization. To objectively evaluate therapeutic effects, hair regrowth was graded on a 7-point scale (−1=spreading, 0=no hair growth, 1=1%~24% hair growth, 2=25%~49% hair growth, 3=50%~74% hair growth, 4=75%~99% hair growth, 5=complete hair growth). Subjects with hair regrowth of point 3 or more (hair regrowth 50% or more) after

4 months of treatment were categorized as responders. The degree of the patients' subjective satisfaction about treatment were recorded on 4 levels (excellent, good, moderate, poor) at the final visit. Investigators evaluated adverse events, such as, pruritus, erythema, blisters, lymph node enlargement, or hyperpigmentation, at every treatment session.

### Assessment of changes in cytokine levels

Levels of IFN-gamma, IL-2, IL-12, and IL-10 were measured at 1 week, 2 months, and 4 months after DPCP sensitization.

### Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 22.0 (IBM Co., Armonk, NY, USA), and statistical significance was accepted for $p$-values $<0.05$. Fisher's exact test was used to compare clinical characteristics including sex, family history, severity, comorbidities, and adverse events after DPCP treatment between responders and non-responders. The independent sample t-test was used to compare ages, disease duration, and SALT score between the two groups.

Results of cytokine concentrations at 1 week, 2 months, and 4 months after DPCP sensitization are presented as means and standard deviations of three independent experiments. The Mann-Whitney U-test was used to compare the each cytokine level at 1 week, 2 months, and 4 months after DPCP sensitization between responder group and non-responder group. The Wilcoxon signal rank test was used to compare the differences of cytokine levels in total subjects, regardless of two groups, at each time interval, and it also used to compare the differences in cytokine levels of each group after DPCP treatment.

**Table 1.** Demographic data of the patients treated with DPCP

| Subject no. | Sex | Age (yr) | Family history of AA | Disease duration (mo) | Severity* | SALT score | Comorbidity |
|---|---|---|---|---|---|---|---|
| 1 | M | 32 | − | 17 | $S_3$ | 68 | - |
| 2 | F | 4 | + | 38 | $S_1$ | 21 | - |
| 3 | F | 54 | − | 50 | $S_3$ | 58 | Thyroid cancer |
| 4 | M | 42 | − | 2 | $S_2$ | 44 | - |
| 5 | M | 33 | + | 17 | $S_2$ | 39 | - |
| 6 | M | 34 | − | 192 | $S_1$ | 17 | - |
| 7 | M | 55 | − | 7 | $S_2$ | 45 | Hypertension |
| 8 | F | 53 | − | 12 | $S_1$ | 10 | - |

DPCP: diphenylcyclopropenone, M: male, F: female, AA: alopecia areata, SALT: Severity of Alopecia Tool. *$S_0$=no hair loss, $S_1$=<25% hair loss, $S_2$=25%~49% hair loss, $S_3$=50%~74% hair loss, $S_4$=75%~99% hair loss, $S_5$=100% hair loss.

**Table 2.** Summary of clinical results of the patients treated with DPCP

| Subject no. | Sex | Age (yr) | Hair regrowth assessment by physicians* | | Hair regrowth assessment by patient | Side effect | Respond to DPCP treatment† |
|---|---|---|---|---|---|---|---|
| | | | 2 months | 4 months | | | |
| 1 | M | 32 | 1 | 3 | Excellent | - | R |
| 2 | F | 4 | 2 | 4 | Excellent | - | R |
| 3 | F | 54 | 0 | 4 | Good | - | R |
| 4 | M | 42 | 1 | 1 | Moderate | - | NR |
| 5 | M | 33 | 0 | 1 | Moderate | Bulla at 10 wk | NR |
| 6 | M | 34 | 1 | 3 | Good | - | R |
| 7 | M | 55 | −1 | −1 | Poor | Bulla at 14 wk | NR |
| 8 | F | 53 | 1 | 1 | Good | - | NR |

DPCP: diphenylcyclopropenone, M: male, F: female, R: responder, NR: non-responder. *−1=spreading, 0=no hair growth, 1=1%~24% hair growth, 2=25%~49% hair growth, 3=50%~74% hair growth, 4=75%~99% hair growth, 5=100% hair growth. †Responder: hair regrowth assessment score ≥3 (hair regrowth ≥50%) at 4 months after DPCP treatment, non-responder: hair regrowth assessment score <3 (hair regrowth <50%) at 4 months after DPCP treatment.

## RESULTS

### Demographic data

This study was performed on eight AA patients; five men and three women. Mean age was 38.4 years and mean duration of disease was 42 months. The range of SALT score was 10 to 68 (mean score, 37.8). Two of the eight patients had a family history of AA. Two patients had a comorbidity, that is, thyroid cancer and hypertension. No patient had an autoimmune disease (Table 1). No significant difference was observed between responders and non-responders in terms of age, family history, duration of disease, comorbidity status, or disease severity ($p>0.05$). It showed that responder group had longer disease duration before DPCP treatment than non-responder group ($p=0.029$).

### Objective assessment

Therapeutic effectiveness was evaluated at 2 months and 4 months after DPCP sensitization. Investigators scored the therapeutic effectiveness by 7 steps according to the area of hair regrowth. At 4 months of treatment, the distribution of score was from −1 to 4 (mean score, 2). Four of the eight patients showed greater than or equal to score 3 (greater than or equal to 50% hair regrowth) at 4 months of treatment period and these patients were categorized to the group of responder to DPCP treatment (Table 2, Fig. 1).

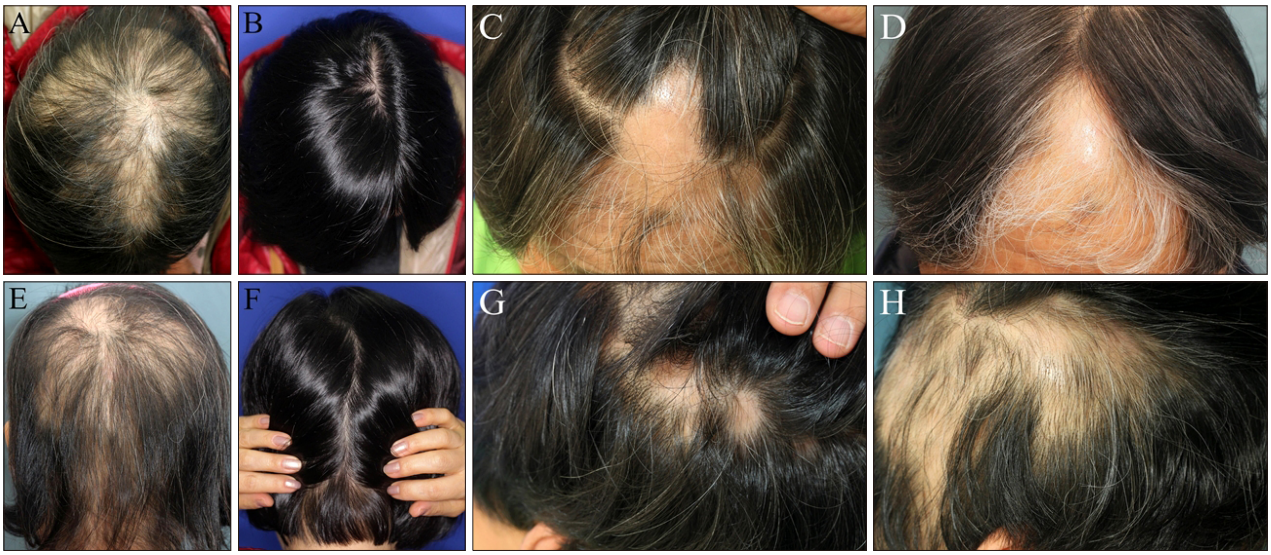


**Fig. 1.** Gross photograph of patient no. 3 (responder) and patient no. 7 (non-responder) at before and 4 months after DPCP treatment. Before treatment (A, E: patient no. 3; C, G: patient no. 7). After 4 months of treatment (B, F: patient no. 3; D, H: patient no. 7).

**Table 3.** Comparison of the cytokine levels between responder and non-responder groups at 1 week, 2 months, and 4 months

| Cytokine (pg/ml) | Respond to DPCP treatment† | 1 week | | 2 months | | 4 months | |
|---|---|---|---|---|---|---|---|
| | | Mean±SD | *p*-value | Mean±SD | *p*-value | Mean±SD | *p*-value |
| IFN-γ | R | 0.16±0.15 | 0.13 | 0.32±0.21 | 0.16 | 0.13±0.16 | 0.024* |
| | NR | 0.29±0.18 | | 0.21±0.22 | | 0.31±0.19 | |
| IL-2 | R | 0.02±0.04 | 0.007* | 0.03±0.06 | 0.71 | 0.03±0.04 | 0.93 |
| | NR | 0.17±0.18 | | 0.05±0.06 | | 0.03±0.06 | |
| IL-12 | R | 0.37±0.10 | 0.67 | 0.44±0.15 | 0.59 | 0.35±0.10 | 0.99 |
| | NR | 0.40±0.14 | | 0.39±0.16 | | 0.34±0.13 | |
| IL-10 | R | 0.44±0.19 | 0.078 | 0.25±0.16 | 0.219 | 0.33±0.15 | 0.755 |
| | NR | 0.72±0.40 | | 0.38±0.27 | | 0.32±0.23 | |

Values are presented as mean±standard deviation (SD) for n=4 of the three independent experiments. DPCP: diphenylcyclopropenone, IFN: interferon, IL: interleukin, R: responder (n=4), NR: non-responder (n=4). *$p<0.05$ calculated by Mann-Whitney U-test between responder and non-responder groups. †Responder: hair regrowth assessment score ≥3 (hair regrowth ≥50%) at 4 months after DPCP treatment, non-responder: hair regrowth assessment score <3 (hair regrowth <50%) at 4 months after DPCP treatment.

### Subjective satisfaction assessment

Patients responded as 'excellent' in two patients, 'good' in three patients, 'moderate' in two patients and 'poor' in one patient. The group which had more response to DPCP treatment tended to have higher subjective satisfaction (Table 2).

### Adverse events assessments

No significant adverse event was observed. Minor adverse events, that is, bulla, were observed in two patients at 10 and 14 weeks after DPCP sensitization (Table 2). No significant intergroup difference was evident ($p=0.429$).

### Changes in cytokine levels

Comparing initial cytokine levels after DPCP sensitization, there was no statistically significant difference between responder and non-responder groups in IFN-gamma. However, after 4 months of treatment, IFN-gamma levels showed statistically significant difference between the two groups; IFN-gamma levels were higher in non-responders than those in responders ($p=0.024$). No significant difference was noticed for any other cytokines after 4 months of the treatment (Table 3, Fig. 2).

IL-2 levels significantly decreased between 1 week and 4 months in non-responder group ($p=0.011$; Table 4). IL-10 levels also decreased in non-responders after DPCP treatment ($p=0.041$; Table 4). In total eight patients, IL-2 and IL-10 levels decreased between 1 week and 4 months after DPCP sensitization ($p=0.047$ and $p=0.013$, respectively; Table 5).

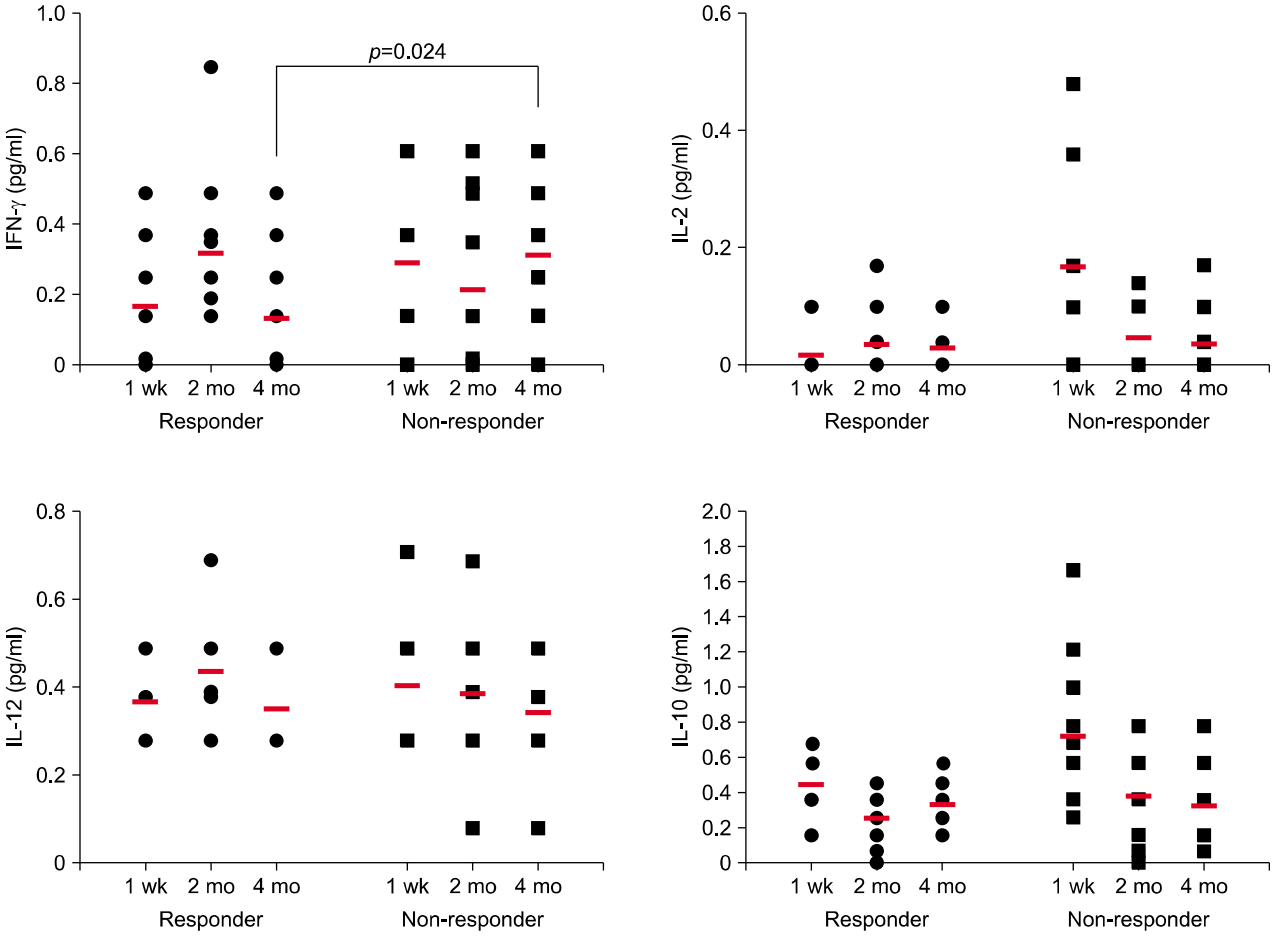


**Fig. 2.** Dot plot showing each value of cytokines in the patients treated with diphenylcyclopropenone (DPCP). Red bar in the graph indicates mean values. Before significant clinical differences appear (1 week and 2 months after treatment), interferon (IFN)-gamma, interleukin (IL)-12, and IL-10 showed no significant difference between responders and non-responders. IL-2 levels at 1 week showed difference between the two groups, however, it did not showed difference at 4 months after treatment. IFN-gamma levels at 4 months after DPCP treatment showed statistically significant differences between responder and non-responder groups ($p=0.024$).

**Table 4.** Comparison of the differences of cytokine levels in each group after DPCP treatment

| Cytokine (pg/ml) | Respond to DPCP treatment† | 1 week | 2 months | 4 months | *p*-value | | |
|---|---|---|---|---|---|---|---|
| | | Mean±SD | Mean±SD | Mean±SD | Between 1 week and 2 months | Between 2 months and 4 months | Between 1 week and 4 months |
| IFN-γ | R | 0.16±0.15 | 0.32±0.21 | 0.13±0.16 | 0.14 | 0.009* | 0.26 |
| | NR | 0.29±0.18 | 0.21±0.22 | 0.31±0.19 | 0.53 | 0.89 | 0.37 |
| IL-2 | R | 0.02±0.04 | 0.03±0.06 | 0.03±0.04 | 0.45 | 0.83 | 0.45 |
| | NR | 0.17±0.18 | 0.05±0.06 | 0.03±0.06 | 0.027* | 0.40 | 0.011* |
| IL-12 | R | 0.37±0.10 | 0.44±0.15 | 0.35±0.10 | 0.33 | 0.13 | 0.77 |
| | NR | 0.40±0.14 | 0.39±0.16 | 0.34±0.13 | 0.72 | 0.39 | 0.34 |
| IL-10 | R | 0.44±0.19 | 0.25±0.16 | 0.33±0.15 | 0.016* | 0.35 | 0.14 |
| | NR | 0.72±0.40 | 0.38±0.27 | 0.32±0.23 | 0.008* | 0.56 | 0.041* |

Values are presented as mean±standard deviation (SD) of changes between each time intervals, for n=4 of the three independent experiments. DPCP: diphenylcyclopropenone, IFN: interferon, IL: interleukin, R: responder (n=4), NR: non-responder (n=4). *$p<0.05$ calculated by Wilcoxon signal rank test in the responder and non-responder group each. †Responder: hair regrowth assessment score ≥3 (hair regrowth ≥50%) at 4 months after DPCP treatment, non-responder: hair regrowth assessment score <3 (hair regrowth <50%) at 4 months after DPCP treatment.

**Table 5.** Comparison of the differences of cytokine levels in total eight patients after DPCP treatment

| Cytokine (pg/ml) | 1 week | 2 months | 4 months | *p*-value | | |
|---|---|---|---|---|---|---|
| | Mean±SD | Mean±SD | Mean±SD | Between 1 week and 2 months | Between 2 months and 4 months | Between 1 week and 4 months |
| IFN-γ | 0.23±0.17 | 0.26±0.22 | 0.22±0.20 | 0.73 | 0.28 | 0.66 |
| IL-2 | 0.09±0.15 | 0.04±0.06 | 0.03±0.05 | 0.12 | 0.51 | 0.047* |
| IL-12 | 0.39±0.12 | 0.41±0.15 | 0.35±0.11 | 0.67 | 0.10 | 0.35 |
| IL-10 | 0.58±0.34 | 0.32±0.23 | 0.33±0.19 | <0.01* | 0.79 | 0.013* |

Values are presented as mean±standard deviation (SD) for n=8 of the three independent experiments. DPCP: diphenylcyclopropenone, IFN: interferon, IL: interleukin. *$p<0.05$ calculated by Wilcoxon signal rank test in total subjects.

## DISCUSSION

AA is characterized clinically by well-circumscribed alopecic patches. In patients with more than 50% of scalp involvement, topical immunotherapy using DPCP or squaric acid dibutylester is recommended[20].

Although the mechanism of DPCP in AA has not been elucidated, it is known that non-specific suppressor T cells infiltrate the region around hair follicles in a delayed hypersensitivity reaction[21]. These infiltrating T cells mainly express $CD8^+$ and $CD1a^+$, and suppress autoreactive T cells. Increases in number of $CD1a^+$ dendritic cells means that the emigration of the antigen presenting cell is disturbed by DPCP application[22]. DPCP treatment affects not only T cells but also the compositions of cytokines around hair follicles[23].

In a study of AA patients treated with DPCP for at least 6 months, no differences were found between the levels of IL-12 and IFN-gamma in peripheral blood mononuclear cells in patients who responded to the treatment as compared with healthy controls, suggesting a loss of upregulation of the activation markers. However, when the disease progressed despite DPCP treatment, the levels of IL-12 and IFN-gamma remained higher than in controls[12]. So it is proposed that these cytokines might be used for the clinical monitoring of long-term DPCP and for the prediction of clinical response to treatment.

Recently, various cytokines have been proposed to contribute to the occurrence of AA. In particular, cytokines related to T helper 1 cells are known to play critical roles[1,9]. IFN-gamma, the main cytokine aberrantly expressed in AA due to $CD4^+$ T helper 1 mediated response, is known to be associated with the extent of AA[1]. Furthermore, it has been suggested T helper 1 cytokines are related to the extent of AA, because IL-2 levels are elevated in extensive AA as well as IFN-gamma levels[10,24].

In one report, IL-12 and IFN-gamma are related to AA activity due to marked elevations of both in peripheral blood mononuclear cells in progressive AA patients, whereas these cytokine levels were not different in controls or in

patients with stable or regressive disease[12]. However, in another study, levels of IFN-gamma, IL-2, and IL-12 were found to be higher in AA patients, regardless of the disease severity[9,10].

In a study using scalp biopsies from patients with alopecia totalis before and after successful DPCP treatment, T helper 1 cytokines, such as, IFN-gamma, IL-1 beta, and IL-2 were found to be elevated in untreated patients, but after DPCP treatment IFN-gamma decreased and IL-2, IL-8, IL-10, and TNF-alpha levels increased. Hoffmann et al.[23] proposed that the T helper 1 cytokine pattern was observed in untreated patients and suggested that IL-10 might be effective at inhibiting T helper 1 response.

IL-10 is known to act as an anti-inflammatory cytokine in association with Treg and T helper 2 cells[10]. After DPCP treatment, IL-10 was reported to be increased in scalp tissues[23], and a continuous increase in the expression of IL-10 in peripheral blood mononuclear cells was observed than controls, whereas this was not observed for IL-12 or IFN-gamma[12]. However, some consider AA relatively restrained in IL-10 knockout mice, which suggests IL-10 acts to both contain and promote sensitivity to AA[25]. On the other hand, it has also been reported that serum IL-10 levels in AA and controls were no different[9,10].

In the present study, early IFN-gamma levels were similar in responders and non-responders. After 4 months of DPCP treatment, IFN-gamma levels were significantly higher in non-responders, whereas IL-2, IL-12, and IL-10 levels showed no significant difference between the two groups after treatment. When we examined cytokine levels after DPCP treatment in all eight patients, IL-2 and IL-10 levels significantly decreased. These observations suggest cytokines related to T helper 1 have attribute AA pathophysiology, and that IFN-gamma is particularly important. The decrease in IL-10 level after DPCP treatment found in the present study, is inconsistent with that reported previously, and thus, we suggest additional studies to conducted to investigate the role of IL-10 during the pathogenesis of AA.

The monitoring cytokine levels in scales has been suggested to monitor various inflammatory diseases[16,17,26]. But until now there is no report analyzed scales from alopecic patch in AA. This is the first study to compared and analyzed cytokine levels in skin scales collected from alopecic patches in AA. The study is undoubtedly limited by its small sample size, by the lack of information on baseline cytokine levels, and by the relatively short follow-up (4 months). Accordingly, we suggest a further larger scale study be conducted over a longer period to confirm our results.

In this study, we wanted to see if the cytokine levels of scales in alopecic patches can predict treatment effects, however, early cytokines did not show difference between responder and non-responder before significant clinical responses appear, indicating that early cytokine analysis could not predict the response of treatments. Meanwhile, after 4 months of treatment, IFN-gamma levels in non-responders were significantly higher than in responders, reflecting clinical differences.

Our observations suggest IFN-gamma reflects the therapeutic response to DPCP in AA patients treated with DPCP. Finally, in our opinion, the non-invasive method used in the present study offers a basis for evaluating various inflammatory skin diseases.

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## CONFLICTS OF INTEREST

The authors have nothing to disclose.

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