

Autoantibodies to Hair Follicles in C3H/HeJ Mice With Alopecia Areata-Like Hair Loss

Desmond J. Tobin, John P. Sundberg,* Lloyd E. King, Jr.,† Dawnalyn Boggess,* and Jean-Claude Bystryn

The Ronald O. Perelman Department of Dermatology, New York University Medical School, New York, New York; *The Jackson Laboratory, Bar Harbor, Maine; and the †Division of Dermatology, Department of Medicine, Vanderbilt University, and Department of Veterans Affairs Medical Center, Nashville, Tennessee, U.S.A.

We have previously described spontaneous but reversible hair loss that clinically and histologically resembles human alopecia areata in a colony of C3H/HeJ mice. Alopecia areata in humans is associated with antibodies to hair follicles. This study was conducted to determine whether C3H/HeJ mice with hair loss have a similar abnormal antibody response to hair follicles. Eighteen C3H/HeJ mice with alopecia, 12 unaffected littermates, and 15 control mice were examined for circulating antibodies to C3H/HeJ anagen hair follicles by indirect immunofluorescence and against extracts of isolated C3H/HeJ and human anagen hair follicles by immunoblotting. Using both procedures, antibodies to anagen hair follicles were present in all C3H/HeJ mice with alopecia but in none of the control mice. The antibodies were also present in some unaffected C3H/HeJ littermates but were absent in mice of an unrelated strain with inflamma-

tory skin disease and alopecia, indicating that their appearance did not result from the hair loss. These antibodies reacted to hair follicle-specific antigens of 40–60 kDa present in murine and human anagen hair follicles. These antigens were also reactive with human alopecia areata antibodies. Some of the antibodies in both C3H/HeJ mice and humans with alopecia areata reacted to antigens of 44 and 46 kDa, which were identified as hair follicle-specific keratins. This study indicates that C3H/HeJ mice with hair loss have circulating antibodies to hair follicles similar to those present in humans with alopecia areata. These findings confirm that these mice are an appropriate model for human alopecia areata and support the hypothesis that alopecia areata results from an abnormal autoimmune response to hair follicles. **Key words:** autoantigen/immunofluorescence/immunoblotting. *J Invest Dermatol* 109:329–333, 1997

Alopecia areata (AA) is a common, reversible disease of hair follicles (HF) characterized by the spontaneous appearance of circumscribed areas of complete hair loss which, when severe, can result in loss of all scalp and body hair. Although the cause of AA is unknown, it is thought to result from an autoimmune response to HF (Bystryn and Tamesis, 1991). This possibility is strongly supported by recent observations that HF express unique antigens not found elsewhere in the skin (Tobin *et al*, 1994a) and that most individuals with AA have an abnormal antibody response to some of these antigens (Tobin *et al*, 1994b).

The absence of a well-characterized animal model for AA has impaired the study of this disease. Several animals develop patchy hair loss resembling AA, including dogs, cats (Conroy, 1979; Muller *et al*, 1983), horses (Conroy, 1979), rats,¹ and nonhuman primates (Conroy, 1979). Unfortunately, these models are poorly characterized, outbred, and/or are not readily available for use.

Recently, spontaneous hair loss that clinically and histologically resembles human AA has been described in aging C3H/HeJ mice (Sundberg *et al*, 1994). Like their human counterparts, affected animals develop spontaneous, reversible, circumscribed patches of nonscarring alopecia that is associated with a perifollicular mononuclear cell infiltrate.

This study, conducted to investigate the pathogenesis of alopecia in these mice, indicates that they have circulating autoantibodies to HF antigens that are similar to those present in human AA. This finding strongly supports the notion that AA is caused by an autoimmune response to HF and indicates that C3H/HeJ mice are an appropriate animal model for this disease.

MATERIALS AND METHODS

Animals Studies were conducted on 18 C3H/HeJ mice with alopecia (17 females, 1 male; mean age 8.5 mo; range 7–11 mo) (Sundberg *et al*, 1994) and 12 age-matched but unaffected littermates (mean age 8.6 mo; range 7–10 mo). Control animals (mean age 7 mo; range 6–9 mo) consisted of C3H/HeJ mice that did not have alopecia and were not littermates of mice with alopecia (5 females), C57BL/6J mice (5 females) with normal hair growth, and C57BL/6J-*Elh*/+ mice (4 females, 1 male) with inflammatory dermatitis associated with mild to severe alopecia. All mice were provided by The Jackson Laboratory (Bar Harbor, ME). Sera were collected from all mice and stored at –80°C until used.

Tissues Anagen HF were obtained from a C3H/HeJ mouse with alopecia and an unaffected age-matched littermate. The mice were anesthetized, depilated (Nair; Carter Products, New York, NY), and monitored for hair regrowth. Skin from areas of re-growing hair (anagen HF) and adjacent skin

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¹ Oliver R, Jahoda CAB, Horne KA, Michie HJ, Poulton T, Johnson BE. The DEBR rat model for alopecia areata. *J Invest Dermatol* 96:97S, 1991 (abstr.).

Reprint requests to: Dr. Jean-Claude Bystryn, Department of Dermatology, New York University Medical Center, Room H-356, 560 First Avenue, New York, NY 10016.

Abbreviations: AA, alopecia areata; HF, hair follicle.

Current address: Dr. Desmond J. Tobin, Department of Biomedical Sciences, University of Bradford, West Yorkshire BD7 1DP UK.

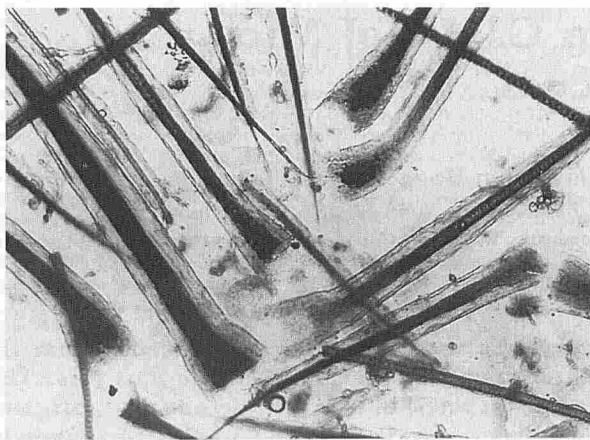


Figure 1. Purified HF obtained from a C3H/HeJ mouse with hair loss. HF were isolated from the dorsal skin in areas of anagen hair growth after removal of the dermis and 0.5% collagenase treatment.

(to serve as a control) was removed surgically. Five-micrometer frozen sections, containing longitudinally sectioned HF, were cut from each specimen for immunofluorescence studies. Immunoblotting studies were conducted on 6-M urea extracts of HF, adjacent epidermis and dermis, and control tissues including human HF, intestine, liver, spleen, and heart, as described previously (Tobin *et al*, 1994b). Briefly, the epidermis was separated from the dermis in 1 M sodium bromide (NaBr) (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C, and HF were isolated by digesting the dermis with 0.5% collagenase Type IV (Sigma) in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Intergen, Purchase, NY) for 1–2 h. The HF released from dermis by this treatment were collected by differential centrifugation at 350 × *g* for 5 min and washed repeatedly with Hank's balanced salt solution to remove contaminating nonfollicular dermal tissue until pure by microscopic examination (Fig 1). Harvested HF were placed immediately in 6 M urea in phosphate-buffered saline (PBS; pH 9.5) with 1 mM phenylmethylsulfonyl fluoride and 1 μg of leupeptin, pepstatin, antipain, and chymostatin per ml (Sigma) overnight at 4°C. Insoluble material was removed by centrifugation at 8800 × *g* for 15 min, and the supernatant was further clarified by centrifugation at 8800 × *g* for 5 min and then stored at –80°C until used. Control tissues including autologous epidermis, intestine, liver, spleen, and heart were similarly extracted, with 6 M urea protein content determined by dye-binding assay (Bio-Rad, Hercules, CA). Human scalp HF were plucked from the parietal scalp of a 30-y-old normal male and similarly extracted with 6 M urea as described previously.

Antibodies to Hair Follicles These were measured by indirect immunofluorescence and immunoblotting. For indirect immunofluorescence, 5-μm frozen sections of longitudinally sectioned skin were obtained from areas of anagen hair growth from an alopecic mouse and an unaffected littermate. The sections were blocked with 10% goat serum in Hank's balanced salt solution for 1 h and incubated individually for 30 min with test

sera diluted 1:50 in 1% goat serum. Sections were washed and then incubated for 30 min with fluorescein-conjugated goat anti-mouse IgG diluted 1:20 in 1% goat serum in Hank's balanced salt solution. Negative control sections were incubated with 1% goat serum in PBS in place of primary serum.

For immunoblotting, 6 M urea-extracted protein of isolated C3H/HeJ anagen HF was separated by sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis (PAGE). Approximately 7 μg protein per 50 μl sample buffer was loaded into each lane of 15 mini-gels. After electrophoretic separation, the HF proteins were electroblotted onto polyvinylidene difluoride microporous membranes (Imobilon; Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in PBS, pH 7.4 (blocking buffer), for 2 h and incubated for 12 h at 4°C with test sera diluted 1:50 in blocking buffer. The membranes were then reacted for 2 h with biotinylated goat anti-mouse IgG monospecific antisera (Organon Teknika, Durham, NC) diluted 1:100, followed by avidin-peroxidase (Organon Teknika) diluted 1:100 for 1 h, and then developed with 4-chloro-1-naphthol. Antibody level was assessed by quantitative densitometry using the Image-1 system to measure band density (Universal Imaging Corporation, Media, PA) (Smith and Sale, 1992) and was expressed as densitometric units.

Selective Immunoprecipitation of Hair Keratins Three micrograms of 6 M urea-extracted protein of murine anagen HF was incubated individually with 2 μl of undiluted sera, or with fetal bovine serum as a negative control, for 16 h at 4°C. Bound proteins were precipitated with protein A-Sepharose for 2 h at 22°C, washed extensively, and boiled at 100°C for 2 min in Laemmli's buffer. Eluted proteins were treated with 5% mercaptoethanol, separated by SDS-PAGE, and immunoblotted with monoclonal antibody AE13 to the 44- and 46-kDa HF-specific keratins (Lynch *et al*, 1986) (kindly provided by Dr. T-T Sun, New York University Medical Center, New York, NY).

Direct Immunofluorescence This was performed by a standard technique, using fluorescein-conjugated goat-anti-IgG+IgM+IgA antiserum (Organon Teknika) diluted 1:40 in 1% goat serum in PBS.

RESULTS

Characteristics of Hair Loss in C3H/HeJ Mice After selective breeding, approximately 15% of C3H/HeJ mice spontaneously develop hair loss that closely resembles human AA by 18 mo of age. Hair loss develops in sharply defined circular areas or diffusely on both dorsal and ventral surfaces. Inheritance is dominant with very low penetrance (Sundberg *et al*, 1994). As in human AA, alopecic lesions (Sundberg *et al*, 1994) contain dystrophic anagen HF surrounded by mononuclear cells consisting primarily of CD8⁺ and CD4⁺ T cells. Although white hair may be spared, all types of hair follicles are involved, and there may be focal hair-shaft defects. As in human AA, the inflammatory cell infiltrate is markedly reduced by intralesional injection of steroids, with subsequent regrowth of hair that may initially be white in the affected site. These mice have no evidence of thyroid dysfunction or an infectious etiology for the alopecia (Sundberg *et al*, 1994).



Figure 2. Indirect immunofluorescence localization of antigens targeted by HF antibodies in C3H/HeJ mice with hair loss. (A) HF antibodies in mouse number 4 react with antigens in the matrix of anagen hair bulb. (B) HF antibodies in mouse 10 react with the outer root sheath. (C) negative reaction with serum of a control A/J mouse with normal hair growth.

Table I. Hair Follicle Structures Targeted by Hair Follicle Antibodies

Diagnosis	Strain	No. of Mice	% Mice With Antibodies to ^a				
			Matrix	Outer Root Sheath	Hair Shaft	Inner Root Sheath	Follicular Papilla
Alopecia	C3H/HeJ	18	72%	62%	22%	11%	11%
Littermates of alopecia mice	C3H/HeJ	12	58%	42%	25%	17%	8%
Normal	C3H/HeJ	5	0%	0%	0%	0%	0%
Normal	C57BL/6J	5	0%	0%	0%	0%	0%
Alopecia	C57BL/6J-Eh/+	5	0%	0%	0%	0%	0%

^a Detected by indirect immunofluorescence using anagen C3H/HeJ mouse skin as substrate.

Detection of Antibodies to Hair Follicles in C3H/HeJ Mice With Alopecia by Indirect Immunofluorescence Antibodies to syngeneic anagen HF were detected at a titer of 50 or greater in 15 of 18 (83%) C3H/HeJ mice with alopecia and in 9 of 12 (75%) unaffected age-matched littermates, but in none of 15 age-matched control mice. The control mice included C3H/HeJ mice ($n = 5$) from the production colony from which the mice with alopecia were obtained, but which were not littermates of affected mice; C57BL/6J mice ($n = 5$) with normal hair growth; and C57BL/6J-Eh/+ mice ($n = 5$) with inflammatory skin disease and hair loss. The antibodies were directed in all cases to the lower one third of anagen HF (Fig 2) and did not react with the upper portion of anagen HF, with telogen HF, or with adjacent autologous epidermis or dermis. The HF structures targeted by these antibodies included the hair bulb matrix (Fig 2), the lower outer root sheath, the hair shaft, the inner root sheath, and/or the follicular papilla (Table I). Antibodies to these structures were present in 72%, 62%, 22%, 11%, and 11%, respectively, of mice with alopecia and in 58%, 42%, 25%, 17%, and 8% of unaffected littermates, but in none of the control mice. The antibodies reacted similarly to HF obtained from alopecia mice and unaffected littermates (data not shown).

Direct immunofluorescence studies were negative apart from rare deposits of Igs in the follicular papilla, which were present in 2 of 18 (11%) C3H/HeJ mice with AA and in 1 of 12 (8%) unaffected littermates, but in none of the control mice.

Identification of Antigens Targeted by Hair Follicle Antibodies Sera of C3H/HeJ mice with alopecia, age-matched unaffected littermates, and control mice were tested by immunoblotting against 6-M urea extracts of isolated anagen HF obtained from a C3H/HeJ mouse with hair loss. The results are illustrated in Fig

3 and summarized in Table II. Antibodies to HF were detected in all 18 (100%) C3H/HeJ mice with alopecia and in 9 of 12 (75%) unaffected littermates, but in only 1 of the 10 (10%) control mice with normal hair growth and in none of 5 C57BL/6J-Eh/+ mice with inflammatory dermatitis and alopecia.

The antibodies were most commonly directed to antigens of 50 kDa and 55 kDa. Antibodies to these two antigens were present in 100% of mice with alopecia and in 75% and 33%, respectively, of unaffected littermates, but in only 10% and 0% of 10 control mice. Antibodies to other antigens of 44/46 kDa and 60 kDa were also present in 36% and 75%, respectively, of mice with hair loss and in 0% and 60% of unaffected littermates, but in none of the control mice with normal hair growth. Occasional mice had antibodies to an antigen of 150 kDa. The average level of antibody to HF antigens in mice with hair loss was usually higher than in unaffected littermates (Fig 3). Similar results were obtained in additional experiments in which sera of six C3H/HeJ mice with alopecia and six unaffected littermates were reacted with HF extract from two other C3H/HeJ mice and three unaffected littermates.

Tissue Specificity of Hair Follicle Antibodies To examine the tissue distribution of the individual HF antigens defined by antibodies in C3H/HeJ mice with alopecia, a panel of four antibody-positive sera was reacted with 6-M urea extracts of freshly isolated autologous anagen HF, autologous adjacent dorsal skin epidermis (containing telogen HF), intestine, liver, spleen, and heart. The tissues were all obtained from the four serum donors, and the extracts were adjusted to the same protein concentration. The results obtained with a representative serum are illustrated in Fig 4. The 44/46-, 50-, 55-, and 60-kDa antigens were expressed only in extracts of anagen HF.

Relation Between Hair Follicle Antibodies in C3H/HeJ Mice With Alopecia and Those in Humans With AA We initially examined whether the HF antibodies in C3H/HeJ mice with hair loss react to antigens also expressed in human HF. C3H/HeJ and control sera were tested by immunoblotting, as described earlier, using extracts of isolated human anagen scalp HF as the antigen source. Murine antibodies to human HF were present in 17 of 18 (94%) C3H/HeJ mice with alopecia and in nine of 12 (75%) unaffected littermates, but in only one of 15 (6%) control mice (data not shown). These antibodies were predominantly directed to 46-, 50-, 52-, and 60-kDa antigens. Some sera also reacted with a 220-kDa antigen, but these antibodies occurred equally in both AA and control mice.

Subsequently, we conducted co-migration experiments in which sera of humans with AA and of C3H/HeJ mice with hair loss were tested concurrently for reaction to human and C3H/HeJ HF. As illustrated in Fig 5, both sets of antibodies reacted with co-migrating murine HF antigens of 40–60 kDa. Similarly, both AA patients and C3H/HeJ mice with alopecia reacted with co-migrating antigens of 40–60 kDa expressed in human HF.

Antibodies in C3H/HeJ Mice With Alopecia Are Directed in Part to Hair Follicle-Specific Keratins Because earlier studies have shown that human AA antibodies react in part with antigens of 44 and 46 kDa, which are immunologically related to

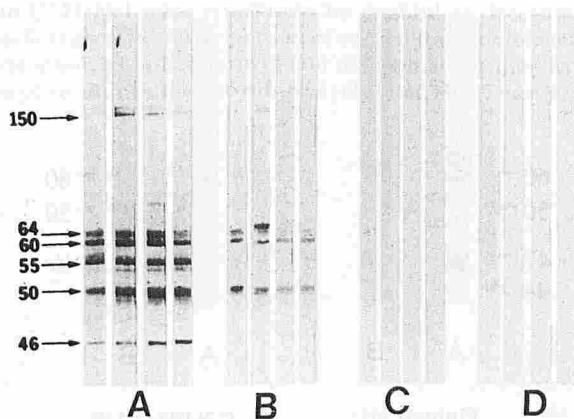


Figure 3. Immunoblot analysis of HF antigens defined by antibodies in C3H/HeJ mice with hair loss. Urea (6 M)-extractable proteins derived from isolated anagen HF of a C3H/HeJ mouse were separated by SDS 8%-PAGE and immunoblotted with individual sera of the following: C3H/HeJ mice with hair loss (lanes A), unaffected littermates (lanes B), control mice with inflammatory dermatitis and alopecia (lanes C), and control mice with normal hair growth (lanes D). Serum diluted 1:50.

Table II. Frequency of HF Autoantibodies in C3H/HeJ Mice With Hair Loss

Diagnosis	Strain	No. of Mice	(% With Hair Follicle Antibodies ^a)			
			44/46 kDa	50 kDa	55 kDa	60 kDa
Alopecia	C3H/HeJ	18	36%	100%	100%	74%
Littermates of alopecia mice	C3H/HeJ	12	0%	75%	33%	60%
Normal	C3H/HeJ	5	0%	10%	0%	0%
Normal	C57BL/6J	5	0%	0%	0%	0%
Alopecia ^b	C57BL/6J-Eh/+	5	0%	0%	0%	0%

^a Measured by Western immunoblotting using 6 M urea extracts of HF from AA mouse as source of extracts.

^b Alopecia secondary to inflammatory dermatitis.

HF-specific keratins, we conducted selective immunoprecipitation experiments to determine whether the murine 44/46-kDa antigens were similarly related to HF-specific keratins. Sera of four C3H/HeJ mice with alopecia and four age-matched C57BL/6J-Eh/+ mice with inflammatory dermatitis and hair loss were incubated with extracts of isolated mouse HF. Bound antigens were precipitated with protein A-Sepharose, eluted, separated by SDS-PAGE, and tested for reactivity with a monoclonal antibody (AE13) to HF-specific keratins. As shown in **Fig 6**, all C3H/HeJ mice with alopecia, but none of the controls, had antibodies that precipitated the 44/46-kDa HF-specific keratins.

DISCUSSION

The most important results of this study are as follows: (i) Abnormal autoantibodies to HF-specific antigens are present in C3H/HeJ mice with AA-like hair loss; (ii) these antibodies are similar to those present in humans with AA; and (iii) they are directed in part to the 44/46-kDa HF-specific keratin. These abnormalities closely mimic those in humans with AA, confirming that these mice are the appropriate model for AA and supporting the hypothesis that AA results from an abnormal autoimmune response to HF.

Human AA affects approximately 1–2% of the population and causes small to large, single to multiple, sharply demarcated areas of complete nonscarring hair loss. In severe cases, AA can progress to complete loss of all scalp and body hair. The hair loss is characterized by a disruption of the anagen stage of the hair cycle (Messenger and Bleehen, 1986) and is associated with a perifollicular/intrafollicular inflammatory cell infiltrate (Todes-Taylor *et al*, 1984). Although the cause of AA is unknown, it is suspected to be an

autoimmune disease. Recently, strong and direct support for this hypothesis has come from the observation that most patients with AA have high levels of IgG antibodies to HF-specific antigens that range in molecular weight from 40 to 60 kDa. Some of these antibodies are directed at the 44/46-kDa HF-specific keratins (Lynch *et al*, 1986) located in the upper cortical cells of the cycling portion of anagen HF (Tobin *et al*, 1996).

Recently, we described a disease of hair loss in a colony of C3H/HeJ mice that resembles human AA (Sundberg *et al*, 1994). As in AA, hair loss in these mice develops spontaneously in well-circumscribed patches and can become diffuse in severe cases; there is no scarring of the skin and no evidence of an infectious cause. Histologically, the changes resemble those in the human disease. The hair loss in both cases is characterized by dystrophic anagen HF surrounded by a mononuclear cell infiltrate consisting predominantly of CD4⁺ and CD8⁺ cells. As in human AA, hair regrowth can occur spontaneously, and the hair may initially be white.

The results of this study indicate that these mice have immunologic abnormalities that mimic those present in the human disease. Almost all C3H/HeJ mice with alopecia had high levels (titer ≥ 50) of circulating antibodies directed against antigens that are specifically expressed in HF and that could be detected by indirect immunofluorescence and immunoblotting. The antibodies appeared to be a primary event rather than secondary to antigens released from damaged HF, as they were also present in 75% of mice of the same strain that had not lost hair, and conversely they were absent in mice of unrelated strains that had severe inflammatory skin disease causing damage to HF and hair loss. As in the human disease, the antibodies in C3H/HeJ mice with hair loss were

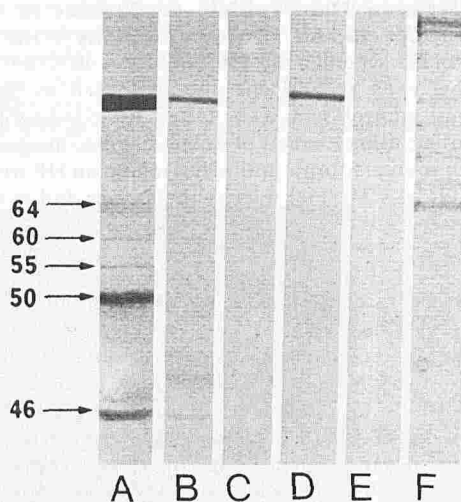


Figure 4. Tissue-specificity analysis of HF antibodies in C3H/HeJ mice with hair loss. Urea (6 M)-extractable proteins of HF (lane A), adjacent epidermis (lane B), large intestine (lane C), liver (lane D), spleen (lane E), and heart (lane F) were separated by SDS 8%-PAGE and immunoblotted with a C3H/HeJ serum containing HF antibodies, diluted 1:50.

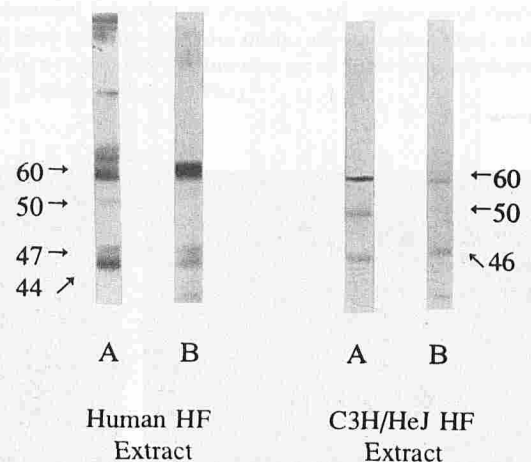


Figure 5. Co-migration of murine and human HF antigens defined by human AA antibodies and sera of C3H/HeJ mice with hair loss. Urea (6 M)-extractable proteins derived from isolated human or murine HF were separated by SDS 8%-PAGE and immunoblotted with human AA (lanes A) or C3H/HeJ serum (lanes B) diluted 1:50.

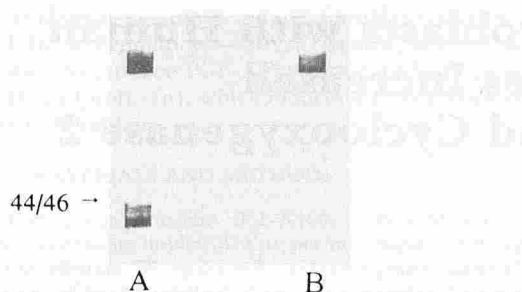


Figure 6. Selective immunoprecipitation of 44/46-kDa HF-specific keratins by antibodies in C3H/HeJ mice with hair loss. Murine HF proteins were immunoprecipitated by serum from C3H/HeJ mice with hair loss (A) and with normal hair growth (B), separated by SDS 8%-PAGE, and immunoblotted with monoclonal antibody AE13 to the 44/46-kDa HF-specific keratins.

directed at antigens that are HF-specific. By indirect immunofluorescence, the antibodies reacted with HF but not with adjacent epidermis or dermis; and by immunoblotting, they reacted with antigens present in extracts of isolated HF, but not in similar extracts prepared from autologous epidermis, dermis, or various internal organs.

The abnormal autoantibody responses to HF present in C3H/HeJ mice with alopecia and in humans with AA appear to be similar based on three sets of observations: (i) The HF antibodies in both mice and humans react with antigens of similar molecular weight (i.e., 44/46, 50, 52–55, and 60 kDa) that co-migrate on SDS-PAGE; (ii) both the mouse and human antibodies react with antigens that are expressed in both murine and human HF; and (iii) in both cases, some of these antibodies react with the 44/46-kDa HF-specific keratins. The antibody response to this particular keratin is unrelated to the “natural” cytoplasmic and keratin antibodies that are present in some normal persons because the “natural” antibodies react to keratins of 56–60 kDa, which are not HF-specific and occur with similar frequency in patients with AA and normal individuals (unpublished data), whereas antibodies to the 44/46-kDa antigen are present only in mice or humans with AA-like hair loss and are HF-specific. Thus, the autoantibodies to HF in C3H/HeJ mice do not appear to be an epiphenomenon produced against normally sequestered antigens released by damage to HF or by some other cause such as inflammation.

The role of the autoantibodies to HF in the pathogenesis of hair loss in C3H/HeJ mice remains to be established. Because these antibodies target the lower portion of anagen (but not telogen) HF, the site involved in hair growth and differentiation, they have the potential to disturb the growth or cycling of HF. Their presence

may explain why lesions in these mice and in humans with AA involve only anagen HF. Although it is always difficult to determine whether autoantibodies are a result or the cause of an autoimmune process, two sets of observations suggest that the HF antibodies in C3H/HeJ mice with hair loss are not a result of the hair loss. One is that the antibodies are absent in mice with severe inflammatory skin disease involving HF and causing hair loss. The other is that these antibodies are found in many C3H/HeJ littermates without hair loss but with an increased propensity to develop AA, indicating that their appearance precedes rather than follows hair loss. These observations suggest that the antibodies may be a cause rather than a result of hair loss. The possibility that cellular immune mechanisms are also involved in pathogenesis, as suggested by the presence of heavy infiltrates of T cells around hair follicles affected by AA, was not studied or excluded.

The correlation between the immunologic abnormalities in these mice and those present in human AA indicates that C3H/HeJ mice are an appropriate model for human AA and strongly supports the hypothesis that AA in humans results from autoimmune responses to HF.

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