

Alopecia Areata in Aging C3H/HeJ Mice

John P. Sundberg, Wade R. Cordy, and Lloyd E. King, Jr.*

The Jackson Laboratory, Bar Harbor, Maine; and *The Department of Medicine, Division of Dermatology, Vanderbilt University and Department of Veterans Affairs Medical Center, Nashville, Tennessee, U.S.A.

A disease closely resembling human alopecia areata was found in a large production colony of C3H/HeJ mice that had no evidence of thyroid dysfunction or an infectious etiology. Alopecia developed diffusely or in circular areas on the dorsal surface. Histologically, the changes in this non-scarring alopecia were limited to anagen follicles that were surrounded by mononuclear cells. This infiltrate, composed primarily of cytotoxic (CD8⁺) and helper (CD4⁺) T cells, was associated with follicular and hair shaft dystrophy. This infiltrate was markedly reduced by intralesional injection of triamcinolone acetonide with subsequent hair regrowth in the affected site.

Pedigree tracing of affected C3H/HeJ mice suggests that this non-scarring alopecia may be an inherited disease. Breeding results of normal haired mice with alopecia areata mice or between alopecia areata mice suggests that this is a

complex polygenic disease with a female predominance at younger ages. Female mice developed the disease earlier than male mice (3–5 versus >6 months), with equal numbers affected by 18 months of age. The relative incidence of alopecia areata in one production colony of C3H/HeJ mice was 0.25% for female and 0.035% for male mice, but selective breeding has raised the frequency to nearly 20%. The frequency in an aging colony selectively bred for inflammatory bowel disease reached 4.7%, with equal sex distribution, for mice over 18 months of age, suggesting that this might be a common aging change in C3H/HeJ mice. This C3H/HeJ mouse disease may prove to be a valuable animal model to study specific subtypes of human alopecia areata. *Key words: animal model/CD4⁺ cells/CD8⁺ cells/breeding studies. J Invest Dermatol 102:847–856, 1994*

Alopecia areata (AA) is a common disease of human beings that is characterized by acute onset of focal, diffuse, or both forms of alopecia. The histologic features consist of a dystrophic anagen or telogen follicle, usually accompanied by a mononuclear cell infiltrate in and around hair follicles, but usually without scarring [1–5]. The mechanisms involved in induction of alopecia areata, changes in coloration of hairs, and regrowth (if it occurs) are poorly understood.

It is impractical or unsuitable to use human volunteers for many types of experimental manipulation. Progress in understanding the underlying mechanisms of this disease and developing new therapeutic regimens are severely hampered by the lack of a readily available, reproducible animal model. Several animal model systems have been proposed, but detailed characterization (other than review of single biopsies) and lack of availability have made them of questionable value. These include comparable syndromes in Dachshund and Miniature Poodle dogs [6,7], Siamese cats [6], Palomino and Appaloosa horses [6], and possibly some non-human primates including stump-tailed macaques, white-fronted capuchin, and spider monkeys [6]. A rat model, the Dundee experimental bald rat (DEBR) has been described, which reportedly has many characteristics of alopecia areata and is believed to be a potentially useful model [8–10]. However, the affected rats described are a hybrid stock derived by a cross between the inbred BD1X and Wistar rats

so they are not inbred, the genetic nature of the defect has not been reported, and these rats are not readily available for study.

Inherited mouse mutations have proved to be extremely useful for defining basic pathogenic mechanisms, at all levels, for a large variety of human diseases [11–13]. In addition to being small and economical to maintain, inbred mice housed in a specific pathogen free (SPF) environment serve as biomedical tools for reproducible studies of human and domestic animal diseases. An inherited mouse mutation with a phenotype resembling AA would be an extremely valuable tool to investigate the fundamental mechanisms of alopecia associated with perifollicular mononuclear cell infiltrates, to determine the genetic causes of alopecia, and to provide a screening system for novel therapeutic approaches to the human disease. A large number of mouse mutations have been reported with various types of alopecia [14] so it was likely that one or more mutations would be found that had similarities to human alopecia areata.

We describe here a spontaneous disease that occurs in a large production colony and a small aging colony of C3H/HeJ mice with clinical and pathologic features closely resembling alopecia areata of human beings. Characterization of the disease process, preliminary genetic studies, and epidemiologic features within the colonies are presented.

MATERIALS AND METHODS

Animals Several large production colonies of C3H/HeJ mice, of various sizes, are maintained at The Jackson Laboratory (Bar Harbor, ME). Once criteria were established for the clinically detectable phenotype, animal technicians were taught to identify phenotypic deviant mice for detailed evaluation. This has provided a readily available resource of affected mice.

Adult mice, up to four per side, were housed in polycarbonate cages (50-in² floor area). For breeding colonies, a pair mating and their offspring were housed in similar cages. Young mice were weaned at 21 d of age. Cages were mechanically washed weekly and filled with an appropriate amount of sterilized white pine shavings. Water bottles were mechanically washed

Manuscript received October 1, 1993; accepted for publication January 14, 1994.

Reprint requests to: Dr. John P. Sundberg, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609-1500.

Abbreviations: AA, alopecia areata; DEBR, Dundee experimental bald rat; IBD, inflammatory bowel disease; SPF, specific pathogen free; T3, triiodothyronine; T4, thyroxine.

weekly and filled with acidified water (pH 2.8–3.2). Food hoppers, built into the cage lid, were filled weekly with a commercial diet (Old Guilford diet 96W for the research colony and 911A for the production colony; Emory Morrison Co., Old Guilford, CT). Cage lids were covered with a non-woven polyester filter. Animal rooms were equipped with controlled light cycles of 12:12 h light:dark.

Histopathologic Studies Mice were euthanized by CO₂ asphyxiation and photographed. Skin was depilated (Nair; Carter Products, New York, NY). Representative portions of dorsal, ventral, ear, muzzle (to include vibrissae), and tail skin, as well as the foot pad, were removed and fixed by immersion in Fekete's acid-alcohol-formalin solution. Fixed tissues were washed in tap water and transferred to 70% ethanol after 12 h of fixation [15]. Tissues were trimmed, processed routinely for histology, sectioned at 6 μ m, and stained with hematoxylin and eosin (H & E), periodic acid Schiff (PAS), Mallory's trichrome, Verhoeff's elastin, and Fontana-Masson for melanin.

Microbiology Ten breeding pairs of C3H/HeJ mice, 23–32 weeks of age, were obtained from production colonies of The Jackson Laboratory. These pairs were selected based on the criteria that at least one mouse of each breeding pair was affected with diffuse ventral alopecia and various degrees of dorsal alopecia. Mice were euthanized by CO₂ asphyxiation; hair was manually plucked. Affected skin from all mice was swabbed with 70% ethanol after shaving and prior to depilation. A 3-mm² area of skin was aseptically removed and homogenized in a Ten Broeck ground glass tissue grinder in sterile physiologic saline. Several drops of this homogenate were placed in tryptose phosphate broth with rabbit serum and incubated at 37°C. If any growth was observed, the medium was streaked onto blood agar plates and the organism identified. The homogenate was also streaked onto the surface of Sabouraud maltose agar plates. These were incubated at 30°C for 21 d. Plucked hairs were streaked and embedded into dermatophyte test media, which were incubated at 30°C for 21 d.

Serum was obtained from the mice and tested by enzyme-linked immunosorbent assay (ELISA) for the following mouse pathogens: pneumonia virus of mice (PVM), Sendai virus, mouse hepatitis virus (MHV), GD VII, minute virus of mice (MVM), ectromelia, lymphocytic choriomeningitis (LCM), reo 3, epizootic diarrhea of infant mice (EDIM), mouse cytomegalovirus (MCMV), mouse adenovirus (MAD), polyoma virus, *Mycoplasma pulmonis*, and *Mycoplasma arthritidis*.

Thyroid Function Blood samples were obtained by retro-orbital bleeding at the time of necropsy from 10 pairs of C3H/HeJ mice with or without alopecia areata and the harvested serum was stored at –20°C. Thyroid function of these mice was assessed by measurement of total thyroxine (T4) and tri-iodothyronine (T3) in serum samples using specific solid-phase radioimmunoassay (RIA) kits for each hormone (ICN Biomedicals, Irvine, CA).

Immunohistochemistry Skin from five 11-month-old, female C3H/HeJ mice with alopecia and five unaffected age-, sex-, and strain-matched mice was placed in OCT compound (Miles, Inc., Elkhart, IN) on a cork and snap frozen in an isopentane bath held in liquid nitrogen. Serial 5- μ m frozen sections were brought to room temperature for 1 h, quenched in acetone for 10 min, air dried for 10 min, and washed three times in 0.1 M trizma buffer (pH 7.2). Slides were then placed in 2% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) in trizma buffer for 10 min, then in 0.25% BSA in trizma buffer to reduce nonspecific staining. The primary antibody (GK 1.5, rat, CD4⁺ T-helper cells, 1:500 [16]; 53-67.2, rat, CD8⁺ cytotoxic T cells, 1:500 [17]. The Jackson Laboratory, Bar Harbor, ME) was applied and slides were incubated in a humidified chamber overnight at 4°C. Following incubation, slides were washed three times in trizma buffer containing 0.3% Superblock (Scy Tek, Logan, UT) as an additional step to reduce nonspecific staining. The appropriate secondary biotinylated antibody (Kirkegaard & Perry, Gaithersburg, MD) was placed on tissues and incubated for 30 min at 37°C. Slides were washed and streptavidin alkaline phosphatase (Kirkegaard & Perry) was applied and sections incubated for 45 min at 37°C. Slides were washed, the chromagen histomark red (Kirkegaard & Perry) was applied, and slides incubated for 30 min at 37°C. Slides were then washed in running tap water, counterstained with hematoxylin, and coverslipped. Trizma buffer replaced the primary antibodies as a negative control. Frozen sections of lymph nodes were used as positive control tissues.

Blood Collection and Preparation for Fluorescence-Activated Cell Sorting (FACS) Immediately prior to CO₂ asphyxiation, peripheral blood was obtained by retro-orbital bleeding from the same 10 mice used for immunohistochemical evaluation of skin. Cells were suspended in FACS medium (Hanks' balanced salt solution without phenol red, 0.1% BSA, and

0.1% sodium azide). Cells were centrifuged (1000 $\times$ g for 10 minutes) and resuspended in FACS medium. Aliquots of 50 μ l containing 10⁶ cells were dispensed into plastic tubes (Becton Dickinson, Brea, MA). For direct labeling, fluoresceinated monoclonal antibodies were added to the tubes (H57-597, hamster, $\alpha\beta$ T-cell receptor, 1:128, Pharmagen, San Diego, CA [18]; HO-13-4, mouse, Thy 1.2, 1:150 [19]; RA3-6B, rat, B220, 1:32 [20], F4/80, rat, macrophages, 1:128 [21]; GK 1.5, rat, CD4⁺ T-helper cells, 1:40 [16]; 53-67.2, rat, CD8⁺ cytotoxic T cells, 1:20 [17]; M5/114, rat, pan class II, 1:20 [22]; UC7-13D5, hamster, $\gamma\delta$ T-cell receptor, 1:2 [23]. The Jackson Laboratory Flow Cytometry Laboratory, Bar Harbor, ME). The solution was mixed thoroughly incubated for 30 min at 4°C, and washed twice with FACS medium. The supernatant was decanted and cells resuspended and washed. Indirect labeling of monoclonal antibody UC7-13D5 was accomplished by incubating for 30 min with fluoresceinated goat anti-hamster immunoglobulin (Ig) at 4°C and washed twice. Labeled cells were resuspended in 0.5 ml of FACS medium and analyzed on the cell sorter (Becton Dickinson).

Pedigree Tracing of Alopecic C3H/HeJ Mice Fourteen alopecic C3H/HeJ female mice were necropsied and confirmed by histologic evaluation to have the alopecia areata-like skin disease. The pedigrees were traced to determine if any of these mice had common ancestors.

Breeding Studies Four sets of breedings were set up. Alopecic C3H/HeJ male and female mice were crossed. Normal C3H/HeJ mice were bred with either an alopecic female or male mouse. Normal female and male mice from pedigreed lines with alopecia were also crossed. Offspring in three successive generations were followed until the mice were at least 1 year of age. Those with alopecia were necropsied and histopathology was done to confirm the presence of lesions compatible with AA.

A second breeding study was set up with clinically normal C3HeB/FeJ mice bred with affected C3H/HeJ male and female mice. C3HeB/FeJ mice in the production colonies have not been observed to develop alopecia with age. The close genetic relationship of the two strains [24] would minimize the effects of background modifying genes. Breeding to an unrelated, unaffected strain would ensure that the mutant genes came only from the C3H/HeJ mice with alopecia areata.

Genetic Quality Control To determine whether or not the alopecia was secondary to genetic contamination or due to a mutation at a locus routinely used to verify genetic quality in C3H/HeJ mice, serum was collected from two female mice with alopecia areata and two age- and sex-matched normal haired mice from one of the production colonies. The mice were assayed for 23 isoenzymes using standard genetic quality control methods [25] and a micro-cytotoxic assay for H-2 [26] to ascertain their genetic integrity.

Due to a mutation that occurred nearly 25 years ago, C3H/HeJ mice respond poorly to lipid A, the immunoreactive structure of the lipopolysaccharide (LPS) molecule, which is found in all Gram-negative bacteria [27]. To determine whether this response was altered in mice with alopecia, age-matched female mice (C3H/HeJ confirmed to have alopecia areata [N = 5], C3H/HeJ normal [N = 2], and C3H/HeSnJ [N = 1]) had splenic lymphocytes removed. Briefly, splenic cultures from C3H/HeJ mice were established in triplicate. To each culture various concentrations of lipopolysaccharide (0, 5, 10, or 20 μ g; Sigma) were added. After 48 h, tritiated thymidine (25 μ Ci/mmol; Amersham Corp., Arlington Heights, IL) incorporation was counted as previously described [28]. C3H/HeSnJ cultures were treated in parallel as a positive control.

Epidemiology Once criteria were established for selection of phenotypic deviant mice with characteristics suggestive of AA, animal technicians working in one of The Jackson Laboratory pedigreed production colonies were trained to identify affected mice. The pedigreed production colony was the only C3H/HeJ colony for which pair, not trio, matings were used to carefully maintain information on the pedigrees, making it possible to trace family lines. The colony studied contained over 10,000 breeding pairs. All retired breeder pairs (4–5 months of age) with alopecia consistent with this disease were removed from the room for evaluation. The total number of breeding pairs was recorded at that time. This was done over a period of 12 months (August 1, 1991–July 31, 1992). From this information it was possible to determine the frequency of new cases in this defined population over a 1-year period. A small subcolony of C3H/HeJ mice was established as part of an independent study,† in which the founders developed inflammatory bowel disease (IBD). These mice were maintained until the founders were over 18 months of age. Mice that developed alopecia were evaluated as

† Sundberg JP, Elson CO, Bedigian H, Sundberg BA, Birkenmeier EH: Inflammatory bowel disease involving the cecum in C3H/HeJ mice (submitted).

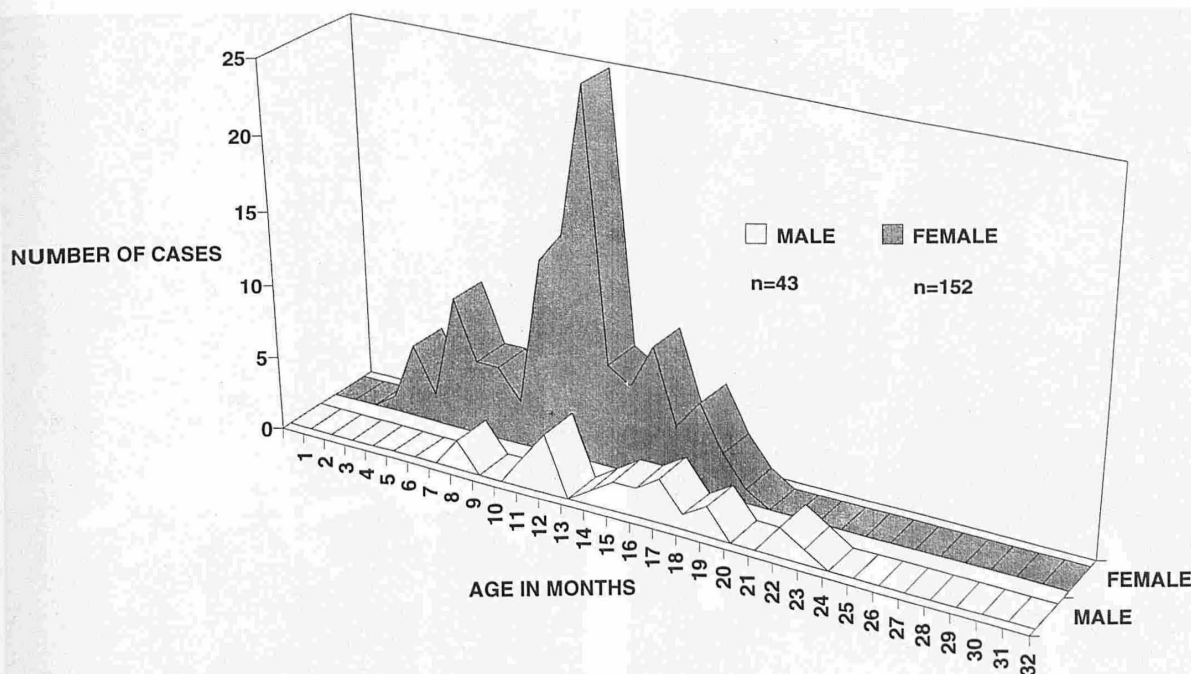


Figure 1. Numbers of mice histologically confirmed to have alopecia areata at the time of necropsy reveals early onset in female compared to male mice.

described above. The frequency of affected mice was calculated for this colony.

Evaluation of Weight Changes with Increasing Age C3H/HeJ retired breeders usually have a slightly rounded abdomen due to fat stores, but these mice are not obese. Mice with alopecia often lacked the fat stores, although they were not cachectic. To determine whether weight loss was a prodromal feature of AA in mice and whether or not mating had any effect on induction of alopecia, the following groups of mice were set up, weighed, and observed weekly. Mice used were from the breeding study. Mice were housed in groups of four of the same sex (F1 female [n = 10], F2 female [n = 10], F1 male [n = 16], and F2 male [n = 10]) or opposite sexes (F1 female [n = 13], F2 female [n = 10], F1 male [n = 13], and F2 male [n = 10]). The date of initial onset of alopecia was noted and the mice were followed for 1 year or longer, with at least 10 mice per group. Animals were added to replace those that died of causes unrelated to the study.

Response to Steroids Two groups of three C3H/HeJ female mice, 11 months of age, with AA were injected intralesionally (ID) with 0.5 mg of triamcinolone acetonide (Kenalog-10; E.R. Squibb & Sons, Inc., Princeton, NJ). Mice were photographed prior to the weekly injections. After 6 weeks, mice were killed and skin was collected for histologic evaluation as described above.

Statistical Analysis Data were analyzed by a one-way analysis of variance test. Differences were considered significant at $p < 0.05$.

RESULTS

Gross Morphologic Features of Development of Alopecia in C3H/HeJ Mice All C3H/HeJ mice that subsequently developed alopecia had a normal-appearing coat of hair or pelage at weaning. There were no visually obvious signs such as difference in body weight or size to indicate either which animals or which body sites were destined to lose hair in the first 8–14 weeks. When the alopecia developed, there were no obvious prodromal changes in hair coloration, thickness, or obvious loss of vibrissae or nail changes. Once the animal technicians began to detect alopecia, it was noted initially that affected female mice lost hair earlier, more rapidly, and to a greater extent than affected male mice (Fig 1). The number of

hair cycles that had occurred prior to the onset of visually obvious alopecia or microscopic changes has not yet been verified. Alopecia initially developed on the medial aspects of the proximal portion of the rear legs. Alopecia of the entire ventral surface and focal or multi-focal sites on the dorsal surface (Fig 2a,b) were subsequently found to be consistent features found most commonly in female C3H/HeJ mice between 5 and 6 months of age and with increasing frequency with age in male C3H/HeJ mice (Fig 1). Up to 7 months of age all of the affected mice were female and 17% developed generalized alopecia that approached complete baldness. There was marked heterogeneity in the sites and regrowth patterns of alopecia not only among the mice but also in the same mouse [29]. A generalized thinning of the pelage was more common than either focal alopecia or complete balding, which made it difficult to detect the wave or progression of the hair cycle except by carefully examining for pigmentary changes. No evidence of loss of skin elasticity, folding, wrinkling, or scarring was noted, but in animals treated with depilatories there was evidence suggestive of skin abrasions due to scratching. When the hair was removed using depilatories, two to three applications for 10–15 min were required for normal mice. In the affected C3H/HeJ mice, usually only one application of depilatory was required to completely remove the hair coat, indicating abnormal keratinization.

Six-month-old female mice observed weekly had persistent alopecia of the ventral surface with waxing and waning of the alopecic foci on the dorsal surface that progressed in most cases to a more generalized alopecia by 10–12 months of age. Preliminary observations indicated that some focal alopecic areas that subsequently regrew became alopecic again in an even larger area in the subsequent hair cycle. These areas were detected because of the loss of pigment in the alopecic area that persisted until an actively growing anagen hair developed during the regrowth stage (data not shown). In most alopecic areas, the skin was hypopigmented even though microscopically there was pigmentary incontinence due to postinflammatory changes. The waves of the hair cycle in unaffected C3H/HeJ mice were seen in pigmentary changes after hair removal by depilation in most areas except over the areas of mammary and accessory

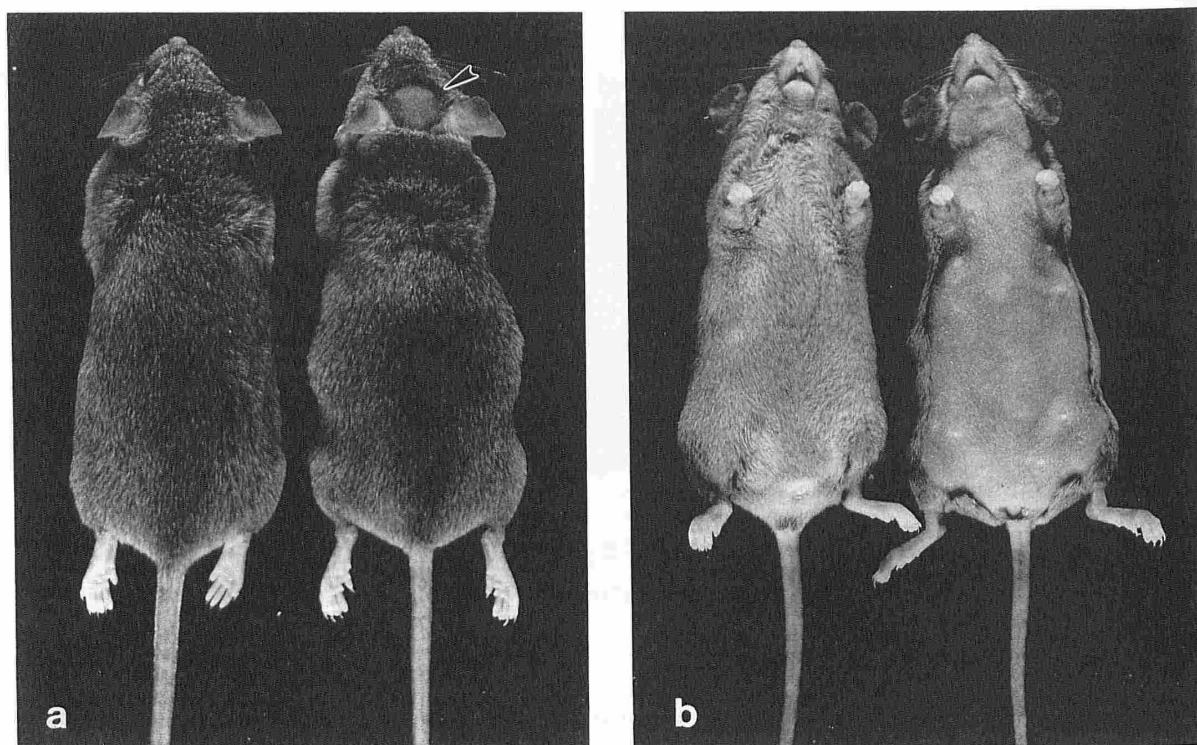


Figure 2. Focal alopecia over the skull (arrowhead) (a) and diffuse ventral alopecia (b) on a 4-month-old affected female (right) compared to an unaffected male (left) mouse.

sexual glands. In affected mice, pigmentary changes (with the exceptions of the sexual gland areas) were detected corresponding to the alopecic area until hair regrew. If the hair coat became very sparse, the skin appeared pink and often irregularly pigmented, because the sites of active anagen follicles, but not vibrissae, were always pigmented. Compared to unaffected mates, some vibrissae were white, shorter, thinner, and fewer in number. The nails were usually not pigmented and no nail deformities were noticed in affected mice [29].

Microscopic Features of Development of Alopecia in C3H/HeJ Mice When C3H/HeJ mice were submitted by the animal technicians as possibly becoming alopecic, random biopsies showed that the visually very subtle lesions that first appeared on the ventral surface had pronounced microscopic changes. The skin in the focal areas of non-scarring alopecia exhibited an abrupt change from widely separated telogen follicles to a contiguous area with thickening of the skin, densely packed anagen follicles, and a perifollicular mononuclear cell infiltrate (Fig 3a–d). The inflammation was very focal, not throughout the dermis, and telogen hair follicles were not affected (Figs 3,4). Siblings or age- and sex-matched controls had no comparable lesions or inflammatory infiltrates.

In the affected anagen follicles, dystrophic hair formation was seen with dyskeratosis, formation of amorphous material in the pilary canal (depilatory artifact?), and adjacent pigmentary incontinence (data not shown). The mononuclear cell infiltrate was present around and within the follicular epithelium in the bulbar regions and in and along the hair follicle epithelium up to the level of the sebaceous glands in late anagen hairs (Fig 4) and around the bulge or site of emergence of an anagen follicle (III–IV) arising from the resting telogen follicle (data not shown). These damaged emerging

follicles correspond to dystrophic earlier stages of anagen (II–IV), but how long these follicles persisted in this state was not determined. There were considerable differences in the extent of mononuclear cell infiltrates, pigmentary incontinence, and follicular dyskeratosis in biopsies from sites of diffuse hair loss and in older animals. However, club hairs and “exclamation point” hair shafts were seen in affected anagen follicles (Fig 4). The number of melanocytes in the hair bulb appeared to be diminished, as did the number of bulbar keratinocytes containing melanosomes. PAS staining showed that at the dermal-epidermal junction there were focally increased areas of amorphous material and the amount of glycogen in the outer root sheath was decreased in areas of active inflammation (data not shown). The ratio of bulbar epithelium to the dermal papillae was variable and not always obviously related to the degree of inflammation. In some specimens there appeared to be follicular edema (spongiosis), perhaps with necrotic keratinocytes associated with mononuclear cells (Fig 5).

Although the most noticeable changes were evident in and around anagen hairs, all different types of body hair were affected to various degrees (Figs 4 and 5). Mononuclear cell infiltrates were not prominent around the vibrissae; however, the cells infiltrated the follicle causing individual cell necrosis, separation of the dermal papilla from the matrix with microvesicle (spongiosis) formation, and destruction of the inner root sheath (Fig 5a–c). Cilia of the eyelids and the large terminal hairs of the tail were also affected to various degrees.

There were no obvious changes in the papillary or reticular dermis and subcutaneous tissues in hematoxylin and eosin-, PAS-, trichrome-, or orcein-stained sections that were not associated with affected anagen follicles. Staining for elastic fibers showed no destruction of these dermal components and no obvious changes were

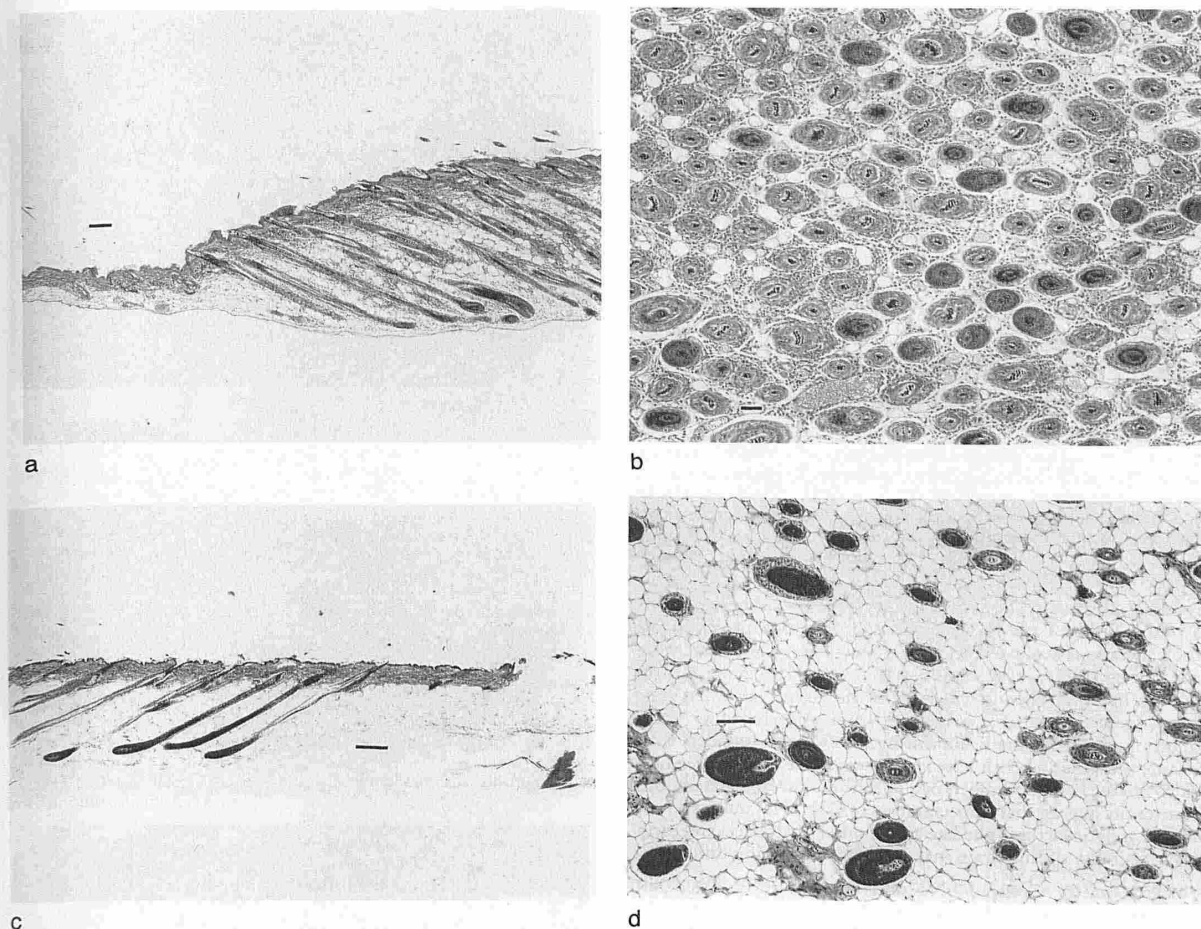


Figure 3. Small normal telogen-like follicles adjacent to the alopecic area (left side, a, H&E; bar, 100 μ m). Anagen follicles (right side, a) are uniformly surrounded by perifollicular mononuclear cell infiltrates. Transverse section of anagen hair region shown in (a) demonstrates perifollicular mononuclear cell infiltrates (b, hematoxylin and eosin; bar, 50 μ m). Comparable areas of unaffected, control skin (c,d) reveal no inflammation in the adipose tissue.

seen in blood vessels not intimately associated with affected anagen follicles.

Microbiology Dorsal skin biopsies taken from the center of the area of alopecia, as well as at the junction of normal-appearing hair, were cultured for aerobic bacteria and fungi. Isolates included *Lactobacillus* spp. (1/9), *Pasteurella pneumotropica* (2/9), *Proteus mirabilis* (1/9), coagulase-negative *Staphylococcus* spp. (1/9), and *Penicillium* spp. One case had both *Pasteurella pneumotropica* and coagulase-negative *Staphylococcus* spp. isolated together. Serologic screenings for antibodies against other mouse pathogens were all negative. PAS-stained sections showed no evidence of pathogenic fungi and fungal cultures of affected hairs were negative.

Thyroid Function The thyroid hormones in serum of affected and non-affected mice were measured by RIA to determine whether disturbances in this hormonal system could be related to the skin and hair pathology described above. Because thyroxine and T3 values did not differ by gender, the data were combined and the following means obtained for affected versus normal littermates: T4 = 7.20 ± 0.50 versus 6.35 ± 0.24 μ g/dl; T3 = 138 ± 5 versus 143 ± 6 ng/dl. Neither T4 nor T3 levels in affected mice were significantly different from those of age- and sex-matched control

mice. No microscopic lesions were evident in thyroid glands of either alopecic or normal C3H/HeJ mice.

Peripheral Blood Lymphocytes and Perifollicular Lymphocytic Infiltrates There were no significant differences in any of the cell populations evaluated in the peripheral blood between the mice with alopecia and mice with normal hair coats by FACS analysis. Immunohistochemical analysis of frozen sections using monoclonal antibodies for CD4⁺ and CD8⁺ cells in and around hair follicles of alopecic mice revealed that the infiltrates were primarily T cells. There was approximately a 1:3 ratio of CD4⁺ to CD8⁺ cells around follicles of affected mice (Fig 6). Unaffected control mice had essentially no CD4⁺ or CD8⁺ cells in their skin.

Pedigree Tracing of Alopecia in C3H/HeJ Mice At the time the alopecia phenotypic deviant had been identified, the C3H/HeJ colony had been inbred for 204–208 generations. Pedigrees of 14 affected females were traced to determine whether any were related. All cases were traced to one common breeding pair at F197. These data suggest that the alopecia areata-like phenotype may have a genetic basis.

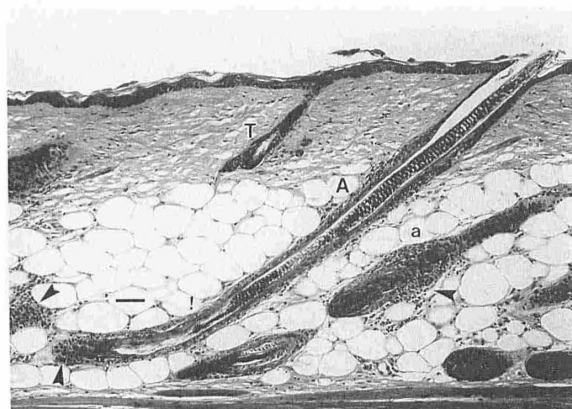


Figure 4. Terminal hair produced by an affected anagen follicle (center, A) with follicular, perifollicular, peribulbar, and intrabulbar mononuclear cell infiltration ("busy bees," arrowheads, a) and an unaffected telogen follicle (T). Tapering and pointed base of the hair is typical of an "exclamation point" type club hair (I, hematoxylin and eosin; bar, 20 μ m).

Breeding Studies The results of the breeding studies are summarized in Tables I and II. Mice with alopecia were present in all three generations evaluated regardless of whether the crosses were made among C3H/HeJ mice or between C3H/HeJ and C3HeB/FeJ mice. Individual pedigrees (data not shown) revealed that generations were skipped that originated from some breeding pairs, suggesting a form of genetic imprinting. Conversely, founders with the same pedigree and both having alopecia at the time of breeding yielded relatively high numbers of alopecic mice in each generation. These results suggest that alopecia areata in C3H/HeJ mice is due to complex genetic factors. The late age of onset of alopecia and early loss of fecundity of this strain makes selective breeding difficult.

Genetic Quality Control No differences were observed between any of the mice with or without alopecia areata tested for 24 markers on Chr 1 (*Idh-1*, *Akp-1*, *Pep-3*), Chr 3 (*Amy-1*, *Car-2*), Chr 4 (*Pgm-2*, *Gpd-1*), Chr 5 (*Pgm-1*), Chr 7 (*Gpl-1*, *Hbb*), Chr 8 (*Es-1*, *Es-11*, *Got-2*, *Gr-1*), Chr 9 (*Apoa-1*, *Trf*, *Mod-1*, *Mpi-1*), Chr 11 (*Es-3*), Chr 14 (*Es-10*), Chr 15 (*Gpt-1*), or Chr 17 (*Glo-1*, *Neu-1*, *H-2^b*). These results indicate that there are no genetic contamination or mutations at the tested loci of the C3H/HeJ mice that develop alopecia with age. Testing for lipopolysaccharide response revealed that all of the C3H/HeJ mice, regardless of the state of their pelage, had reduced responses, whereas the C3H/HeSnJ mouse tested had normal responses. This indicates that this immunologic defect, characteristic of this inbred strain, continues to be present in those individuals with alopecia areata.

Epidemiology of the Development of Alopecia in C3H/HeJ Mice One of the pedigree, pair-mated (equal numbers of age-matched male and female mice) production colonies of C3H/HeJ mice was studied from August 1, 1991 until July 31, 1992. During that time, 106 (0.25%) female and 15 (0.035%) male mice were found to be alopecic. All of these mice were confirmed to have alopecia areata by histologic examination of the skin. All of the mice evaluated were retired breeders at 5 months of age. When animals of increasing age were evaluated, based on age at the time of necropsy, it became apparent that with increasing age, more male mice were affected (Fig 1).

In an aging colony established with mice having a high frequency of IBD, 10 of 206 female (4.8%) and 10 of 221 male mice (4.5%; 4.7% for both sexes) were affected with alopecia areata by 18 months of age. The disease was confirmed by histopathologic evaluation of all cases.

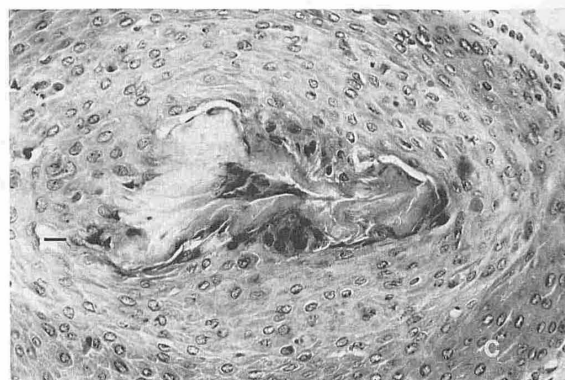
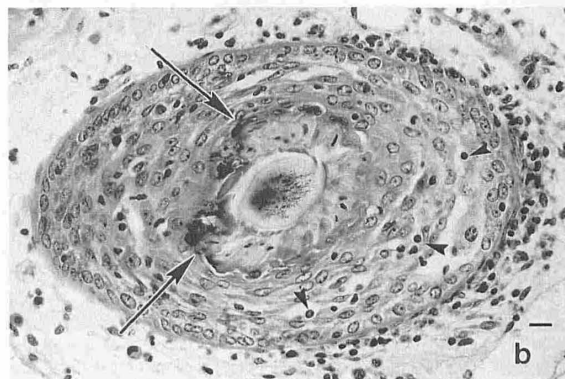
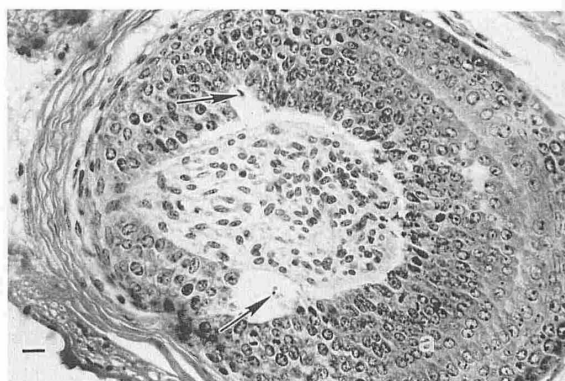


Figure 5. Separation of the dermal papilla from the matrix, with vesicle formation containing necrotic debris (arrow), is found in the bulb of an affected vibrissa (a, hematoxylin and eosin; bar, 10 μ m). Perifollicular and follicular mononuclear cell infiltration (arrowheads) and focal destruction of the inner root sheath (between arrows) is an affected vibrissa (b, hematoxylin and eosin; bar, 10 μ m). Complete destruction of the vibrissa shaft and surrounding inner root sheath (c, hematoxylin and eosin; bar, 10 μ m).

Weight Changes with Age Decreases in body weight did not precede onset of alopecia (Fig 7). Of the female mice, 1–4 (10–40%) in each group developed alopecia (total, eight of 43, 18.6%). The number with alopecia per group of female mice was not significantly different ($p > 0.8$) between any of the four groups, indicating that neither generation nor parity were important factors. The

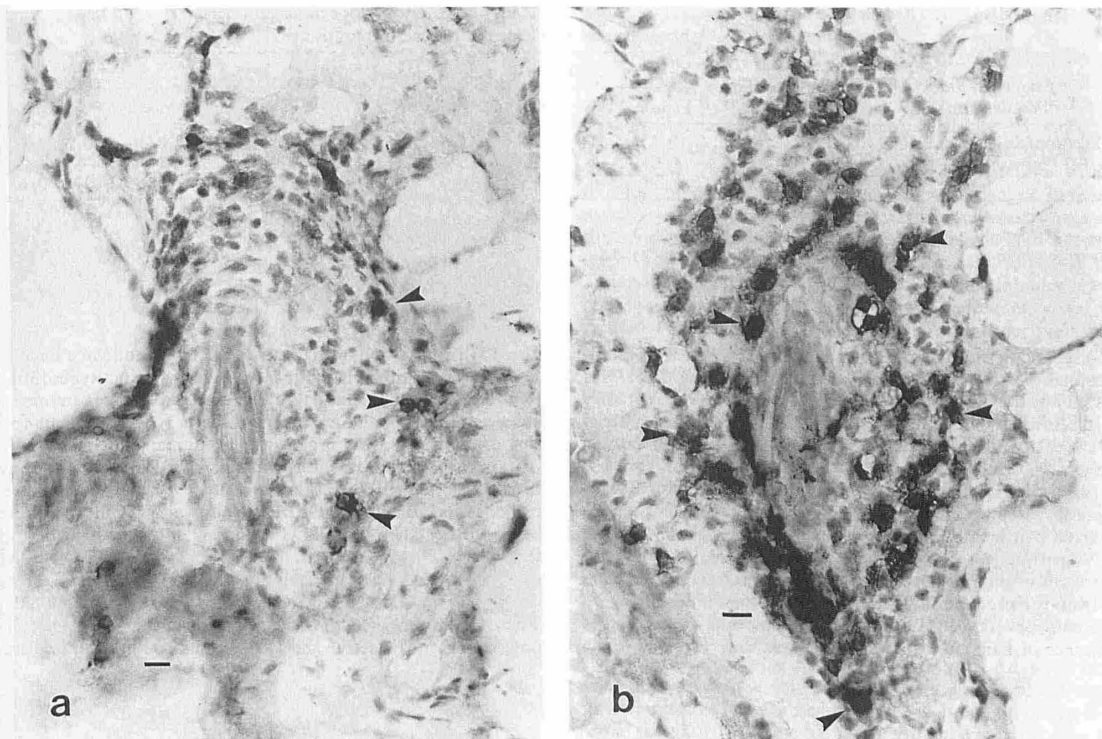


Figure 6. Perifollicular and intrafollicular lymphoid cells consisted primarily of CD4⁺ (arrowheads, *a*, immunohistochemistry) and CD8⁺ (arrowheads, *b*, immunohistochemistry) cells.

age of onset of disease in female mice ranged from 21 to 50 weeks of age (mean of 29 weeks). Most cases became evident at the point where the female mice could no longer be successfully bred by the male mice (Fig 7). One female mouse with late-onset alopecia remained fecund until the onset of alopecia (Fig 7). Only one male mouse developed alopecia at 52 weeks of age, when the study was terminated.

Response to Steroids Mice receiving intralesional injections of phosphate-buffered saline exhibited waxing and waning of lesions, typical of an unmanipulated case or lack of hair growth in the generalized alopecia case. In contrast, mice receiving intralesional triamcinolone acetonide injections developed a short, fine hair coat, even in cases with generalized alopecia in which untreated cases never grow hair. Microscopically, those treated with phosphate buffered saline had mononuclear cell infiltrates in and around anagen follicles, typical of the untreated disease (Fig 8*a*). In contrast, mice injected with steroids had either normal, uninvolved telogen

follicles or biopsies containing anagen follicles with few, if any, inflammatory cell infiltrates, indicative of a positive response (Fig 8*b*).

DISCUSSION

These studies show that both clinically and pathologically the C3H/HeJ mice have a non-scarring alopecia with follicular involvement that most closely resembles the human disease alopecia areata [1–5] and the non-scarring, perifollicular inflammatory alopecia seen in the DEBR rat [8–10]. In numerous studies in normal mice and many other mouse mutations with abnormalities of hair anagen and telogen follicles, consistent findings of mononuclear or lymphohistocytic perifollicular and interfollicular infiltrates are rarely observed [14,29] but are seen in some breeds of horses, dogs, cats, nonhuman primates, and human alopecia areata [1,6,7].

A variety of bacterial and mycotic agents are well-known causes

Table I. Number of C3H/HeJ Mice with Alopecia Areata per Number of Offspring Raised in Three Successive Generations

Parental Features Female × Male	Founding Pairs	F1				F2				F3			
		Female	%	Male	%	Female	%	Male	%	Female	%	Male	%
Alopecia × Alopecia	11	7/44	15.9	3/28	10.7	6/45	13.3	3/45	6.6	2/68	2.9	1/82	1.2
Alopecia × Normal	17	4/41	9.8	3/48	6.3	6/32	18.8	2/33	6.1	0/4	0	0/9	0
Normal × Alopecia	3	1/3	33.3	0/3	0	—	—	—	—	—	—	—	—
Normal × Normal	2	3/24	12.5	0/26	0	2/30	6.7	3/25	12.0	—	—	—	—
Total	33	15/112	13.4	6/105	5.7	14/107	13.1	8/103	7.8	2/72	2.8	1/91	1.1

Table II. Number of C3H/HeJ $\times$ C3HeB/FeJ Mice with Alopecia Areata per Number of Offspring Raised in Three Successive Generations

Parental Features Female $\times$ Male	Founding Pairs	F1 ^a				F2 ^b				F3 ^c			
		Female	%	Male	%	Female	%	Male	%	Female	%	Male	%
C3H/HeJ Alopecia $\times$ C3HeB/FeJ Normal	2	0/5	0	0/7	0	1/25	4	1/30	3.3	0/4	0	0/6	0
C3HeB/FeJ Normal $\times$ C3H/HeJ Alopecia	3	4/33	12.1	2/44	4.5	0/36	0	0/45	—	—	—	—	—
Total	5	4/38	10.5	2/51	4.0	1/61	1.6	1/75	1.3	0/4	0	0/6	0

^a Age range 5–9 months.^b Age range 1–7 months.^c Age range 2 months.

of follicular and perifollicular inflammation, which must be eliminated as factors in the pathogenesis of alopecia in aging C3H/HeJ mice. Many of the production colonies at The Jackson Laboratory were established by Cesarean derivation and are housed in strict quarantine to maintain their specific pathogen-free status. Extensive quarterly monitoring of all mouse rooms and representative mice is done to verify the microbiologic status of mice within rooms. Routine bacterial and mycotic cultures were prepared from skin biopsies and plucked hair from C3H/HeJ mice with and without alopecia areata. No pathogens were isolated nor were any visualized in histologic sections of skin using special stains in the mice cultured or in others from the production and breeding colonies.

In humans, several forms of alopecia areata occur in which specific clusters of hairs are affected to the extreme form, alopecia

universalis, in which all hair types are involved. Mice have more hair types than humans, with at least eight distinct types [30]. It is important to note that all eight hair types are affected in mice with alopecia areata, including the vibrissae, a specialized hair follicle that is a somatosensory organ with no human equivalent [30].

In immunoperoxidase studies of the DEBR rat skin, there was a marked increase in T cells around affected hair follicles. The peripheral blood of affected DEBR rats showed a significant difference between the CD4⁺ and CD8⁺ subpopulations of control and affected rats. However, the DEBR rat study was staged into three categories: pre-lesion, active-lesion, and late-lesion. No difference in the CD4⁺:CD8⁺ ratio was observed in the pre-lesional rats. There was a significant increase in circulating T-helper (CD4⁺) populations in the active and late-lesional rats [8]. Limited studies

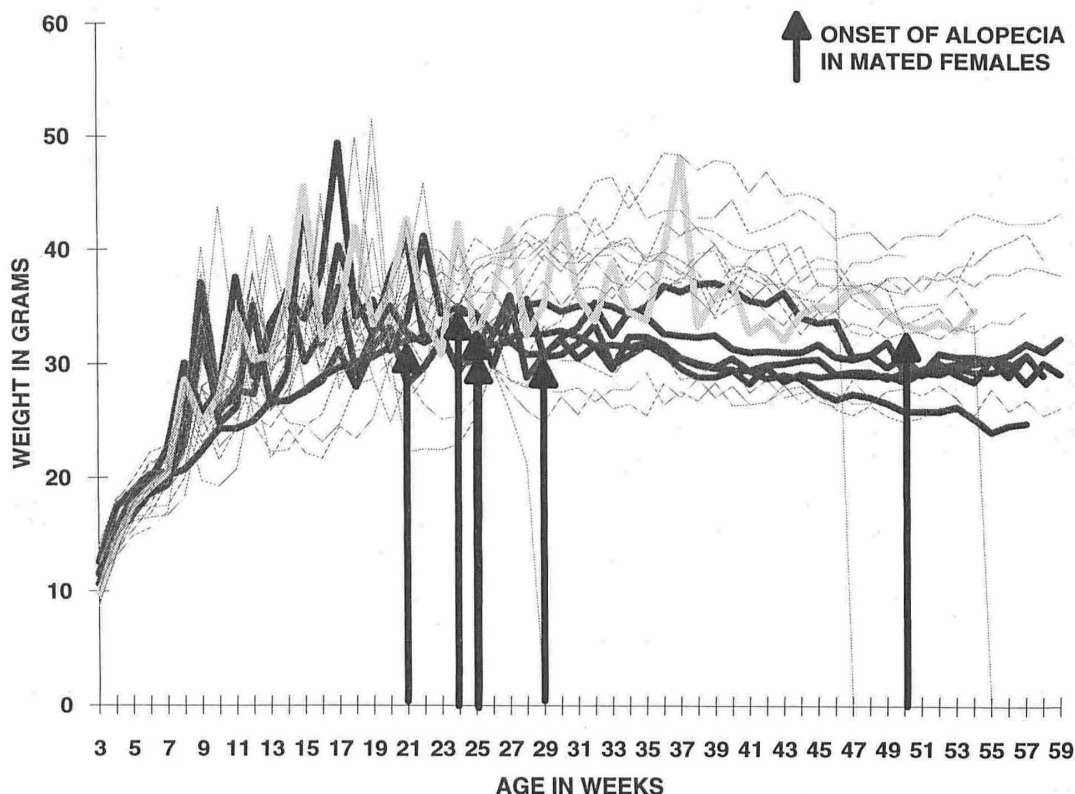


Figure 7. Onset of alopecia areata (arrows) was associated with loss of fecundity. Peaks indicate pregnancies. Weight changes did not precede onset. Solid dark lines, weights of mice with alopecia areata. Dashed lines, weights of clinically normal mice.

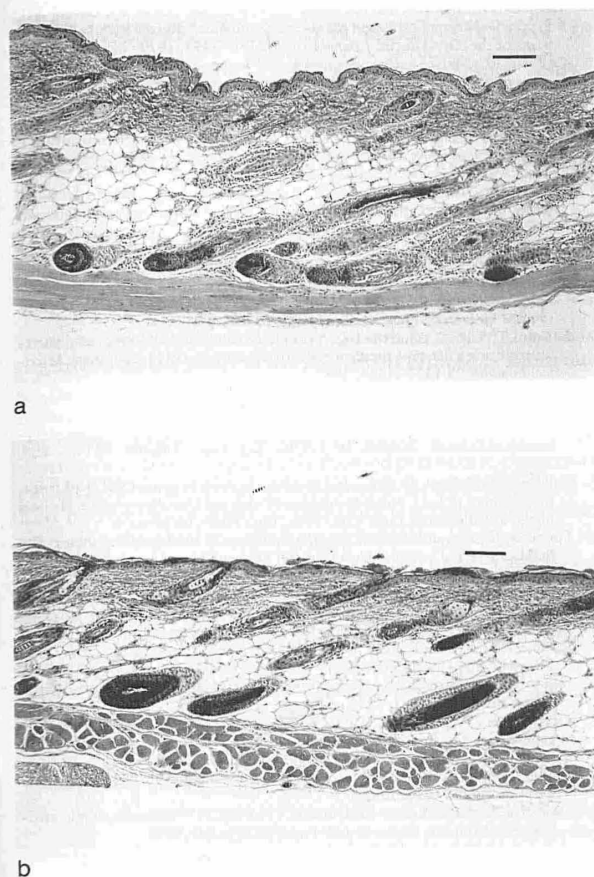


Figure 8. Lymphocytic infiltrates were greatly reduced from alopecia areata mice that received intraslesional phosphate buffered saline (a) compared with mice that received 6 weekly intraslesional injections of triamcinolone (b, hematoxylin and eosin; bar, 100 μ m).

on human AA have also shown imbalances in the CD4⁺:CD8⁺ ratio in the peripheral blood. Two independent studies demonstrated a marked decrease in the CD8⁺ population of peripheral blood in human AA [31,32]. In addition, it has been shown that there is a CD4⁺:CD8⁺ ratio of 4:1 in the peribulbar infiltrate of the hair follicles of humans with AA [32]. In the present study, peripheral blood studies indicated that there were no significant differences between controls and AA mice for any of the cell populations evaluated. When the skin was examined, CD4⁺ and CD8⁺ cells were localized, by immunohistochemistry, to be in and around alopecic mouse anagen follicles in a ratio of approximately 1:3. This ratio is opposite that reported in human AA [31,32] and the rat model [8]. These mouse studies were done based on age groups rather than by clinical staging of the disease. As indicated above, staging of AA in humans and DEBR rats yields different results and staging studies will need to be done in C3H/HeJ mice that develop AA. Alternatively, underlying abnormalities of the C3H/HeJ immune system, which are documented [27], may be reflected in the distorted CD4⁺:CD8⁺ ratio. More detailed studies are warranted in all three species to define these observations. Intraslesional injections of steroids in mice with AA resulted in decreased intra- and perifollicular lymphocytic infiltration and resumption of hair growth, in-

dicating that lymphocytes are involved in the pathogenesis of AA in these mice.

C3H/HeJ mice are an inbred strain of mutant mice (205 generations when the alopecia phenotypic deviants were identified) that are defective in their response to lipopolysaccharides [27,32]. The fecundity of females decreases dramatically by 4 months of age, giving this strain one of the shortest breeding periods of inbred strains [24]. The onset of detectable alopecic lesions in affected females corresponded to the period of rapid decline in fecundity, making selective breeding difficult. However, we determined that the frequency of alopecia areata in C3H/HeJ was 0.25% female and 0.035% male mice in a large production-based breeding colony. The frequency increased to almost 5% in C3H/HeJ mice selectively bred for IBD and aged to 18 months. The frequency was further increased in some lines to over 18% in mice selectively bred for alopecia areata. Pedigrees of 14 affected females were traced to determine whether any were related and all cases identified to date were traced to one common breeding pair. These data suggest that alopecia areata in mice has a genetic basis and that it may be a widespread aging characteristic in the C3H/HeJ inbred strain. This appears to be a complex polygenic trait in this inbred strain. Increased familial incidence for human alopecia areata suggests that genetic factors have a causative role [34,35]. In one study of human patients, 60% were female. Of these patients with alopecia areata, 2% of their children and 7% of their parents had the disease [34]. Selective breeding, which can easily be done with inbred laboratory mice but not humans, was able to increase the frequency of disease and may prove to be an important tool for modeling the genetics of human alopecia areata.

The observation that C3H/HeJ mice with AA were in better condition than haired, fat, retired breeders of the same age suggested that weight loss or failure of excessive weight gain might be prodromal signs for AA. This did not prove to be the case. The weight differential became evident after the onset of alopecia. However, the weekly evaluation of over 80 male and female mice revealed that the mean age of onset of alopecia was 29 weeks in females. Male mice did not develop alopecia within the study period. It also revealed that breeding versus virginity were not important factors in the female mice.

Alopecia areata in humans has been associated with a variety of disorders of supposed autoimmune cause [5]. Muller and Winkelmann [35] reported thyroid disease as being statistically related to alopecia areata (8% of 736 patients), whereas a study by Main *et al* [36] failed to confirm this observation. Furthermore, although Galbraith and Pandey [37] found that 22 of 52 patients (18.2%) with alopecia areata had antithyroid antibodies, they found a comparable frequency (17.1%) in their reference population. Thyroid function, as observed by serum T3 and T4 levels, in aging C3H/HeJ mice with alopecia areata was normal in this study. No histologic abnormalities of the thyroid glands were observed. Other autoimmune-based endocrinopathies such as diabetes mellitus [38–40] and gonadal disease [41,42] have also been correlated with alopecia areata in humans. Although gonadal histologic lesions have not been identified in affected C3H/HeJ mice, onset of alopecia in mice corresponds to termination of reproductive activity, suggesting future avenues for investigation.

Alopecia that develops in aging C3H/HeJ mice has many features that resemble alopecia areata in humans and may be a useful animal model to study this disease. The results of one study support the concept that human alopecia areata is not a single pathologic entity [37]. Because the mouse model is on an inbred background and therefore will yield a homogeneous system, its true value will be realized when it is accurately correlated to a specific subset of human alopecia areata patients [43]. Because of the polygenic nature of the mouse disease, it will probably continue to be very difficult to establish lines that yield predictable numbers of affected offspring. However, numerous affected animals can be obtained at a predictable and regular level at institutions such as The Jackson Laboratory, where large research and production colonies of C3H/HeJ mice are maintained.

This work was supported by grants from the National Alopecia Areata Foundation (LEK, JPS) and the National Institutes of Health (AR40324 and DK44240, JPS; DK26518, LEK) and research funds from the Department of Veterans Affairs (LEK).

Wade Cordy participated in The Jackson Laboratory Summer Student Program during 1992. He was supported by the George I. Alden Trust, The Burroughs Wellcome Fund, Lucille P. Markey Charitable Trust, Grant Bio-9000833 from the National Science Foundation's Research Experiences for Undergraduate Program, and the Lowell and Frances Hyams Brentano Scholarship Fund.

The authors wish to thank Drs. W. Beamer, M. Davisson, and E. Russell for their review, guidance, and interpretation of the breeding studies. We also thank C. Vallee, D. Boggess, W. Watermann, L. Edison, T. Duffy, and A. Higgins for their technical assistance with this project, W. Beamer for running the T3 and T4 assays, R. Bates for the LPS response testing, E. Sargent for the genetic quality control assays, B. Sundberg for assistance with the graphics, C. Basso for help with the pedigree search, and Susan J. Grindle for providing affected mice from production colonies.

REFERENCES

- Ackerman AB: *Histologic Diagnosis of Inflammatory Skin Diseases*. Lea & Febiger, Philadelphia, 1978, pp 696–701
- Tosti A: Alopecia areata: more on pathogenesis and therapy. *Dermatologica* 178:61–63, 1989
- Nelson DA, Spielvogel RL: Alopecia areata. *Int J Dermatol* 24:26–34, 1985
- Lever WF, Schaumburg-Lever G: *Histopathology of the Skin*, 7th ed. JB Lippincott Co., Philadelphia, 1990, pp 223–224
- Mitchell AJ, Krull EA: Alopecia areata: pathogenesis and treatment. *J Am Acad Dermatol* 11:763–775, 1984
- Conroy JD: Alopecia areata. In: Andrews EJ, Ward BC, Altman NH (eds.). *Spontaneous Animal Models of Human Disease, Vol II*. Academic Press, New York, 1979, pp 30–31
- Muller GH, Kirk RW, Scott DW: *Small Animal Dermatology*, 3rd ed. WB Saunders Co., Philadelphia, 1983, pp 589–592
- Michie HJ, Jahoda CAB, Oliver RF, Poulton TA: Immunobiological studies on the alopecic (DEBR) rat. *Br J Dermatol* 123:557–567, 1990
- Michie HJ, Jahoda CAB, Oliver RF, Johnson BE: The DEBR rat: an animal model of human alopecia areata. *Br J Dermatol* 125:94–100, 1991
- 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
- Winter RM: Malformation syndromes: a review of mouse/human homology. *J Med Genet* 25:480–487, 1988
- Davisson MT: The Jackson Laboratory Mouse Mutant Resource. *Lab Anim* 19:23–29, 1990
- Sundberg JP: Mouse mutations: animal models and biomedical tools. *Lab Anim* 20:40–49, 1991
- Sundberg JP, Shultz LD: Inherited mouse mutations: models for the study of alopecia. *J Invest Dermatol* 96:95S–96S, 1991
- Sundberg JP, Roop RD: Optimizing fixation and immunohistochemical staining of mouse skin. *J Cytochem Histochem* (in press)
- Dialynas D, Wilde D, Marrack P, Pierres A, Wall K, Havran W, Otten G, Loken M, Pierres M, Kappler J, Fitch F: Characterization of the murine antigenic determination, designated L3T4a by functional T-cell clones appears to correlate primarily with class II MHC antigen reactivity. *Immunol Rev* 74:30–52, 1983
- Ledbetter J, Herzenberg L: Xenogeneic monoclonal antibodies to mouse lymphoid differentiation. *Immunol Rev* 47:63–71, 1979
- Kubo TR, Born W, Kappler JW, Marrack P, Pigeon M: Characterization of a monoclonal antibody which detects all murine $\alpha\beta$ T-cell receptors. *J Immunol* 142:2736–2742, 1989
- Marshak-Rothstein A, Fink P, Gridley T, Raulet DH, Bevan MJ, Gelfer ML: Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J Immunol* 122:2491–2497, 1979
- Coffman RL: Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. *Immunol Rev* 69:5–23, 1982
- Austyn JM, Gordon S: F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *J Immunol* 11:805–815, 1981
- Bhattacharya A, Dorf ME, Springer TA: A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: Evidence for I region gene duplication. *J Immunol* 127:2488–2495, 1981
- Ezquerria A, Cron R, McConnell T, Valas R, Bluestone J, Coligan J: T-cell receptor δ -gene expression and diversity in the mouse spleen. *J Immunol* 145:1311–1317, 1990
- Green MC, Witham BA: *Handbook on Genetically Standardized JAX Mice*, 4th ed. The Jackson Laboratory, Bar Harbor, ME, 1991, pp 10, 15, 35
- Green MC: 1989. Catalog of mutant genes and polymorphic loci. In: Lyons MF, Searle AG (eds.). *Genetic Variants and Strains of the Laboratory Mouse*, 2nd ed. Oxford University Press, Oxford, 1989, pp 12–403
- Shiroishi T, Sagai T, Moriaki K: A simplified micro-method for cyto-toxicity testing using a flat-type titration plate for the detection of H-2 antigens. *Microbiol Immunol* 25:1327–1334, 1981
- Vetvicka V: C3H/HeJ mice. In: Rihova B, Vetvicka V (eds.). *Immunological Disorders of Mice*. CRC Press, Boca Raton, 1991, pp 154–171
- Leiter EH, Beamer WG, Shultz LD: The effect of immunosuppression on streptozotocin-induced diabetes in C57BL/KsJ mice. *Diabetes* 32:148–155, 1983
- Sundberg JP, Vallee CM, King, Jr. LE: Alopecia areata in aging C3H/HeJ mice. In: Sundberg JP (ed.). *Mouse Mutations with Skin and Hair Abnormalities: Animal Models and Biomedical Tools*. CRC Press, Boca Raton (in press)
- Sundberg JP, Hogan ME: Hair types and subtypes in the laboratory mouse. In: Sundberg JP (ed.). *Mouse Mutations with Skin and Hair Abnormalities: Animal Models and Biomedical Tools*. CRC Press (in press)
- Lutz G, Niedecken H, Bauer R, Kreysel H: Two-color flow cytometry analysis in alopecia areata. *Dermatologica* 178:64–66, 1989
- Perret C, Wiesner-Menzel L, Happle R: Immunohistochemical analysis of T-cell subsets in the peribulbar and intrabulbar infiltrates of alopecia areata. *Acta Derm Venereol* 64:26–30, 1984
- Staats J: Standardized nomenclature for inbred strains of mice: eighth listing. *Cancer Res* 45:945–977, 1985
- van der Steen P, Traupe H, Happle R, Boezeman J, Strater R, Hamm H: The genetic risk for alopecia areata in first degree relatives of severely affected patients. *Acta Dermatol Venereol (Stockh)* 72:373–375, 1992
- Muller SA, Winkelmann RK: Alopecia areata. An evaluation of 736 patients. *Arch Dermatol* 88:290–297, 1963
- Main RA, Robbie RB, Gray ES, Donald D, Horne CHW: Smooth muscle antibodies and alopecia areata. *Br J Dermatol* 92:389–393, 1975
- Galbraith GMP, Pandey JP: Km1 allotype associated with one subgroup of alopecia areata. *Am J Hum Genet* 44:426–428, 1989
- Taniyama M, Kushima K, Ban Y, Kaihara M, Nagakura H, Sekita S, Katagiri T, Sueki H: Case report: Simultaneous development of insulin dependent diabetes mellitus and alopecia areata universalis. *Am J Med Sci* 301:269–271, 1991
- Aw TC, Cheah JS: Diabetes mellitus presenting with alopecia areata totalis. *Lancet* II:268, 1978
- Schmitt GM, Lohaus A, Salewski C: Kontrollüberzeugungen und Patienten Compliance: Eine empirische Untersuchung am Beispiel von Jugendlichen mit Diabetes mellitus, Asthma bronchiale und Alopecia areata. *Psychother Med Psychol* 39:33–40, 1989
- Brown AC, Pollard ZF, Jarrett WH: Ocular and testicular abnormalities in alopecia areata. *Arch Dermatol* 118:546–554, 1982
- Brown AC: Autoimmune gonadal disease and alopecia areata. In: Brown AC, Crounse RG (eds.). *Hair, Trace Elements, and Human Illness*. Praeger Publ., New York, 1980, pp 313–333
- Sundberg JP: Conceptual evaluation of animal models as tools for the study of diseases in other species. *Lab Animal* 21:48–51, 1992