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Higher concentrations of dithranol appear to induce hair growth even in severe alopecia areata

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Abstract

Alopecia areata (AA) is the commonest autoimmune cause of non-scarring alopecia. Topical treatments including corticosteroids and irritants maybe beneficial. Studies report variable hair regrowth with dithranol (anthralin) but all used low concentrations (0.1–1.25%) and inconsistent measurements of AA severity. We report retrospective data (2005–2014) of 102 patients who had failed ultra-potent topical steroids and were referred to a specialist hair clinic for treatment with dithranol up to 3%. The severity of alopecia areata tool was used and participants graded as mild (<25%), moderate (>25 to 75%), and severe (>75%) hair loss. Compared with baseline any and at-least 50% hair regrowth [72%, 68%, 50% and 61.5%, 48.4%, 37.5%, in mild, moderate and severe AA respectively] occurred in all groups (median treatment duration 12 months). Twenty-nine patients (28.4%) were discharged with complete regrowth; with no difference in proportions in severity groups (33.3%, 29%, and 21.9%) but in the period to discharge [7.9, 6.3, and 29.4 months (p-values <.05)] for mild, moderate, and severe AA. Treatment trials of 12 months with dithranol at higher concentrations may be an option in patients who failed potent topical or intralesional steroids) regardless of AA severity. Randomized trials (of less staining formulations) of dithranol are warranted.

KEYWORDS

alopecia areata, dithranol (anthralin), dithranol staining, irritant

1 | INTRODUCTION

Alopecia areata (AA) presents with well-defined patches of hair loss with normal underlying skin. Focal or patchy AA is the commonest form of the disease and is reported to spontaneous regrowth in up to 50% of patients. Increasing severity and worsening prognosis for regrowth is associated sequentially with other variants of AA [ophiasis (hair line), totalis (entire scalp), and universalis (body hair)] (Alkhalifah et al., 2010). Spontaneous recovery may occur, as may relapses or disease progression (Garg & Messenger, 2009). On histology AA is characterized by peribulbar lymphocytic inflammatory infiltrates resulting in a "swarm of bees" appearance (Wasserman et al., 2007). It is suggested that these inflammatory cells, mostly T cells, cause growth arrest primarily in hair bulbs in the anagen phase of the hair cycle. The hair follicle itself is preserved (Garg & Messenger, 2009; Tang, Lui, et al., 2003; Tang, Sundberg, et al., 2003). The pathogenesis is incompletely understood but AA is regarded as an organ-specific autoimmune disease. The lifetime risk of AA is around 1.7% and the prevalence is equal across race and gender.

Topical treatments include steroids and in resistant cases immunotherapy (with squaric acid dibutylester or diphenylcyclopropenone) and irritants (dithranol) have been used. Dithranol (anthralin), more commonly used in psoriasis has been used in AA. In one case series concentrations of dithranol were increased until a mild contact dermatitis was attained; "cosmetically good" response was reportedly seen in 75% of patients with patchy alopecia and 25% of patients with alopecia totalis (Schmoeckel et al., 1979). However, most subsequent studies (also small and uncontrolled) failed to demonstrate similar efficacy. The only randomized study compared 4 groups (each with 20 participants: dithranol 0.25%, tretinoin, steroid and vehicle reported regrowth of 35%, 50%, 70%, and 20%, respectively). These studies used inconsistent assessment of alopecia severity and treatment improvement; further, all used low concentrations (0.5%, range 0.1–1.25%) (Das et al., 2010; Deshpande et al., 2011; Fiedler et al., 1990; Fiedler-Weiss & Buys, 1987; Nelson & Spielvogel, 1985; Sasmaz & Arican, 2005; Schmoeckel et al., 1979; Torchia & Schachner, 2010) of dithranol (Table 1).

TABLE 1 Published studies reporting the use of dithranol (anthralin) in the treatment of alopecia areata

Reference	Year	Study design	Sample size	Comparative intervention	Dithranol, %	Outcome
Schmoeckel et al.	1979	Case series	32	None	0.2–0.8	"[Cosmetically good": in 75% with patchy alopecia vs. 25% alopecia totalis
Nelson and Spielvogel	1985	Case series	10 (11)	None	0.1–0.5	4 cases stable, 4 spontaneous improvement and dithranol patches, 2 cases progressed despite treatment
Fiedler-Weiss and Buys and Torchia and Schachner	1987 and 2010	Case series	68	None	0.5–1	25% had a cosmetic response but in this group of responders, only 71% had a sustained response
Das et al.	2010	Controlled trial nonrandomized	80	Three additional groups (20 each) Dithranol Topical steroids Topical tretinoin petroleum jelly	0.25	"Regrowth . . . defined >60% coverage of bald patch" in 3 months and was: 35% dithranol group 70% topical steroid group 55% tretinoin group 20% control group
Torchia and Schachner	2010	Case report	1	Topical steroids Half scalp treatment	0.1	Dithranol: left side, no improvement Topical steroids: right side, regrowth after 4 months.
Sasmaz and Arican	2005	Randomized controlled trial (2 assessors).	31	Azelaic acid	0.5	Complete response: 53.3% azelaic acid vs. 56.2% dithranol $p > .05$
Fiedler et al. and Olsen et al.	1990 and 2004	Case series	45 (51)	None, combination: 5% minoxidil 0.5% dithranol	0.5	5 cases (11%) cosmetically significant response sustained in 4/5 responders
Deshpande et al.	2011	Uncontrolled case series	15	None combination: oral? Betamethasone 2–5% minoxidil 1.15% dithranol	1.15	6 months: 11/15 (73%) cosme significant 1 partial response 3 no response.

2 | METHODS

Retrospective data from the Groote Schuur Hospital Hair Clinic (2005–2014) was analyzed. This is a referral clinic for both children and adults with hair loss. Dithranol treatment is used for AA in a similar manner as for treatment of psoriasis (i.e., 0.5% dithranol in lygols is applied to patches of hair loss for 5 min, wiped-off with paper towel till all residue is removed and then washed thoroughly with liquid soap. The dithranol contact time is increased by 5 min every 2 days to a maximum of 30 min then continued until the 80 gram supplied runs out. The concentration is then sequentially increased to 1%, 2%, and 3% dithranol (with each concentration increase, the whole process starts again from the beginning at 5 min contact time increased to 30 min until the 80 gram supply is finished). Once at 3% dithranol the contact time is kept at 30 min per day until termination of treatment. Scalp staining is an expected common sign of treatment compliance and not a reason to stop treatment. Regional lymphadenopathy is not uncommon and resolves on stopping treatment. Pre-treatment counselling is essential to reduce the chance of symptomatic irritation. Proper care should be taken to wipe the dithranol away with paper towel before washing the hair thoroughly. At the slightest discomfort, treatment should be interrupted, restarted 2–3 days later and contact time dropped to the last comfortable one.

Disease severity was assessed at baseline and every visit using percentages estimated from the severity of AA tool (Olsen et al., 2004). Patients were graded as mild (<25%), moderate (25–75%), and severe (>75%) alopecia.

Statistical analyses were performed using STATA Version 13.0 (StataCorp., LP, College Station, TX, USA). The distributions of patients by selected characteristics and severity are represented as number and percentage. The chi-squared test or Fisher's exact test was used to establish associations between patient characteristics and improvement in severity of hair loss. Multiple logistic regression was used to calculate the odds ratios (ORs) for the relationship between general improvement in hair loss or at-least 50% improvement in hair loss and specific patient characteristics. All significance tests were two-tailed, and significance was defined at the 5% alpha level with 95% confidence intervals (CIs).

The study was approved by the ethics committee of the University of Cape Town and informed written consent was obtained.

3 | RESULTS

A total of 102 patients were attended for the study period; 73 females and 29 males including 30 children (<12 years) (Table 1). At baseline, proportions were equally distributed among mild, moderate, and severe AA. Compared with baseline AA severity, females were more likely than males to have general improvement (0.041); however, the difference was not significant for at-least 50% improvement (*p*-value .124). All severity groups experienced regrowth. Although there was a trend toward better hair regrowth from dithranol in the mild compared with the severe group, this was not statistically significant [general improvement: 72%, 68%, and 50% ($p = .141$); and at-least 50% hair regrowth: 61.5%, 48.4%, and 37.5% ($p = .128$) for mild, moderate, and severe, respectively]. The odds for better improvement were higher in mild versus severe disease. Further the odds for general (but not at-least 50%) improvement were better in females than males. Twenty-nine patients (28.4%) had improvement significant enough for discharge (complete hair regrowth) during the study period, median treatment of 12.1 months. There was no difference in proportions that improved enough for discharge (33.3%, 29%, and 21.9%); however, the follow-up period to discharge was significantly different [7.9, 6.3, and 29.4 months (*p*-values <.05)] for mild, moderate, and severe disease, respectively. The median follow-up to improvement for the whole cohort was 11.7 months.

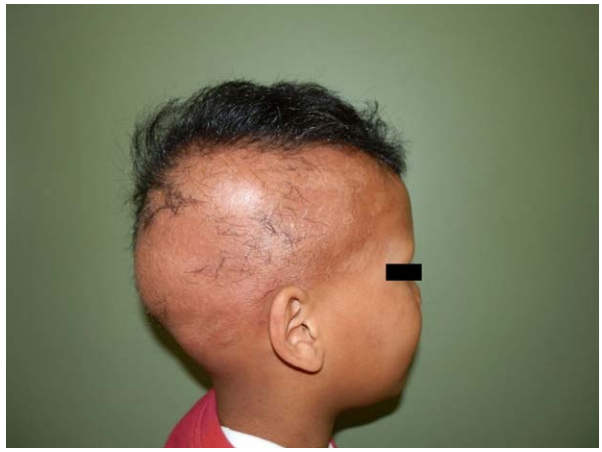

FIGURE 1 Severe alopecia areata in a child after initiating treatment; note dithranol staining

4 | DISCUSSION

The study suggests that mild (<25%), moderate (25–75%), and severe (>75%) AA may all improve on dithranol therapy. However, patients with severe AA take much longer to respond. Dithranol is an irritant and it is important to council patients (parents) to increase the contact time and concentration from 0.5% to 3% carefully. It cannot be over emphasized dithranol should be wiped off until the paper towel is clean and then the scalp washed thoroughly with liquid (or dish washing) soap which is better than shampoo at removing the oily dithranol residue which can cause irritation. Following these instructions allows up to 3% dithranol to be used without significant side effects that warrant discontinuation of treatment even in children (Figure 1, 2). However, the staining is a noticeable marker of dithranol use and maybe severe enough to warrant use of a wig or head covering—usually already worn by patients with severe AA. Dithranol may induce lymphadenopathy which resolves on stopping treatment.

Two animal models, the C3H/HeJ mice and the Dundee experimental bald rat (DEBR), have alopecia comparable to human AA (Tang, Sundberg, et al., 2003). Dithranol was reported to have a significant therapeutic effect in both (with 64% of C3H/HeJ mice and all DEBR rats responding). The mechanism of action of dithranol may be related

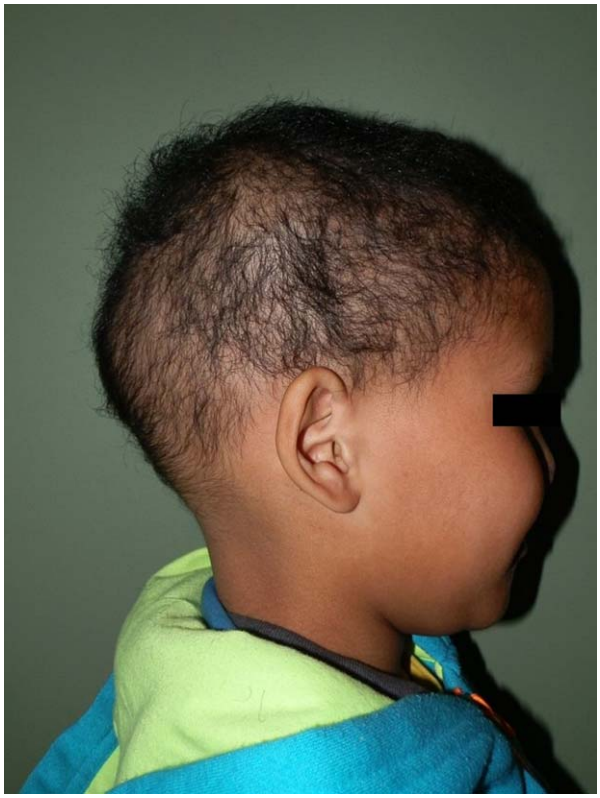

FIGURE 2 Complete regrowth with treatment; note staining resolves completely within a few weeks of stopping dithranol

to changes in cytokine expression. In C3H/HeJ mice with AA response to dithranol is associated with a decrease in the pro-inflammatory cytokines TNF-α and TNF-β; a decrease was also seen in DEBR rat skin treated with dithranol. Interestingly, DEBR rat skin treated with dithranol is also associated with increased levels of IL-1α, IL-1β and IL-1R_a but levels of these cytokines are unchanged in similarly treated C3H/HeJ mice skin (Tang, Sundberg, et al., 2003). The lymphocytic infiltrate associated with AA in DEBR rats was predominantly composed of CD8 cells and although in treated skin, there was no reduction in CD8 cells, their distribution was markedly altered that is more uniformly distributed in the dermis rather than concentrated around the "follicular periphery" and "intrafollicular peribulbar area" as in controls (Tang, Sundberg, et al., 2003).

The management of AA remains challenging and there is conflicting evidence regarding the efficacy of dithranol in the treatment of AA. However, most studies are very small case series, the concentrations of dithranol used was consistently low (0.5% (0.1–1.25%)) (Schmoeckel et al., 1979) and severity AA was not measured to assess treatment outcome (Table 1). Another confounder is the recognition that mild disease may resolve spontaneously. The British AA guidelines state: "... study from Japan reported that spontaneous remission within 1 year occurred in 80% of patients with a small number of circumscribed patches of hair loss, data from secondary and tertiary referral centres are less favourable indicating that 34–50% of patients will recover within 1 year" (Messenger et al., 2012). The patients in this study attended a tertiary hospital referred by general practitioners and dermatologists. These were patients who had not spontaneously recovered and had also failed ultra-potent topical and/or intra-lesional steroids; thus were more likely to have longstanding and/or resilient disease.

The retrospective nature of the study makes it impossible to quantify adverse effects, and recurrence of alopecia. The study is also limited by the lack of a comparison treatment group; however, the sample size of 102, is the largest of published dithranol for AA studies. We used objective measures of hair loss severity and found high concentration of dithranol to induce hair regrowth in all severity groups of patients. A third of patients in all groups were discharged with complete hair regrowth although the response took longer in severe AA. Treatment trial periods of 1 year may be adequate in patients who have not responded to potent steroids. Our study suggests that dithranol should not be discarded as a treatment option in AA; it is likely that modern technology could improve its formulation and reduce staining. Randomized controlled trials with less staining formulations of dithranol are warranted to elucidate the efficacy of dithranol in AA.

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Restoration of hair growth in mice with an alopecia areata-like disease using topical anthralin

Tang L, Cao L, Sundberg JP, Lui H, Shapiro J. Restoration of hair growth in mice with an alopecia areata-like disease using topical anthralin. Exp Dermatol 2004: 13: 5–10. © Blackwell Munksgaard, 2004

Abstract: Anthralin is a widely used topical anti-psoriatic drug that may have an immunomodulating effect on alopecia areata (AA) as it does in psoriasis. The aims of the present study were to investigate the effects of anthralin on hair growth in balding C3H/HeJ mice affected by an AA-like disease and to study the underlying mechanisms. Affected C3H/HeJ mice were treated daily for 10 weeks on half of the dorsal skin with 0.2% anthralin and the contra-lateral side was treated with the vehicle ointment. The percentage of surface hair coverage and hair density was graded weekly for both sides and hair growth indices were calculated using these two variables. Hair regrowth was observed in 9/14 mice on the treated sides. Four mice displayed near complete replacement of normal density and length hairs. All the vehicle-treated sides showed either no change or continued hair loss. An RNase protection assay (RPA) showed that expression of tumor necrosis factor-α (TNF-α) and -β were inhibited by anthralin upon successful treatment. It appears that anthralin may be an effective therapy for C3H/HeJ mice with AA and certain cytokines may be involved in the therapeutic effects of anthralin on restoring hair regrowth in AA-affected C3H/HeJ mice.

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Key words: C3H/HeJ mice – topical therapy – cytokine

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Introduction

Alopecia areata (AA) is a common inflammatory disease of the hair follicle affecting 1.7% of the population that results in patchy to complete hair loss (1). While not life threatening, AA is nonetheless serious as it may cause severe psychological problems, social ostracism, and even loss of employment (2). Although AA is widely regarded as an autoimmune disease (3,4), the precise cause of the disease is unknown. Peri-follicular and intra-follicular inflammatory cell infiltrates are histologic characteristics of AA. These infiltrating cells are mainly activated T-lymphocytes with an admixture of macrophages and Langerhans' cells that appear to preferentially attack anagen hair bulbs within active lesions of AA (5,6). A wide range of treatments has been utilized in AA, including contact sensitizers, immunomodulators, and biological response modifiers (7). Treatment efficacy is variable and unpredictable and most of the treatments have untoward side-effects. The treatment of AA with topical anthralin has been reported to be effective by some investigators (8–10), but not by others (11). Cosmetically acceptable hair regrowth has been reported in 25% of the AA patients treated with 0.5–1.0% anthralin cream (7).

C3H/HeJ mice develop a diffuse, non-scarring alopecia with clinical and pathologic features similar to human AA (12,13). In AA-affected C3H/HeJ mice, a normal coat of agouti hair is initially present, and hair loss typically occurs as early as 4 months of age in females and 6–12 months in males. The incidence of hair loss can be as high as 20% in some colonies of mice aged 18 months or older. The hair loss phenotype has been reproduced by full-thickness AA-affected skin grafts onto histocompatible mice (14). The C3H/HeJ animal model provides the means whereby new

Abbreviations: AA: alopecia areata; DPCP: diphenylclopropenone; DEBR: Dundee experimental bald rat; HGI: hair grow index; RPA: RNase protection assay; SADBE: squaric acid dibutylester; TNF-α: tumor necrosis factor-α; TNF-β: tumor necrosis factor-βGM-CSF: granulocyte macrophage colony stimulating factor; IL-6: interleukin-6; IL-8: interleukin-8; DEPC: diethyl pyrocarbonate; TGF-β1: transforming growth factor-β1.

forms of treatment can be developed, tested, and analyzed with respect to mechanism of action. Drug-induced hair regrowth has not yet been extensively studied in this AA model. We and other investigators have shown that contact immunotherapy using diphenylcyclopropenone (DPCP) or squaric acid dibutylester (SADBE) stimulates hair growth in these mice (15–20). Here we report the efficacy of anthralin on AA in C3H/HeJ mice.

Anthralin remains one of the most effective and most widely used therapeutic agents for psoriasis (21). Its anti-psoriatic therapeutic efficacy may be related to the induction of cutaneous inflammation via the expression of cytokines and cell adhesion molecules (21–24). At the molecular level, anthralin inhibits pro-inflammatory cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) produced by activated monocytes and epidermal keratinocytes (21,23). The mechanism of anthralin's potential beneficial effect in AA is speculative at this time. AA is believed to be an autoimmune disease and a number of pro-inflammatory cytokines have been indicated in the pathogenesis of AA including IL-1β, TNF-α, and IFN-γ (25–27). The inhibitory effects of anthralin on the production of the pro-inflammatory cytokines might be the mechanism responsible for hair regrowth in AA. To determine whether anthralin modulates cytokine expression in the C3H/HeJ mice, we examined various cytokines using an RNase protection assay (RPA). Our data support that TNF-α and TNF-β are both decreased by anthralin upon successful treatment. The possible roles of these cytokines in restoring follicular activity in AA are discussed.

Materials and methods

Animals

Seven spontaneous C3H/HeJ mice with AA (6 females and 1 male, mean age of 9 months, age range 8–12 months) and 7 grafted mice (all females, mean age of 7 months, average 10 weeks after grafting) were housed in groups of two or three and kept in a controlled environment with an alternating 12 h day/night cycle. Hair loss ranged from large bald areas on the flanks and heads to almost complete loss of head and body hair. The study was approved by University of British Columbia Animal Care Committee.

Drug treatment

Topical anthralin ointment was prepared by the Vancouver Hospital Pharmacy as a 0.2% ointment with 3% salicyclic acid in hydrophilic petrolatum. Concentrations of anthralin higher than 0.2% were tested but severe dermatitis was observed. So, 0.2% anthralin ointment was chosen for the study. Vehicle control was identical except for the absence of anthralin. All mice were treated on half of the dorsal skin by an even application of anthralin ointment. The contra-lateral side was treated identically with the vehicle ointment. Once daily topical applications were applied for 5 days, 2 days off, repeated for a total of 10 weeks (70 days). During treatment, mice were weighed and photographed weekly. After 10 weeks of treatment, mice were killed by CO_2 asphyxiation and necropsied.

Hair growth grading

The percentage of surface hair coverage and hair density was graded weekly for both anthralin-treated and control sides. For each side, the percentage of hair surface coverage was estimated. The quality and density of the hair were assessed and graded on a four-points scale (Grade 0: no hair; Grade 1: stubble, short, broken hairs; Grade 2: sparse, intermediate length hairs; Grade 3: normal length and density hairs). The percentage of hair coverage was multiplied by the quality/density grade for each area. The products were added together to give the total hair growth index (HGI) for each side of the animals. Using this method, the hair growth index may thus range from 0 (no hair) to a maximum of 300 (100% normal hair).

RNA extraction and RNase protection assay

Two whole skin tissue samples were collected from each mouse, one from the treated and another from the control side after 10 weeks of 0.2% anthralin treatment. Hairy skin was shaved before the biopsies were taken. Total RNA was prepared from the whole skin using Trizol™ solution (Invitrogen, Carsbad, CA, USA) and polytron homogenization according to the manufacturer's instructions. RNA was precipitated with isopropanol and re-dissolved in diethyl pyrocarbonate (DEPC)-treated water. Yield and purity were assessed by UV spectrophotometry and denatured agarose gel electrophoresis. The yield was approximately 100 μg of total RNA per 100 mg of mouse skin.

RPA were performed using PharMingen's RiboQuant® Multi-Probe Ribonuclease Protection Assay System (PharMingen, San Diego, CA, USA). Template sets were designed by PharMingen. The following gene sequences were included in the templates: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, IL-1 receptor antagonist (IL-1Ra), TNF-α, TNF-β, IFN-γ, and two housekeeping genes, L32 and GAPDH. Total RNA (15 μg) was used for each RPA assay. Probe synthesis, hybridization, and RNase treatment were performed according to the manufacturer's instructions. ^{32}P[d-UTP] was used to label the probe (Pharmacia, Piscartaway, NJ, USA). The 'RNase-protected' fragments were resolved on 6% polyacrylamide gel containing 4 M urea and the dried gel was exposed to X-ray film for various times. The signals were detected by autoradiography. The corresponding bands were quantitated by densitometry analysis. The levels of cytokine expression were normalized to that of L32 from > the same sample. The ratio of treated over control was used to determine the difference in the gene expression between two sides. The ratio of greater than 2 or less than 0.5 was considered as increased or decreased, respectively. Anything between was valued as unchanged.

Results

After 10 weeks of treatment, the average HGI was significantly higher on the treated sides than the control sides ($P = 0.036$, Student's t-test) (Fig. 1). Before the onset of treatment, the average HGI was 94 ± 49 for both treated and control sides. At the completion of the study, the average HGI

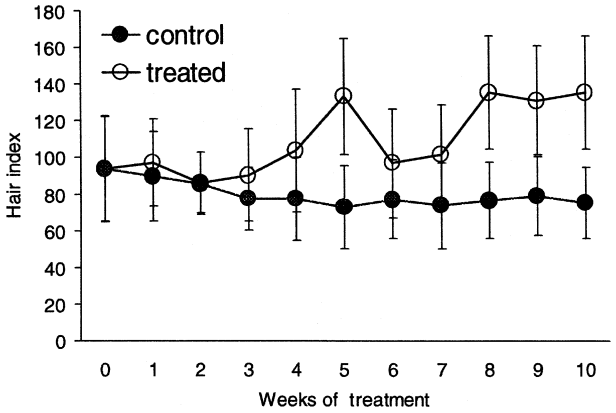

Figure 1. Anthralin increases hair growth indices in AA-affected C3H/HeJ mice. Each data point represents the average hair growth index of 14 mice minus the baseline hair growth index at week 0.

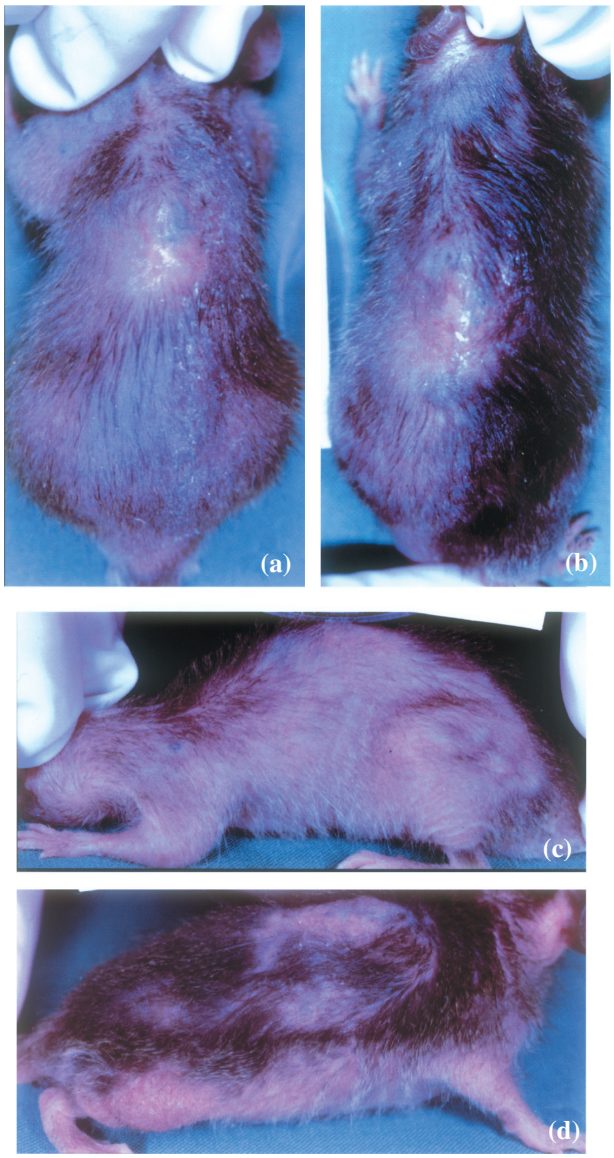

Figure 2. A female C3H/HeJ mouse was successfully treated unilaterally with anthralin. There was no difference between the two sides in hair density 1 week after the onset of the treatment (a). After 10 weeks, significant terminal hairs were regrown on the anthralin-treated right side (b and d), while the vehicle-treated left side (b and c) had diffuse alopecia.

increased to 135 ± 65 on the treated sides vs. 74 ± 32 on the control sides. Nine out of 14 mice exhibited hair regrowth on the treated sides over 10 weeks of treatment ($P < 0.05$). Hair regrowth was observed as early as 1 week after the initiation of treatment in one mouse. The rest of the responding mice showed renewed hair regrowth on the treated sides between weeks 3–5 and this continued consistently in four mice. By week 10, there was extensive hair replacement with normal hair density and quality on the anthralin-treated sides (Fig. 2). In these four mice, the hair regrowth indices reached above 200 on the treated sides, while on the control sides the hair growth indices were less than 100. The other five responders showed repeated hair loss and regrowth over 10 weeks of treatment (Fig. 1). Overall, the average hair indices were significantly higher on the treated sides ($P < 0.05$) than that of control sides except for the 7th week (Fig. 1). During that week, 4/5 responders lost hair from the treated sides; however, the hair regrew on the treated side after week 7 in these animals. Of the five non-responders, there was either no hair regrowth as compared with the control side or hair was lost on both sides. One non-responding mouse lost more hairs on the anthralin-treated side than the control side. On the vehicle-treated sides at the end of this study, the hair either remained unchanged or continued to demonstrate hair loss compared with baseline for the other 13 mice. One of the responders showed a slight increase in HGI on the control side. The photographs of a representative positive responder are shown in Fig. 2. Throughout the study, all mice maintained constant body weights. In anthralin-treated skin, local dermatitis with erythema and pigmentation were observed as early as 2–3 days after starting the treatment, and continued for the duration of the study. These effects were not observed on the vehicle-treated sides.

Histopathologic examination, carried out at the end of the study on anthralin- and vehicle-treated sides, revealed that the overall number of $CD4^+$ and $CD8^+$ infiltrates was not significantly different between the two sides. On the control sides, infiltrating lymphocytes (both $CD4^+$ and $CD8^+$ cells) were mainly present around the follicular periphery and also penetrated the intra-follicular peribulbar area. In contrast, on the anthralin-treated

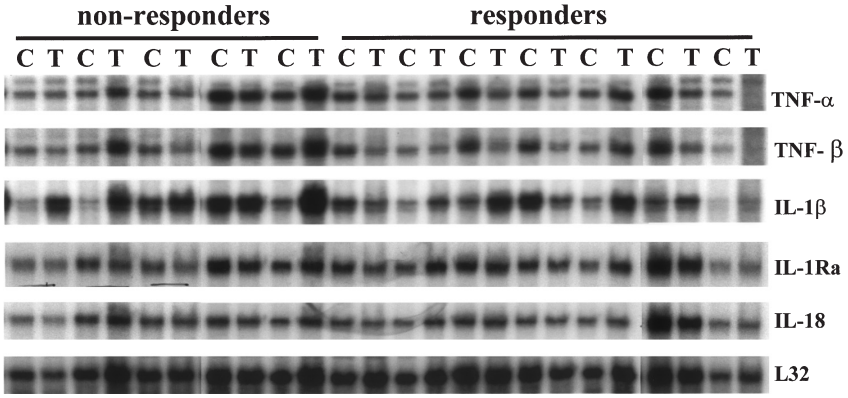

Figure 3. Cytokines are modulated by anthralin in AA-affected C3H/HeJ mice. Total RNA was extracted from 12 C3H/HeJ mice after 10 weeks of anthralin treatment. Two skin tissue samples were collected from each mouse, one from the treated side (T) and one from the control side (C) after 10 weeks of anthralin therapy. An RNase protection assay was performed using the templates indicated to the right. IL-1β was increased by anthralin in the majority of all the mice tested. The expressions of TNF-α and TNF-β were correlated to mice's response to anthralin treatment. L32 was used as an internal control and showed no significant difference between samples.

sides, these infiltrated lymphocytes were distributed more uniformly in the dermis (data not shown).

To explore the possible molecular mechanisms of anthralin's therapeutic effects on hair restoration in AA-affected C3H/HeJ mice, RPA was performed on the biopsies collected from 12 mice. Among these 12 mice, 5 were non-responders and 7 responders. The pro-inflammatory cytokine IL-1β was induced by anthralin in 8 mice, 4 responders and 4 non-responders (Fig. 3, Table 1). The expression patterns of TNF-α/β were quite different. The majority of the responders displayed decreased expression of TNF-α (5/7) and TNF-β (5/7) on the treated sides. In contrast, only one non-responder showed a decreased expression of TNF-α and two exhibited a decreased expression of TNF-β. The other two abundantly expressed cytokines in mouse skin, IL-18 and IL-1Ra, did not show any significant differences between the two sides. The rest of the cytokines in the template sets including IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IFN-γ, were not consistently detected in the mouse skin using this assay.

Table 1. Summary of cytokine expression affected by anthralin

Cytokines	Change	Non-responders (5)	Responders (7)
IL-1β	increased	4	4
	decreased	0	2
	unchanged	1	1
TNF-α	increased	2	1
	decreased	1	5
	unchanged	2	1
TNF-β	increased	2	1
	decreased	2	5
	unchanged	1	1

The levels of IL-1β, TNF-α and TNF-βexpression were normalized to that of L32 from the same sample.

Discussion

Previous trials using anthralin on AA patients have been performed with some success (8), and the current study confirms the potential efficacy of topical anthralin in restoring hair growth in C3H/HeJ mice with AA-like hair loss. In the preliminary animal experiments, we compared the efficacy of 0.1% anthralin with that of 0.2% anthralin and found that 0.2% was more effective (data not shown). Topical application of 0.2% anthralin led to hair regrowth on the treated sides in 9 out of 14 mice and the vehicle-treated sides remained unchanged or exhibited continued hair loss, suggesting that anthralin had a localized effect. We noticed that the grafted mice did not respond to the treatment as well as the spontaneous AA-affected C3H/HeJ mice. The hair growth indices of the control sides of the spontaneous AA mice did not change while the overall hair indices in the grafted mice were lower at the end of the experiment (data not shown). This might be because that most of the grafted mice were still actively losing hair during the experiment. They were in active disease compared with end-stage disease in chronically affected mice. Overall, efficacy rates of anthralin (64%) on mice were comparable to that of DPCP (62.5%) (18). The efficacy rates of SADBE (75%) (15) and tacrolimus (79%) (17) were slightly better on this mouse AA model. In our experiences, mechlorethamine has been the most effective treatment on AA-affected mice (19).

Some mice lost hair from the treated sides around weeks 6 and 9 that then grew back when

Table 2. Comparison of cytokine expression mediated by different therapies in alopecia areata

Cytokines	Animal: DEBR rat Therapy: anthralin	Animal: C3H/HeJ AA mouse Therapy: anthralin	Animal: C3H/HeJ AA mouse Therapy: meclorethamine	Alopecia areata patients Therapy: DPCP
IL-1α	↑	UC	UC	NT
IL-1β	↑	UC	UC	↑
IL-1Ra	↑	UC	UC	NT
IL-2	UC	ND	UC	↑
IL-8	NT	NT	NT	↑
IL-10	↑	ND	UC	↑
IL-12	UC	UC	↓	NT
MIF-1	NT	NT	UC	NT
IL-18	UC	UC	UC	NT
IFN-γ	↓	ND	↓	↑
TNF-α	↓	↓	↓	↑
TNF-β	NT	↓	↓	NT
Reference	unpublished, (20)	this study	(19,20)	(25)

↑: increased; ↓: decreased; UC: unchanged; NT: not tested; ND: not detected
[1]Only decreased in the responding mice, no change in the non-responders.

the treatment continued to the end of week 10 (Fig. 1). The cause of the transient relapse in these responders after hair regrowth was unclear. Oxygen-free radicals generated from anthralin are believed to be central to its anti-inflammatory and anti-proliferative effects. However, these same free radicals are responsible for inflammation of non-affected areas (21,23), and it is therefore difficult to determine which effects are primary. In AA therapy, free radicals generated from anthralin might be the mechanism of anti-inflammatory action for clearing the infiltrative lymphocytes. Yet it is also possible that these free radicals may have attacked fully developed hair follicles thereby leading to hair loss. We have found that the viability of lymphocytes and different follicular cells (follicular keratinocytes and follicular dermal fibroblasts) were equally affected by anthralin *in vitro* (data not shown). This indicates that the mechanism is not simply a selective cytotoxicity against infiltrating lymphocytes around the hair follicle.

The mechanism of anthralin on hair growth is currently unknown. AA is believed to be an autoimmune disorder and various cytokines have been suggested to be associated with the condition. We have shown in this study that IL-1β is mainly stimulated by anthralin, while TNF-α/β are inhibited in the majority of the responding mice by anthralin treatment. Most likely, overexpression of Il-1β by anthralin is associated with anthralin's inflammatory side-effects. The correlation between the TNF-α/β and anthralin's efficacy is very intriguing as suppression of these cytokines have been associated with the improvement of various autoimmune conditions (28,29). In a human study, an aberrant *in situ* expression of IFN-γ, IL-2 and IL-1β occurs in untreated AA, and successful topical immunotherapy (DPCP) is followed by increased mRNA levels of transforming growth factor-β1 (TGF-β1), IL-2, IL-8, IL-10 and TNF-α as well (25). The discrepancy can be due to either different therapies or different approaches used. We used RPA which is a more quantitative and accurate assay compared which RT-PCR. Our group has performed a similar experiment on another rodent model, the Dundee experimental bald rats (DEBR). All the rats (a total of 15) responded to anthralin therapy. The pattern of cytokine expressions in rat skin was different. IL-1α/β, IL-10 and IL-1Ra were increased by anthralin treatment in all the rats tested. TNF-β was not consistently detected from the rat skin. TNF-α and IFN-γ were reduced by anthralin treatment (Tang and Shapiro, data not published). The cytokines modulated by different therapies in two animal models and patients are summarized in Table 2. Whether different cytokine profiling is associated with the different responses between these two animal models needs to be investigated further. Alternatively, each rodent species may represent a different type or subtype of AA with different mechanisms involved.

In conclusion, anthralin is a promising therapeutic reagent for rodent models with AA. It appears that downregulation of TNF-α/β may be responsible for anthralin's therapeutic effects in the mouse AA model. Further study is needed to explore the mechanisms of anthralin on hair regrowth in rodent animal models.

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