Immunohistological Evaluation of Alopecia areata Treated with Squaric Acid Dibutylester (SADBE)

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Johansson E, Ranki A, Reunala T, Kianto U, Niemi K-M. Immunohistological evaluation of alopecia areata treated with squaric acid dibutylester (SADBE) Acta Derm Venereol (Stockh) 1986; 66: 485–490.

A total of 19 patients with alopecia areata volunteered for serial biopsies of the scalp skin during SADBE treatment. Regrowth of terminal hair was seen in 12 of the 19 patients on the side of the scalp treated with SADBE for a minimum of four months, but not on a control side treated with sodium lauryl sulphate (SLS). Immunocompetent cells were characterized with ANAE staining and monoclonal antibodies in biopsy specimen showing marked peribulbar and perivascular inflammatory cell infiltrates. Inflammatory cell sub-classes were repeatedly evaluated during SADBE treatment. No specific alterations in lymphocyte subclasses and macrophages were seen in relation to the hair growth response. In immunofluorescent studies it was found that in patients with regrowth of hair, immuno-globulins, fibrin and complement, appeared in the hair bulb and along the basement membrane during the treatment. Results were negative in patients without regrowth of terminal hair and in SLS-treated skin. As we could not demonstrate that any cell-mediated mechanism was involved, we suggest that SADBE may induce terminal hair growth through as yet uncharacterized mediators. *Key words: Alopecia; Immunocompetent cells; Immunofluorescence studies.* (Received March 10, 1986.)

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There is increasing, although for the most part circumstantial, evidence that immunological factors are important in the pathogenesis of alopecia areata (1, 2). The type of immune mechanism involved, however, is not known. So far a specific anti-hair follicle antibody has not been found. Immunofluorescent studies of the presence of immunoglobulins or complement components or both in the hair follicles have given conflicting results (3, 4, 5, 6, 7).

A lymphohistiocytic infiltration around and invading the hair bulb has been demonstrated (8, 9) and the inflammatory cells have been shown to consist mainly of T-lymphocytes (10, 11, 12, 13). The role of cell-mediated immune response has further been expressed by the fact that contact-sensitizing agents such as dinitrochlorbenzene (DNCB) and squaric acid dibutylester (SADBE) may induce hair growth in patients with alopecia areata (14, 15, 16, 17).

It has been suggested that contact hypersensitivity reaction might induce a functional change in the local T-cells, thus resulting in the regrowth of hair (18, 15). In this study we have examined the effect of squaric acid dibutylester (SADBE) treatment on the inflammatory cell infiltrate and on the immunofluorescent findings in the scalp skin of patients with alopecia.

PATIENTS AND METHODS

Patients

Nineteen patients volunteered for the study. The clinical data of the patients are presented in Table I. Altogether, nine of the patients have had several attacks of alopecia.

Table I. *Clinical data on the 19 patients with alopecia*

AA = alopecia areata, AT = alopecia totalis, AU = alopecia universalis

Type of alopecia	No.	F	M	Age in years mean (range)	Duration of alopecia in years mean (range)	Duration of mean (range)
AA	6	4	2	37 (31–44)	10 (2–35)	4 (2–5)
AT	7	4	3	31 (20–53)	8 (2–25)	3 (2–4)
AU	6	0	6	32 (14–55)	8 (2–22)	3 (2–5)
Total	19	8	11	31 (14–55)	8 (2–35)	3 (2–5)

SADBE treatment

The patients were sensitized according to the same method as has been described for DNCB and slightly modified by us (19, 20).

All the patients who volunteered for the study could be sensitized. The following concentrations of SADBE in acetone were prepared for treatment purposes: 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001 %. At the beginning the concentrations were adjusted according to the patient's response to the challenge test.

The treatment was continued every week and concentrations were selected so that mild contact dermatitis was maintained on the treated side of the scalp. The other side of the scalp was treated with aqueous solution of 10% sodium laurylsulphate (SLS) as often as was necessary to achieve a mild erythema and was continued until a clear difference between the sides could be observed. Both sides were then treated with SADBE. Clinical evaluation of the hair growth was carried out monthly. Biopsy speciments of the scalp skin were taken before the treatment, as soon as regrowth of terminal hair was observed and, in the cases with no response, before treatment was terminated. Biopsies were also taken from the SLS-treated scalp skin.

Immunohistological studies

Biopsy specimens were processed for routine haematoxylin-eosin staining, and for ANAE (alpha-naphthyl-acetate esterase) and immunoperoxidase staining. For immunofluoresent studies, the speciments were snap-frozen and cut in cryostat (4 to 5 μm). At least five sections were examined for in vivo bound immunoglobulins (IgA, IgG, IgM) complement (IC1q, C_3) and fibrin using FITC conjungated antisera (Behringswerke, Marburg, F.R.G.).

Inflammatory cell subclasses were further evaluated in biopsy speciments showing marked follicular or peribulbar infiltrates in routine histological examinations. At the beginning of the study the ANAE marker was used to characterize T-lymphocytes (dot-like staining), B-lymphocytes (negative staining) and macrophages (diffuse staining) (21). For demonstration of ANAE the specimens were fixed in Baker's formol calcium and processed as previously described (22). Later on, serial biopsies of four patients undergoing the treatment were also studied with monoclonal (OKT-3, OKT-4, OKT-8 and OKT-6) antibodies (Ortho Pharmac. Corp., Raritan, New Jersey) and the immunoperoxidase method. Specimens were snap-frozen in liquid nitrogen and stained with the appropriate monoclonal antibody and avidin biotin peroxidase method (Vectasin, Vector Lb., Burlingame, California) as previously desribed (12).

For the demonstration of intracellular Ig-containing cells the biopsy specimens were fixed in Bouin's fluid, processed in paraffin sections, incubated with rabbit antihuman IgA, IgG or IgM (Dakopatts, Copenhagen, Denmark) and stained with peroxidase-anti-peroxidase (PAP, Dakopatts).

RESULTS

In 12 of the 19 patients, growth of terminal hair was seen on the side of the scalp treated with SADBE after a treatment period of four to 19 months. On the SLS-treated side thin vellus hairs were occasionally observed. Permanent regrowth of hair, when both sides of the scalp were treated with SADBE, was achieved in eight patients.
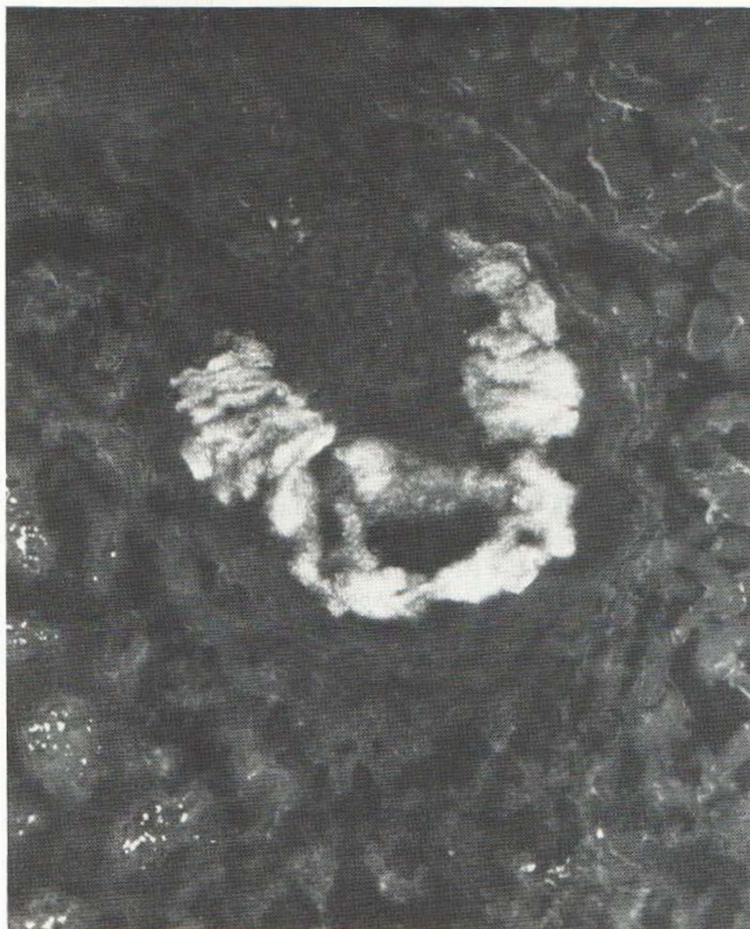
Fig. 1. Complement (C_3) deposition in the hair bulb area of SADBE treated alopecia patients. Direct immunofluorescence; original magnification ×60.

Immune deposits

In the biopsy specimens taken before SADBE treatment minimal deposits of immunoglobulin IgM were found in two and C_3 in one of 19 cases examined. During the treatment period a positive finding was noted in another eight patients (Table II). The most common finding was the appearance of fibrin (ten patients) and C_3 (seven patients) in the hair bulb (Fig. 1) and along the basement membrane zone. No C1q deposits were seen. Eleven of these 12 patients showed a terminal hair growth which turned to be permanent in eight cases (Table II). None of the patients with a negative immunofluorescence finding showed a permanent growth of hair. Immune deposits were not found in the 12 biopsy specimens taken from the SLS-treated scalp skin.

Histological examinations before therapy showed peribulbar and periadnexal inflammatory cell infiltrates in five of six cases with AA, in five of seven cases with AT and in four of six cases with AU, whereas mononuclear cell infiltrates within follicle bulb epithelium were seen in three cases of AA and in one case of both AT and AU. Serial biopsies were available in 15 cases in seven of which the peribulbar inflammatory cell infiltrate had decreased or disappeared during the treatment. Five of these seven showed regrowth of terminal hair, which in four of them was permanent. The infiltrates remained stationary on two of the eight patients with permanent hair growth; one could not be evaluated.

Table II. *Results of direct immunofluorescence studies of the scalp skin during SADBE treatment*

In parenthesis the number of cases with terminal hair growth in each group

Type of alopecia	No. of cases	Number positive for *Immunoglobulins*				
		A	G	M	C_3	Fibrin
AA	6	0	1	2	4	5 (6)
AT	7	0	1	2	4	2 (4)
AU	6	0	1	2	1	2 (2)

Perivascular inflammatory cell infiltrates were seen before treatment in 11 cases and disappeared in four cases without any correlation to the terminal hair growth.

Inflammatory cell subclasses

Before SADBE treatment about half of the cells in the perivascular and peribulbar monoclear cell infiltrates showed dot-like staining, indicating that they were T-lymphocytes. The other half of the infiltrating lymphocytes were ANAE-negative, indicating that they were B-lymphocytes or possibly activated T-lymphocytes. About one third of all inflammatory cells expressed diffuse cytoplastic ANAE staining, which is typical of monocytes/macrophages. A decrease in the relative number of perivascular and peribulbar ANAE positive-lymphocytes during SADBE treatment was seen in four of eight patients with permanent hair growth. Increased numbers of T-cells were seen in three cases and in one there was no change. A uniform change in the number of ANAE negative-lymphocytes could not be seen. Serial biopsy specimens could be evaluated immunohistologically during SADBE treatment in five of the 11 patients without permanent hair growth. A decrease in the number of infiltrating ANAE positive-cells was seen in three cases and an increase in the number of ANAE negative-cells in three.

Immunoglobulin-containing cells could be examined in 12 specimens altogether. Before treatment some IgA- and IgG-containing cells were found. During SADBE treatment the number of Ig-containing cells decreased in four of seven patients with permanent regrowth of hair, whereas only one of four patients without response to the treatment showed a decrease in the number of Ig-containing cells. In four patients the inflammatory cell infiltrate could be characterized with monoclonal antibodies. Most of the cells were OKT-3 and OKT-4 positive, indicating that they were helper/inducer T-lymphocytes. The mononuclear cells invading the hair follicles were mostly OKT-4-positive but somewere also OKT-8-positive (supressor/cytotoxic cells). During SADBE treatment the relative number of infiltrating helper/inducer cells increased. There was no difference in the distribution of OKT-3, OKT-4 or OKT-8-positive cells within the infiltrates in the three cases that responded to the treatment and in one that did not respond.

DISCUSSION

Nineteen patients with AA, AT or AU were treated with SADBE and SLS. Regrowth of terminal hair which could be induced only with SADBE, was permanent in eight cases. Immune deposits in the hair bulb or along the follicular basement membrane zone were found to correlate with regrowth of terminal hair. The most common finding was the appearance of fibrin and complement. None of the patients with a negative immunofluorescence finding showed permanent hair growth and immune depostis were not found in the biopsy specimens taken from the SLS-treated scalp skin.

Reports on the presence of immune deposits in the scalp skin in AA have been contradictory. Some investigators have found complement, mostly C_3 and occasionally C_5 and C_9 (1, 4, 6) whereas others have not been able to confirm these results (3, 7). Some investigators have also found complement in normal scalp skin and it has been suggested that it is somehow connected with the hair cycle especially with the active growing phase (5).

Before treatment a rather sparse perivascular or peribulbar inflammatory cell infiltrate was seen in most of the cases, and sometimes they were both seen. In accordance with previous reports, inflammatory cells were seen to invade the hair bulb, mostly in the form of AA-type alopecia (1, 12). In some biopsy specimens taken during SADBE treatment the cell infiltrate was found to diminish or disappear with the regrowth of terminal hair, suggesting an active role of the local inflammatory cells in the disease process.

Before therapy inflammatory cell infiltrate was found to consist mainly of T-and B-lymphocytes, but macrophages were also seen. Some B-lymphocytes were also shown to be stimulated to Ig-containing plasma cells, which seemed, however, to decrease during SADBE treatment. A previous study recorded that in all types of alopecia the number of T-cells was greater peribulbarly than it was perivascularly (12). As a potent contact sensitizing agent, SADBE was expected to act through an efffect on especially T-lymphocytes (23, 24). However, it was found that in patients responding with permanent hair growth there was neither a consistent change in the number of ANAE positive lymphocytes, nor clear difference from the lymphocyte response in non-responding scalp skin. Although in some instances, activated T-cells may turn ANAE negative (25) no clear correlation of the amount of ANAE negative cells to the hair growth could be seen.

In the few patients examined with monoclonal antibodies, no clear change in the T-cell subclasses during the treatment was seen either, and further studies with monoclonal antibodies were not considered relevant.

PUVA treatment which is shown to alter the distribution and function of T-lymphocytes and antigen presenting cells (25, 26, 32, 33) induces hair growth in about 70% of the alopecia cases (28, 29). In our previous studies it was found that in patients treated with SADBE or PUVA the regrowth of hair correlated significantly with an increase in an active subpopulation of peripheral T-lymphocytes (active E rosette-forming cells) whereas the total number of T-cells decreased (E. J., unpublished data). Activated T-lymphocytes are known to produce soluble factors, lymphokines, regulating the immune response. It is possible that some of these factors also act on growth factors for follicular epithelial cells (30) and thus explain the connection between T-cell and hair growth responses.

The findings that immune deposits were present in the hair bulb and along the follicular basement membrane zone in all cases responding to the treatment, suggests that a local immune response might be a prerequisite for the regrowth of terminal hair. The deposition of complement and fibrin cannot be due merely to a local inflammatory process with increased vasopermeability, as a similar contact dermatitis was elicited in the non-responders and they did not show these deposits.

Further investigations are needed to estimate the significance of immune deposits induced by SADBE and the role of immunocompetent cells in the peribulbar infiltrate.

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