Successful Treatment of Alopecia Areata-Like Hair Loss with the Contact Sensitizer Squaric Acid Dibutylester (SADBE) in C3H/HeJ Mice

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A type of hair loss closely resembling human alopecia areata has been described in C3H/HeJ mice. In order to test the assumed analogy with human alopecia areata, we investigated the efficacy of treatment with the contact allergen squaric acid dibutylester. In 12 C3H/HeJ mice with alopecia areata an allergic contact dermatitis was induced and elicited weekly on one side of the back by topical applications of squaric acid dibutylester. Overt hair regrowth was observed only on the treated side of the back in nine of 12 mice. Histopathologic examination revealed a change in the distribution of the inflammatory infiltrate from a dense perifollicular lymphocytic infiltrate around the mid and lower regions of hair follicles in untreated skin to a uniform presence in the upper dermis in treated skin. Immunohistomorphometric studies revealed that treatment with squaric acid dibutylester increased the $CD4^+/CD8^+$ ratio from approximately 1:2 in untreated alopecia areata to 1:1 in treated alopecia areata. Additional immunohistochemical investigations showed an aberrant expression of major histocompatibility complex class I, major histocompatibility complex class II and intercellular adhesion molecule 1 on keratinocytes of the mid and lower parts of hair follicles in untreated alopecia areata. In successfully treated skin ectopic major histocompatibility complex class I and II expression was clearly reduced, whereas intercellular adhesion molecule 1 expression showed only minor changes. In conclusion, alopecia areata-like hair loss in C3H/HeJ mice responded to treatment with the contact sensitizer squaric acid dibutylester analogous to human alopecia areata. Moreover, successful treatment changes the aberrant expression of major histocompatibility complex class I and II in a way similar to that observed in human alopecia areata. These observations support the concept that alopecia areata-like hair loss in C3H/HeJ mice can be utilized as an appropriate model for the study of human alopecia areata. ***Key words: CD4/CD8/major histocompatibility complex class I/major histocompatibility complex class II. J Invest Dermatol 113:61–68, 1999***

Alopecia areata (AA) is a common human disease with a lifetime risk of 1.7% in the general population (Safavi *et al*, 1995). It is characterized by a reversible patchy hair loss that may sometimes lead to complete baldness. Histopathologic features of AA include perifollicular lymphocytic infiltrates involving the anagen hair follicles with subsequent miniaturization of these follicles (Gollnick and Orfanos, 1990). The pathogenesis of AA is poorly understood, but a peribulbar and intrabulbar accumulation of T lymphocytes (Perret *et al*, 1984) and an aberrant expression of intercellular adhesion molecule 1 (ICAM-1) and HLA-DR molecules on follicular keratinocytes and dermal papillae (Hamm *et al*, 1988; Nickoloff and Griffiths, 1991) provide evidence that an immune process is involved (Baadsgaard, 1991).

Induction and periodic elicitation of an allergic contact dermatitis is currently the most effective mode of treatment (Hoffmann and Happle, 1996). The mode of action, however, by which topical immunotherapy induces hair regrowth in AA remains to be elucidated. For obvious reasons, many therapeutic strategies cannot be tested in human patients with AA. Hence, a suitable animal model would be helpful to investigate the mechanism of the effect of topical immunotherapy and to develop new therapeutic approaches that should be more specific than the induction of an allergic contact dermatitis.

Several animal species have been reported to develop hair loss resembling human AA, including dogs, cats, horses, rodents, and nonhuman primates (Conroy, 1979; Michie *et al*, 1991; Sundberg *et al*, 1995; McElwee *et al*, 1998). In the larger species, AA is poorly characterized, the animals are outbred, and they are not readily available for study which makes them of little practical use as research models. A type of reversible hair loss, that closely resembles human AA both clinically and histopathologically, has been described in aging C3H/HeJ mice (Sundberg *et al*, 1994). This disease arises spontaneously with a

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Abbreviations: AA, alopecia areata; DP, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; SADBE, squaric acid dibutylester; B, bulbar region of the hair follicle; M, mid part of the hair follicle; U, upper part of the hair follicle; D, interfollicular dermis.

Table I. Primary antibodies, sources, host species, and dilutions

Antigen	Clone	Source	Species	Dilution
CD4 (L3T4)	RM4–5	Pharmingen	Rat	1:500
CD8 (Lyt-2)	53–6.7	Southern Biotechnology	Rat	1:500
MHC Class I	ER MP 42	BMA	Rat	1:200
MHC Class II	ER-TR 3	BMA	Rat	1:500
ICAM-1	KAT-1	ATCC	Rat	1:50

low frequency in mice beginning at about 6 mo of age, and in some colonies the expression frequency can approach 20% by 18 mo of age. Affected animals first develop patchy hair loss on both ventral and dorsal surfaces. Spontaneous hair regrowth occurs in 2%–3% of affected mice whereas 10% of affected mice develop subtotal or total hair loss. Histopathologic examination shows that these mice develop nonscarring alopecia with dystrophic anagen hair follicles surrounded by mononuclear cell infiltrates with a predominance of $CD8^+$ lymphocytes and small numbers of $CD4^+$ cells. Telogen hair follicles are not affected. Recently, circulating autoantibodies to hair follicle antigens, similar to those present in human patients with AA, have been found in C3H/HeJ mice with AA (Tobin *et al*, 1997).

In order to determine whether this mouse disorder can be treated in the same way as human AA we treated AA affected C3H/HeJ mice with the contact allergen squaric acid dibutylester (SADBE). Successfully treated skin was examined for histopathologic and immunologic changes comparable with those observed in humans.

MATERIALS AND METHODS

Animals Studies were conducted on 16 C3H/HeJ mice (15 females, one male) with AA-like hair loss (Sundberg *et al*, 1994). Twelve mice were treated by topical immunotherapy, and four mice received the vehicle alone. The group that received topical immunotherapy consisted of five mice with large bald patches and seven with total hair loss on their dorsal skin. In the control group, three mice had patchy and one had subtotal hair loss. Mice were provided by the Jackson Laboratory (Bar Harbor, ME). Animals were housed, one per cage, with controlled light cycles of 12–12 h light/dark and fed with acidified water (pH 2.8–3.2) and conventional diet (altromin 1324, Altromin GmbH, Lage, Germany) *ad libitum*.

Treatment Twelve mice were sensitized on a 1.0 × 1.0 cm area of the left side of the back with 2% SADBE in acetone. Starting 1 wk later, we treated the left half of the dorsal skin by weekly topical applications of 0.1%, 0.5%, or 1% SADBE in acetone. The right side of the back remained untreated. The concentration and total volume of the contact allergen was chosen individually until a moderately severe contact dermatitis, lasting for 2–3 d, was induced. Treatment was started with the lowest concentration (0.1% SADBE) and thereafter escalated individually until a moderately severe dermatitis occurred. The appropriate concentration of SADBE was maintained until the end of treatment. In this way one mouse was treated with 0.1% SADBE, six mice with 0.5% SADBE and five mice with 1% SADBE. Treatment was continued until complete regrowth on the treated side was achieved, but no longer than 5 mo (two mice were treated for 7 wk, three mice for 11 wk, two mice for 13 wk, two mice for 14 wk, two mice for 19 wk, one mouse for 21 wk). Four control mice were treated by weekly topical applications of acetone on the left side of the back for 10 wk. Morphologic changes were examined and documented daily. Photographs were taken before, during, and after treatment. At the end of treatment two investigators who were not involved in the treatment of mice categorized the animals as responders or nonresponders according to macroscopic examination. Only those mice showing hair regrowth limited to the treated side were regarded as successfully treated.

Histopathologic examination At the end of treatment mice were anesthesized and their dorsal skin was shaved. Afterwards the animals were killed by cervical dislocation. Representative portions of both treated and untreated skin were removed and snap-frozen in liquid nitrogen. Eight to 10 μm vertical sections were stained with hematoxylin–eosin and examined by light microscopy. In order to determine the localization of perifollicular and intrafollicular infiltrates their presence in the following four compartments was analyzed and compared in treated and untreated skin of every mouse: B, in and around the bulbar and suprabulbar region of the hair follicle; M, along the mid part of the hair follicle between bulb and sebaceous gland; U, along the upper part of the hair follicle above the sebaceous gland; D, in the interfollicular space of the dermis (**Fig 5**).

Immunohistochemistry Untreated and treated skin was snap-frozen in liquid nitrogen for immunohistochemistry immediately after the animals were killed. Frozen vertical sections, 8–10 μm thick, were fixed in acetone for 10 min at –20°C and air dried. Between all incubation steps sections were washed three times for 5 min with Tris-buffered saline (0.05 M, pH 7.6). Non-specific binding was blocked by application of an avidin–biotin blocking kit solution (Vector Laboratories, Burlingame, CA) as well as by 2% bovine normal serum and 2% goat normal serum in Tris-buffered saline. Thereafter, the slides were incubated with the primary antibody, diluted in Tris-buffered saline containing 2% normal bovine serum at room temperature for 1 h. Sources, host species, and concentrations of primary antibodies used are listed in **Table I**. After washing, sections were incubated with the secondary, biotinylated goat anti-rat antibody (Dianova, Hamburg, Germany), diluted 1:150 in Tris-buffered saline containing 4% mouse normal serum and 2% goat normal serum for 30 min at room temperature. After washing a routine staining method for avidin–biotin complex labeled with alkaline phosphatase was used (Vector Laboratories, Burlingame, CA) and counterstained with Mayer's hematoxylin. Negative controls included replacement of primary antibody by normal rat IgG.

For major histocompatibility complex (MHC) class I and II, and ICAM-1 the intensity and distribution of staining was assessed by use of the following scoring system: *no expression*; *mild expression*, weak or intermediate intensity of staining of the minority (<50%) of hair follicles; *moderate expression*, weak staining of the majority (>50%), or intense staining of the minority of hair follicles; *severe expression*, intermediate or intense staining of the majority of hair follicles. To analyze anatomic and functional different regions of the hair follicle, four parts of it were assessed separately: P, dermal papilla; B, bulbar and suprabulbar region; M, mid part of hair follicle between the bulb and sebaceous gland; U, upper part of hair follicle above the sebaceous gland. In the epithelial compartments, staining patterns of inner (IRS) and outer (ORS) root sheath were examined separately. Immunoreactivity of the dermis was assessed qualitatively. In this way, MHC class I and II and ICAM-1 expression was compared in skin of untreated and treated AA as well as in normal anagen skin obtained from three unaffected C3H/HeJ mice. Because of the heterogeneous expression of the assessed surface molecules, a comparison was made in each individual mouse before and after therapy.

Histomorphometry This technique was performed to determine the number of perifollicular and intrafollicular $CD4^+$ and $CD8^+$ lymphocytes. Video images of the entire length of longitudinally cut hair follicles were stored by means of a Lucia M image analysis system (Nikon, Düsseldorf, Germany). Perifollicular and intrafollicular immunoreactive cells were counted on digital images at 10-fold magnification using LUCIA M software version 2.995β (Nikon). At least five intact longitudinal hair follicles were analyzed in serial sections for $CD4^+$ and $CD8^+$ lymphocytes on their entire length below the sebaceous gland. The mean cell numbers of positive cells per hair follicle served as a basis for calculating the $CD4^+/CD8^+$ ratio before and after therapy for every mouse.

Statistical analysis Statistical significance of unilateral hair regrowth was estimated using the Mann–Whitney U test. The changes in the number of perifollicular $CD4^+$ and $CD8^+$ cells before and after treatment as assessed by histomorphometry were analyzed by t test for paired samples.

Figure 1. AA-like hair loss in C3H/HeJ mouse was successfully treated unilaterally with SADBE. Distinct hair regrowth on the left side of the back treated with SADBE for 15 wk. The untreated right side served as a control and remained bald.

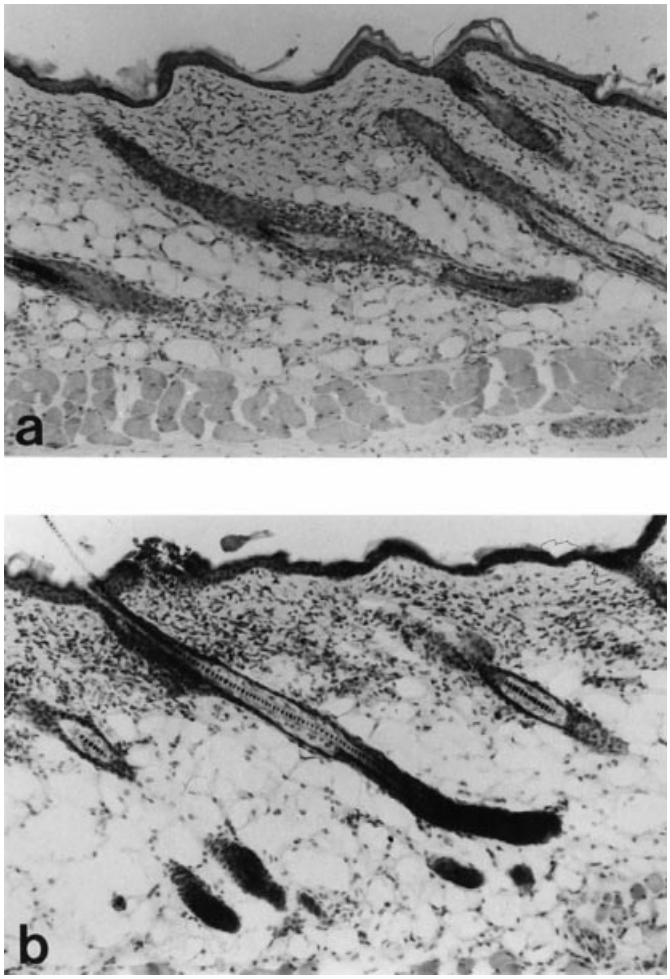

Figure 2. After treatment the lymphocytic infiltrates no longer show a predilection for the perifollicular area at the mid region of hair follicles but are diffusely distributed throughout the dermis. Staining of cryostat sections with hematoxylin and eosin. (*a*) Dystrophic anagen hair follicle with dense perifollicular lymphocytic infiltrate between the bulb and sebaceous gland (compartment M) in untreated AA. (*b*) Widespread lymphocytic infiltrate in perifollicular and interfollicular dermis in successfully treated skin.

RESULTS

Unilateral hair regrowth occurred after SADBE treatment After elicitation with the individually determined concentration of SADBE all mice treated with SADBE developed a mild to moderately severe contact dermatitis strictly limited to the treated side of the back. The allergic response was characterized by erythema 1 d after application of SADBE in five of 12 mice. After 2 d in all mice increasing scaling and excoriations lasting for 3 d were noted. Subsequently, the signs of contact dermatitis decreased until the next treatment.

In nine of 12 mice a dark pigmentation of some areas of the treated side appeared after 3–12 wk of treatment whereas the untreated side remained pink or gray. Shortly thereafter definite hair regrowth was observed initially in the pigmented regions and subsequently in a normal density. The untreated side did not show any pigmentation or hair regrowth (**Fig 1**). Hair regrowth continued during the following 4 wk of treatment. These nine of 12 mice were classified as successfully treated ($p \leqslant 0.01$).

In two of 12 mice with patchy alopecia, pigmentation and regrowth occurred on both sides but were more pronounced on the treated side. One of 12 mice did not show any regrowth after 5 mo of therapy. In this mouse a severe contact dermatitis with subsequent scarring had developed after application of 1% SADBE. Four of four control mice did not show any eczema or unilateral hair regrowth.

Lymphocytic infiltrates predominate in untreated skin around the lower parts of hair follicles and in treated skin in the dermis Histopathologic examination revealed that untreated skin (**Figs 2*a*** and **5*b***) contained anagen hair follicles showing a dense perifollicular and sometimes intrafollicular lymphocytic infiltrate with an admixture of some granulocytes. Most of the affected hair follicles were reduced in size and showed hair shaft dystrophy as well as pigmentary incontinence. In five of nine mice the infiltrate was present in the bulbar and suprabulbar region and along the hair follicle up to the sebaceous gland (compartment B and M), in four of nine hair follicles it was exclusively present along the hair follicle between bulb and sebaceous gland (compartment M). In successfully treated skin (**Figs 2*b*** and **5*c***) the inflammatory infiltrate consisting of lymphocytes and neutrophils showed a rather diffuse distribution throughout the interfollicular space of the dermis (compartment D) and a less pronounced presence along the mid and upper parts of the hair follicles (compartment M and U) in seven of nine mice (**Table II**). In two of nine successfully treated mice a diffuse dermal infiltration was similarly present but the perifollicular infiltrate remained unchanged. Most of the hair follicles were in anagen and of normal size. The epidermis showed consistent thickening and orthokeratosis and parahyperkeratosis. Pigmentation was apparent in the interfollicular epidermis with no restriction to the hair follicle. Occasional pigmentary incontinence was noted in the dermis. Skin biopsy of the mouse that did not show any hair regrowth revealed scarring features in the upper dermis.

$CD4^+/CD8^+$ ratio increased after SADBE treatment In untreated skin $CD8^+$ cells predominated over $CD4^+$ cells in the perifollicular lymphocytic infiltrate with a mean $CD4^+/CD8^+$ ratio of 1:2. Furthermore, $CD8^+$ cells were localized mainly in the center of the infiltrate in close vicinity to, or within, the hair follicle epithelium (**Figs 3*b*** and **5*b***). $CD4^+$ cells were more numerous in the periphery of the infiltrate (**Figs 3*a*** and **5*b***). After SADBE treatment, the number of infiltrating $CD4^+$ and $CD8^+$ cells decreased (**Figs 3*c*, *d*** and **5*c***), which was due to a reduction of $CD8^+$ cells, whereas the number of $CD4^+$ cells had slightly increased (**Table III**). A mean $CD4^+/CD8^+$ ratio of 1:1 after SADBE treatment reflects a substantial change within perifollicular inflammatory infiltrates.

Hair follicle epithelium in mouse AA showed aberrant expression of MHC class I (Figs 4*a*, *b* and 5*a*, *b*) In unaffected anagen skin of all three healthy control mice a moderate MHC class I expression was found in the ORS of the upper part of the hair follicle above the sebaceous gland (U). The infrasebaceous parts of the hair follicle epithelium (B, M) were negative for MHC class I. The dermal papilla (P) showed a mild MHC class I expression. In AA skin the MHC class I expression of the upper part U was severe in all mice. Moreover, there was a severe expression of MHC class I on the ORS of the suprabulbar infrasebacous part (M) of all mice. In addition, a mild expression on the IRS of part M was seen in four of nine animals. In the bulbar region (B) a severe expression on the ORS for MHC class I was found in six of nine cases, a mild expression in one of nine and no expression in two of nine. The IRS of part B was negative in all animals. The dermal papilla showed a moderate or severe expression in seven of nine cases. The inflammatory infiltrate itself showed only weak MHC class I positivity in some cells.

Expression of MHC class I on hair follicle epithelium decreased after SADBE treatment (Figs 4*c* and 5*c*) When the degree of MHC class I expression in the three epithelial hair follicle compartments B, M, and U in each mouse before and after therapy was compared, a reduction or total absence of staining of part B and M, but no difference in the staining of part U was found in eight of nine mice. In one of nine mice no difference in MHC class I expression before and after therapy was noted (**Table II**). The dermal papilla (P) showed a reduced or absent staining for MHC class I in five of nine cases. As before therapy the perifollicular infiltrate showed only partial and faint expression.

MHC class II was expressed ectopically on hair follicle epithelium in murine AA (Figs 4*d*, *e* and 5*a*, *b*) In normal control mice a moderate or severe expression of MHC class II was found on the follicular epithelium of the suprasebaceous part U of the hair follicle. In the lower parts B and M only single cells situated in the connective tissue sheath showed expression of MHC class II (**Fig 4*d***). The dermal papilla P was negative in one of three control mice, and in two of three MHC class II expression was mild or moderate. In AA mice the MHC class II expression increased to a severe expression in the upper part U and the ORS of part M in all animals, a moderate or severe expression on the IRS of part M in six of nine mice and a mild to severe expression on the ORS of part *B* in eight of nine mice. The dermal papilla showed a mild or moderate staining for MHC class II in all cases. The infiltrating cells were stained very strongly for MHC class II.

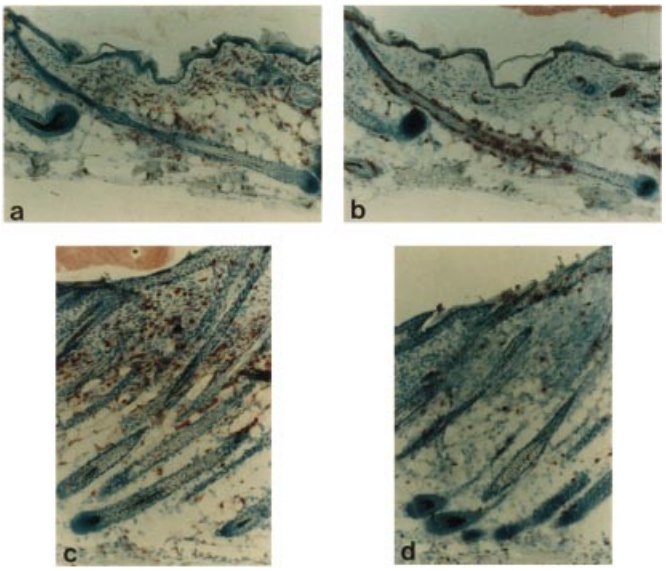

Figure 3. CD8$^+$ T cells predominate over CD4$^+$ T cells in the lymphocytic infiltrate of untreated mouse AA, and are reduced after treatment with SADBE. Immunohistochemical staining for CD4 (*a*, *c*) and CD8 (*b*, *d*) of untreated (*a*, *b*) and treated (*c*, *d*) murine AA. (*a*) Localization of CD4$^+$ cells mainly in the periphery of the infiltrate. (*b*) High number of CD8$^+$ cells, localized predominantly in the center of the infiltrate in close vicinity to, or within, the hair follicle epithelium. (*c*) Increased number of CD4$^+$ cells in the dermis and around upper parts of hair follicles. (*d*) Distinct reduction of perifollicular CD8$^+$ cells.

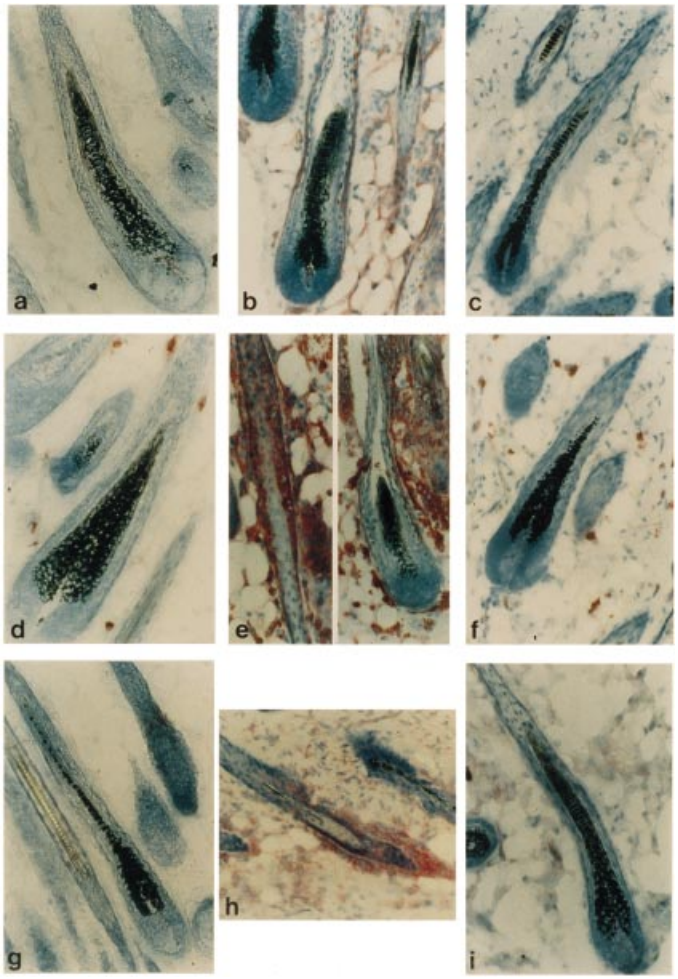

Figure 4. Aberrant expression of MHC class I and II and ICAM-1 in hair follicle epithelium is present in untreated mouse AA, whereas MHC class I and II expression is downregulated by SADBE treatment. Immunohistochemical staining for MHC class I (*a–c*), MHC class II (*d–f*), and ICAM-1 (*g–i*) of skin of healthy control mice (*a*, *d*, *g*), untreated mouse AA (*b*, *e*, *h*), and successfully treated skin (*c*, *f*, *i*). (*a*) No expression of MHC class I on keratinocytes of bulbar region (B) and weak expression on dermal papilla in a control mouse (P); (*b*) positive staining for MHC class I on keratinocytes of bulb (B) and dermal papilla (P) in untreated AA; (*c*) no staining for MHC class I in parts B and P after treatment; (*d*) only single MHC class II positive cells in the connective tissue sheath in a control; (*e*) positive staining for MHC class II on hair follicle epithelium of parts B and M in untreated AA; (*f*) negative staining for MHC class II after treatment; (*g*) no ICAM-1 expression in hair follicle bulb of a control; (*h*) intense staining for ICAM-1 of follicle epithelium of dystrophic anagen hair follicle, part M, in untreated AA; (*i*) no ICAM-1 expression on keratinocytes of part B in treated skin, weak staining of dermal papilla. Notice the intense positive staining for MHC class I and II and ICAM-1 of lymphocytes in untreated AA (*b*, *e*, *h*).

Expression of MHC class II on hair follicle epithelium decreased after treatment (Figs 4*f* and 5*c*) A longitudinal comparison of MHC class II expression on the follicular epithelium before and after topical immunotherapy in each mouse revealed a reduced or absent expression in part B and M after therapy in six of nine cases (**Table II**), whereas no difference could be observed in part U. Staining of the dermal papilla (P) was reduced or absent in only four of nine cases. The dermis contained a large number of cells that were strongly positive for MHC class II.

ICAM-1 is expressed on the epithelium of affected hair follicles (Figs 4*g*, *h* and 5*a*, *b*) In normal control mice there was no expression of ICAM-1 on the follicular epithelium, and the expression on the dermal papilla was only weak. In AA skin staining for ICAM-1 was mainly found in part M of the hair follicle in all animals with a mild to severe expression on the ORS in nine of nine mice and a mild expression on the IRS in six of nine. By contrast part U showed only a weak expression in all cases and on the ORS of part B ICAM-1 was mildly positive in only three of nine cases. The dermal papilla showed an expression of varying degree in eight of nine cases and no staining in one of nine mice. Many cells of the inflammatory infiltrate were positive for ICAM-1.

ICAM-1 expression in the suprasebaceous hair follicle epithelium increased after treatment (Figs 4*i* and 5*c*) An increased expression of ICAM-1 in the suprasebaceous part U was observed in five of nine mice after therapy. A reduced or absent ICAM-1 expression in part B or M could only be found in four of nine mice, whereas no change could be seen in five of nine mice (**Table II**). Staining of the dermal papilla was reduced or absent in four of nine cases. In the lower part of the dermis there was a large number of cells that were ICAM-1 positive.

DISCUSSION

The results of our study show that in C3H/HeJ mice with AA-like hair loss treatment with the contact sensitizer SADBE dissolved in acetone induces hair regrowth whereas control animals treated with the vehicle alone remained bald. Moreover, in order to rule out the possibility that hair regrowth was due to spontaneous remission, unilateral treatment was performed as described for topical immunotherapy in humans (Happle and Echternacht, 1977; van der Steen *et al*, 1991; Shapiro, 1993; Hoffmann and Happle, 1996). We were able to show that hair regrowth was restricted to the treated half of the dorsal skin in nine of 12 mice. In two mice with patchy hair loss we were not able to exclude a spontaneous remission, a well-known problem in human patients with this form of AA (Hoffmann and

Table II. Number of mice in whom SADBE treatment induced a change in the distribution of inflammatory infiltrates or a reduced aberrant expression of MHC class I and II, ICAM-1 in the lower parts B and M of the hair follicle

	Inflammatory infiltrate		MHC class I	MHC class II	ICAM-1
Changed distribution from around lower hair follicle to dermis	7/9	Reduced aberrant staining of the lower hair follicle	8/9	6/9	4/9
No change in distribution	2/9	No reduction of staining of the lower hair follicle	1/9	3/9	5/9

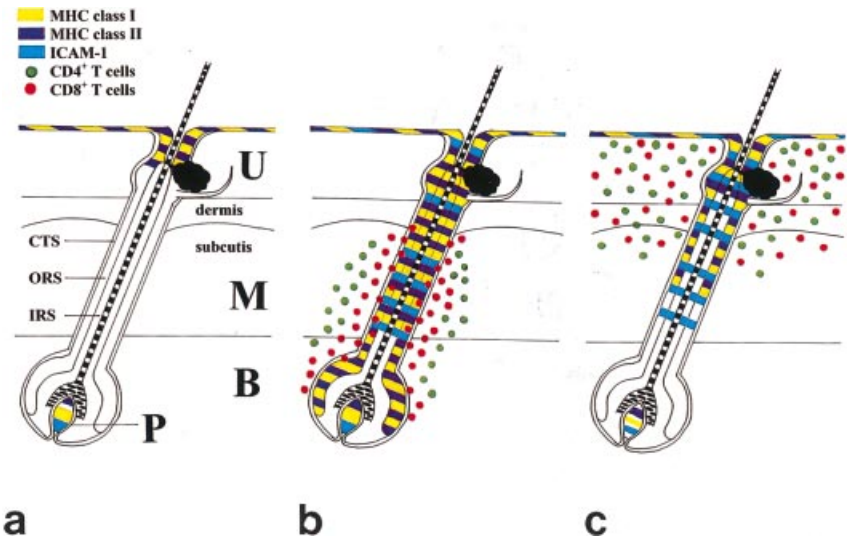

Figure 5. The perifollicular infiltrate of $CD4^+$ and $CD8^+$ T cells shows a shift from around lower hair follicles to dermis after SADBE treatment and this is accompanied by reduced abnormal expression of MHC class I and MHC class II on the epithelium of lower hair follicles. (*a*) Normal control mouse: MHC class I and II are only expressed in part U of the hair follicle epithelium. ICAM-1 is not expressed. (*b*) Untreated mouse AA: $CD8^+$ T cells predominate and are localized in the center of the perifollicular lymphocytic infiltrate. $CD4^+$ T cells in the periphery. MHC class I and II expression involves part U and M as well as the ORS of part B of hair follicle epithelium. ICAM-1 expression mainly involves part M and is weak on part U. Note the close spatial relationship between MHC class I and II and ICAM-1 expression and the lymphocytic infiltrate. (*c*) Successfully treated AA: $CD4^+$ and $CD8^+$ T cells in perifollicular and interfollicular dermis. MHC class I and II, and ICAM-1 are present in part U and M (MHC class I and II only in ORS of M). CTS, connective tissue sheath, SC, sebaceous gland, P, dermal papilla, U, upper, suprasebaceous part of hair follicle, M, mid part of hair follicle between sebaceous gland and hair bulb, B, bulbar and suprabulbar part of hair follicle.

Table III. Changes in the number of perifollicular infiltrating $CD4^+$ and $CD8^+$ cells before and after SADBE treatment[a]

	$CD4^+$ cells	$CD8^+$ cells	$CD4^+$ + $CD8^+$ cells	$CD4^+/CD8^+$ ratio
Before treatment	21.1 ± 3.9	36.4 ± 7.7	57.5 ± 9.1	1:1.9 ± 0.4 (1:2)
After treatment	23.2 ± 4.7	24.9 ± 8.5[b]	48.1 ± 12.4	1:0.95 ± 0.3 (1:1)[b]

[a]Cell numbers of positive cells per hair follicle and $CD4^+/CD8^+$ ratio are given as the mean ± SEM.
[b]$p \leq 0.05$.

Happle, 1996). Because mice treated with vehicle alone did not show any clinical signs of eczema, it could further be excluded that hair regrowth was caused by an irritant contact dermatitis.

Similar to humans treated with the contact sensitizer SADBE (Happle *et al*, 1980), mice can be sensitized with 2% SADBE, and challenged with individually varying concentrations of the substance, resulting in a contact dermatitis that lasts for several days. Owing to the hair cycle-dependent pigmentation of murine skin, erythema could not be observed in all mice whereas it is a major feature of contact dermatitis in humans. Scaling and itching as observed 2–3 d after treatment in humans, with a spontaneous resolution during the following 5 d, was observed after a similar period of time in mice as a reliable sign of successful induction of contact dermatitis. Hence, induction and elicitation of allergic contact dermatitis after SADBE treatment show similar dynamics in C3H/HeJ mice and humans. As an analogy to human AA treated unilaterally with SADBE (Happle *et al*, 1980), an allergic contact dermatitis was observed exclusively on the treated side of the back in all mice.

The period of weekly elicitation of contact dermatitis necessary to induce hair regrowth tends to be shorter in mice (4–12 wk) than in humans (8–12 wk) (Hoffmann and Happle, 1996). This may be explained by a shorter murine hair cycle. Darkening of the treated skin precedes hair regrowth in all mice. Pigmentation reflects the onset of anagen and initiation of a new hair cycle. The association of cutaneous darkening with the onset of anagen is well documented in mice (Chase *et al*, 1953; Chase, 1954; Paus *et al*, 1990). Although untreated AA skin and treated skin in the initial phase of hair regrowth both contain only anagen hair follicles, untreated AA skin showed a lighter color than treated skin. This difference may be due to a damage of hair bulb melanocytes in untreated AA hair follicles as it has been described in human AA (Tobin *et al*, 1990, 1991) and a normalized hair bulb pigmentation in treated skin. It cannot be ruled out, however, that the observed pigmentation is caused by postinflammatory pigmentary incontinence that was also seen microscopically. A postinflammatory hyperpigmentation is a rare but well-known side-effect of AA treatment with a contact allergen in humans (Hoffmann and Happle, 1996). A severe contact dermatitis with the development of crusts, as observed in one mouse, can be taken as another side-effect that may occur in humans as well, especially in the beginning of treatment (van der Steen *et al*, 1991; Hoffmann and Happle, 1996).

Histopathologic examination after therapy showed typical features of contact dermatitis. The change of the inflammatory infiltrates from around the mid and lower hair follicles to a rather diffuse distribution throughout the interfollicular space of the dermis and a less pronounced presence along the mid and upper parts of the hair follicles reflects a downregulation of the perifollicular lymphocytic infiltration after a long period of treatment with a contact sensitizer.

The perifollicular $CD4^+/CD8^+$ ratio in untreated murine AA was 1:2. This predominance of $CD8^+$ cells was previously noted by Sundberg *et al* (1994) who even found a $CD4^+/CD8^+$ ratio of 1:3. Moreover, we observed an affinity of $CD8^+$ cells to the center of the infiltrate in close vicinity to the hair follicle epithelium, whereas $CD4^+$ cells were mainly found at the periphery of the infiltrate. Hence, a pathogenetic interaction between $CD8^+$ T cells and the hair follicle epithelium may be assumed, whereas the infiltration of $CD4^+$ T cells may be a secondary phenomenon. Our results show that treatment with a contact sensitizer modulates the composition of the perifollicular lymphocytic infiltrate resulting in a change of the $CD4^+/CD8^+$ ratio from 1:2 to 1:1, due to a reduction of $CD8^+$ cells. A modulatory effect on the composition of the perifollicular infiltrate by treatment with a contact sensitizer has likewise been shown in humans (Happle *et al*, 1986). Differences between humans and mice arise from the fact that the perifollicular lymphocytic infiltrate in human AA consists mainly of $CD4^+$ T cells, resulting in a $CD4^+/CD8^+$ ratio of 4:1 in progressive, and 2:1 in stable untreated AA (Perret *et al*, 1984; Happle *et al*, 1986), or ranging between 5.3:1 and 1.5:1 as observed by other groups (Peereboom-Wynia *et al*, 1986; Macdonald Hull *et al*, 1991). After treatment with the contact allergen the $CD4^+/CD8^+$ ratio was decreased to 1:1 in human AA, presumably due to an increased number of $CD8^+$ cells, whereas this ratio increased in murine AA due to a reduction of $CD8^+$ cells. Whether this difference is meaningful concerning the immunologic pathways of treatment with a contact allergen remains unclear. Functional changes independent of the categorization into $CD4^+$ and $CD8^+$ cells, such as an altered profile of cytokine production of these cells, may be more important (Hoffmann *et al*, 1994).

Our immunohistochemical studies reveal that murine hair follicles affected by AA show a pathologic expression of MHC class I and II and ICAM-1. The lack of MHC class I and II expression on mid and lower hair follicle epithelium that we have observed in normal mice is consistent with the findings of other studies (Paus *et al*, 1994, 1998). Absence of ICAM-1 expression on keratinocytes in normal murine skin has also been described by other authors (Goebeler *et al*, 1990). In contrast, murine hair follicles affected by AA show aberrant expression of MHC class I and II and ICAM-1 on almost all parts of the follicular epithelium, and this was especially pronounced on the ORS of the middle part of the hair follicle between the bulb and sebaceous gland. The parts of the hair follicle that showed abnormal expression of surface molecules were surrounded by a lymphocytic infiltrate. Aberrant expression of HLA-ABC (MHC class I), HLA-DR, and HLA-DQ (MHC class II), and ICAM-1 has been described in human AA by several authors (Messenger and Bleehen, 1985; Bröcker *et al*, 1987; Hamm *et al*, 1988; Khoury *et al*, 1988; Gupta *et al*, 1990; Nickoloff and Griffiths, 1991; McDonagh *et al*, 1993). Differing from our observations in murine skin, these surface molecules were predominantly expressed on keratinocytes of the hair bulb, whereas staining of the subinfundibular epithelium was observed less often. This different localization of MHC class I and II and ICAM-1 expression is consistent with the differences between mice and men regarding the localization of perifollicular lymphocytic infiltrates. Lymphocytic infiltrates and aberrant expression of MHC class I and II and ICAM-1 are mainly found in the subinfundibular region of the hair follicle in mice whereas human AA is characterized by a peribulbar lymphocytic infiltrate and abnormal expression of surface molecules in this region. Whether the aberrant expression of one or more of these surface molecules is an initial event or only an epiphenomenon, as suggested by Khoury *et al* (1988), remains to be elucidated. But even if upregulation of surface molecule expression should follow after the lymphocytic infiltration, e.g., by cytokine induction, it might be an important pathogenetic factor in the maintenance

of the immune reaction affecting the hair follicle in AA. Anyhow, a close spatial correlation of aberrant surface molecule expression and lymphocytic infiltrate could be demonstrated in both human and mouse AA.

Our results show that after SADBE treatment there is a conspicuous reduction of aberrant MHC class I expression on hair follicle keratinocytes in mouse AA and a less marked but still distinct reduction of ectopic MHC class II expression. These findings are consistent with the down-modulation of abnormal HLA-ABC and HLA-DR expression as observed in human AA after treatment with the contact allergen diphenylcyclopropenone (Bröcker *et al*, 1987). In mouse AA the pronounced down-regulation of aberrant MHC class I expression after treatment can be explained by a reduced number of $CD8^+$ cells as compared with their abundance in untreated AA, especially in the center of the inflammatory infiltrates. This may reflect a specific interaction between $CD8^+$ T lymphocytes and MHC class I positive hair matrix keratinocytes in the pathogenesis of murine AA. Similar pathogenetic implications of an interaction between cytotoxic T lymphocytes and MHC class I expression on the hair follicle have been suggested in human AA (Bröcker *et al*, 1987). Whether this interaction is an initial event or an epiphenomenon is still unclear in both mouse and human AA.

The abnormal ICAM-1 expression was not downregulated by treatment with SADBE but rather was found to be increased on upper parts of the hair follicle. In contrast to the MHC class molecules, ICAM-1 was not expressed on the suprasebaceous part of the hair follicle in untreated AA, and for this reason we regard this upregulation as an inherent feature of allergic contact dermatitis (Goebeler *et al*, 1990). Because there is no further change in ICAM-1 expression on the hair follicle, especially not on those parts that are affected by the lymphocytic infiltrate, ICAM-1 expression does not seem to play an important functional part in the effect of SADBE treatment in these mice. The part of ICAM-1 expression during treatment with a contact sensitizer in humans is likewise poorly understood. ICAM-1 has been described to be expressed less frequently in AA patients who did not respond to diphenylcyclopropenone treatment, but no statistically significant decrease of ICAM-1 expression was observed in responders after therapy (Shapiro *et al*, 1993).

MHC class I and II and ICAM-1 expression on the dermal papilla does not seem to be momentous in mouse AA because it was likewise found in normal control mice, and because it was reduced after therapy in only about 50% of the mice. Moreover, the observed positive staining may simply be due to a less effective blocking of endogenous alkaline phosphatase activity, which is known to be very high in the dermal papilla of murine skin (Handjiski *et al*, 1994). Although some authors have described ectopic HLA-DR, HLA-DQ, and ICAM-1 expression in the dermal papilla in human AA (Messenger and Bleehen, 1985; McDonagh *et al*, 1993), most investigations of aberrant MHC class I and II and ICAM-1 expression in human AA focused on hair follicle keratinocytes rather than on the dermal papilla (Hamm *et al*, 1988; Bröcker *et al*, 1987; Khoury *et al*, 1988; Gupta *et al*, 1990; Nickoloff and Griffiths, 1991).

In conclusion, we have shown that AA-like hair loss in C3H/HeJ mice is characterized by aberrant surface molecule expression similar to those observed in human AA. Analogously, this murine disorder can be successfully treated with a contact sensitizer. The immunohistologic changes during therapy are comparable with those described in human AA treated with a contact sensitizer. Whether the slight differences in the localization of the perifollicular lymphocytic infiltrate and the reversed $CD4^+/CD8^+$ ratio are meaningful, remains to be proven. Anyhow, our study supports the concept that the hair disorder of these mice can be taken as a model of human AA in order to elucidate the underlying pathogenesis of the disease, to study the mode of action of current treatment, and to develop new therapeutic approaches.

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