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The C3H/HeJ mouse and DEBR rat models for alopecia areata: review of preclinical drug screening approaches and results

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Abstract

The C3H/HeJ inbred mouse strain and the Dundee Experimental Bald Rat (DEBR) strain spontaneously develop adult onset alopecia areata (AA), a cell mediated disease directed against actively growing hair follicles. The low frequency of AA and the inability to predict the stage of AA as it evolves in the naturally occurring C3H/HeJ model of AA can be converted into a highly predictable system by grafting full thickness skin from AA affected mice to normal haired mice of the same strain. The rat DEBR model develops spontaneous AA at a higher frequency than in the mouse model but they are more expensive to use in drug studies due to their larger size. Regardless of the shortcomings of either model, these rodent models can be used successfully to screen novel or approved drugs for efficacy to treat human AA. Since the pathogenesis of AA follows the canonical lymphocytic co-stimulatory cascade in the mouse AA model, it can be used to screen compounds potentially useful to treat a variety of cell mediated diseases. Efficacy of various agents can easily be screened by simply observing the presence, rate, and cosmetic acceptability of hair regrowth. More sophisticated assays can refine how the drugs induce hair regrowth and evaluate the underlying pathogenesis of AA. Some drugs commonly used to treat human AA patients work equally as well in both rodent models validating their usefulness as models for drug efficacy and safety for human AA.

Keywords

alopecia areata; treatment; animal model; review; diphenylcyclopropanone; squaric acid dibutyl ester; dinitrochlorobenzene

Introduction

Alopecia areata (AA) is a relatively common disfiguring human cell mediated disease that targets hair follicles in the actively growing (anagen) phase of the hair cycle. It has a complex genetic basis (1) and is often associated with other systemic cell mediated diseases

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Conflict of Interest

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(2). AA is relatively common in the general population, with a lifetime risk of 1.7% (3). Human AA involves patchy hair loss from any hair-bearing region of the body that may progress to total body hair loss. AA may wax or wane on different sites, or the same site, or frequently it will spontaneously resolve with no treatment. AA most commonly affects the scalp but other body regions may also be affected. Extensive or total hair loss involving the scalp is termed alopecia totalis (AT). AA affecting sites in addition to the bald scalp is termed AT/AU or alopecia universalis (AU) if the entire body is affected. This spontaneous, patchy, potentially reversible, non-scarring, hair loss has been the focus of medical research for over 100 years (4). Only recently however, with access to many new biomedical and molecular tools, have concerted attempts been made to understand the susceptibility, activation, pathogenesis, and treatment of AA (5). Progress in understanding the pathogenesis and genetics of AA and developing new therapies was severely hampered until relatively recently due to the lack of appropriate animal models.

In the last decade, AA-like diseases have been recognized in a variety of mammals, in addition to humans, including laboratory rodents, dogs, cats, horses, cattle, and non-human primates (2,6,7). A feather loss syndrome in chickens also has many characteristics of AA (8). The most extensively studied mammalian models are spontaneous AA-like diseases in the C3H/HeJ inbred mouse strain (Fig. 1-3), the Dundee Experimental Bald Rat (DEBR; Fig. 4) and, to a lesser extent, several inbred and congenic mouse strains (6,7,9-16). These models have been used effectively to work out the immunologic mechanisms and genetic basis of AA, as well as to test the efficacy and safety of drugs known to work to varying degrees on humans with AA. These studies showed that AA is a cell mediated disease targeting anagen stage hair follicles. AA progresses through the canonical lymphocyte costimulatory cascade (17), but many other aspects of its pathogenesis have yet to be elucidated. In particular, the critical first step, identifying the disease initiating antigen(s), has not yet been determined. Regardless, having 2 homologous rodent model systems makes it possible to systematically test compounds for therapeutic efficacy and safety. Recently completed longitudinal gene expression profiles in the mouse model identified many gene pathways and potential drug targets making future efficacy screening studies analytical rather than empirical (Sundberg et al., unpublished data).

Concerns remain that the C3H/HeJ and DEBR rat AA models are not the best for human AA (Table 1), although most researchers in the field do consider them to be the best tools currently available, especially for novel drug efficacy trials (18). It is always difficult to compare monomorphic rodent models maintained on inbred backgrounds (genetically identical) in highly controlled, pathogen free environments with human patients who have NONE of these advantages. Human AA, like many other diseases that affect this species, consists of numerous very similar diseases lumped together clinically, which makes comparisons to the rodent models controversial at best for those who do not recognize these fundamental differences. The rodent models provide a distinct advantage over human patients as they are highly controlled models and can be reproduced to generate adequate numbers to generate reproducible results.

A number of drugs used to treat human and rodent AA with various degrees of efficacy were tested in several different laboratories. How the efficacy and safety of these drugs were assessed as well as the methodology and formulation of these agents have not been systematically investigated. In order to demonstrate that these rodent AA models AA can be effectively used to screen therapeutic agents for AA, we have summarized the methodology, routes of administration, and results of these trials. This summary is to encourage the use of these rodent AA models to test drugs that are approved by the United States Food and Drug Administration (FDA) for other indications (off-label use) and agents found by analysis of gene array (transcript) data to be potential new therapies for human AA. The ultimate goal

of using these rodent models of AA is to devise effective and safe new treatments for human AA patients.

Therapeutic approaches to alopecia areata

Many drugs have been tried in small to large clinical trials on human AA patients (19,20). The most effective agents are classified as: contact sensitizers, immunosuppressive agents, monoclonal antibodies to block specific molecular pathways, alkylating agents, and hair growth promoters. We summarize the results of these agents used in the rodent AA models below (Table 2).

Contact Sensitizers used as immunotherapy agents

Topical immunotherapy with contact sensitizers is considered the most effective therapeutic modality (21,22). The contact sensitizers used are all effective to treat human AA patients with various rates of success (23-26). Contact sensitizers that have been used include diphencyprone (DPCP), squaric acid dibutylester (SADBE), and dinitrochlorobenzene (DNCB) that is no longer used due to its potential mutagenic properties. Topical sensitization to treat AA relies on four characteristics of the contact sensitizer for efficacy and safety: predictable immunomodulation (sensitization of the immune system), absence from the natural environment, lack of cross-reactivity with other substances, and safety (27).

The mechanism by which contact sensitizing agents act to induce hair re-growth is unknown but the hypothetical phenomenon of “antigenic competition” has been proposed. An immune reaction to a given antigen may inhibit the development of the immune response to another unrelated antigen (28). One of the more popular theories is that they redirect the inflammatory response in AA away from the hair follicle and direct it towards the exogenous chemical sensitizer (28). Other hypotheses include a tolerization of AA-specific T cells and the induction of a non-permissive milieu for these cells, or induction by contact sensitizing agent treatment alters trafficking/homing of leukocytes in the affected skin (29,30). Other mechanisms suggest contact sensitizers alter the AA-associated cytokine profile and that this non-specifically mediates beneficial effects on AA (29,30).

Squaric acid dibutylester (SADBE)

Freyschmidt-Paul et al. (31) reported that AA-like hair loss in C3H/HeJ mice responded to treatment with SADBE analogous to human AA. Hair regrowth was observed only on the treated side of the dorsal skin in 9 of 12 mice. Histopathologic examination revealed a change in the distribution of the inflammatory 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 SADBE altered the CD4⁺/CD8⁺ ratio from approximately 1:2 in untreated AA to 1:1 in treated AA. SADBE treatment also changed the aberrant expression of major histocompatibility complex class I and II in a way similar to that observed in human AA. A similar report by Gardner (32) showed 9 of 11 experimental mice with AA regrew hair on the treated side only and that this was associated with a reduction in peri/intrafollicular inflammatory cell infiltrates, hair follicle dystrophy, melanin incontinence/clumping, and an increase in the numbers of hair follicles in full anagen.

Diphencyprone (DPCP)

Diphencyprone (DPCP) was reported to be a highly variable, but effective, topical immunotherapy for extensive human AA (25,26,33-35). However, the mechanisms of DPCP action to induce hair growth in AA patients are still unknown (23). Topical DPCP treatment for AA is effective, well-tolerated, and provides prolonged therapeutic benefits in treatment

responsive patients. The two rodent AA models were used to determine if topical DPCP was efficacious (36,37). Similar to human AA, DPCP induced hair regrowth on the treated skin regions in the majority of the animals. Mice with AA that regrew hair exhibited intrafollicular infiltration of CD4⁺ and CD8⁺ T lymphocytes prior to hair regrowth. Successful treatment with DPCP was associated with a reduction of the peribulbar lymphocytic infiltrate and development of inflammation in the upper dermis. In rats, there was a paucity of lymphocytic infiltrates in both the untreated and the treated skin and thus no significant shift of lymphocyte localization was apparent. These two models of AA can be used as a tool to further our understanding of human AA and the therapeutic actions of DPCP (36).

Dinitrochlorobenzene (DNCB)

Dinitrochlorobenzene exerts a significant immunomodulatory effect on antigen presenting cells *in vitro* and *in vivo* (38,39). The effect of topically applied DNCB appears to be systemic in nature, with cellular immune responses appearing at distant sites following DNCB application (40,41). Dendritic cells exposed to DNCB *in vivo* and *in vitro* stimulate CD8⁺ T-cells and natural killer cells (42,43). A significant finding was that topical DNCB application did not result in change of CD4⁺ T-cells counts, on the contrary, CD8⁺ T-cells and natural killer cells counts increased significantly which was presumed via dendritic cell modulation (44). Dearman and Kimber (45) proposed that topical DNCB stimulates Th1 responses in mice. One study obtained more than 75% hair regrowth at the end of 20 weeks of DNCB therapy in 65% of treated human AA patients. However, Summer et al. (46) reported that DNCB was mutagenic against *Salmonella typhimurium* in the Ames test and should no longer be used. DNCB was tested at one point in DEBR rats with a hair re-growth response, however, the inflammatory response was so severe that the investigators abandoned the study after a few applications. The work was never published but results were listed in a review (15).

Recombinant mouse interleukin 4 (IL4)

Recent studies confirmed that in human AA Th1 reactions are dominant (18). Intraperitoneal injections of recombinant IL4 were used successfully to suppress delayed type hypersensitivity reactions (47). To determine if this approach would have an effect on spontaneous C3H/HeJ mouse AA, intralesional injections of mouse recombinant IL4 were given daily for 3 weeks which significantly restored hair growth which persisted during the 2 month observation period (18).

Gene therapy with T-box 21 (*Tbx21*)

T-box 21 is a transcription factor involved in Th1 differentiation, the dominant immunological process in AA as mentioned above. TBX21 binds to and augments the expression of interferon gamma (IFNG), which is significantly upregulated in AA skin. Using the spontaneous C3H/HeJ mouse model, antisense *Tbx21* oligonucleotide was significantly more effective for resolving AA than the non-sense oligonucleotide. Furthermore, the cationized gelatin-conjugated *Tbx21* siRNA intralesional injections were more effective than naked siRNA. Resolution of AA without recurrence persisted during the 2 month observation period. These results suggest that molecular downregulation of the Th1 response can be effectively accomplished, at least in the mouse model, and that modifications in formulation and delivery can improve efficacy (18).

Immunosuppressive Treatment

Cyclosporin A (CsA)

Cyclosporin A (CsA), a cyclic endecapeptide, is a T cell-specific immunosuppressant and, due to its lack of bone marrow toxicity, has assumed a leading role in the therapy of organ transplant rejection (48). CsA is used to treat severe psoriasis and cell mediated diseases such as systemic lupus erythematosus. More recently, it has been used experimentally to treat AA (49,50). A common side effect of oral CsA is induction of hypertrichosis suggesting that CsA may be an effective treatment for different forms of hair loss, not just for the treatment of AA, but also for androgenetic alopecia (AGA). It also stimulates mouse telogen follicles to enter anagen (51,52). CsA inhibits T cell activation by inhibition of phosphatase 3, catalytic subunit, alpha isoform (PPP3CA, obsolete name: calcineurin) (53). Because CsA is a specific inhibitor of lymphocyte activation, it may be useful in treating patients with AA (50). However, whether CsA is an effective treatment for AA is controversial (49). Recent studies in mice suggest that CsA may induce hair growth by inhibiting PPP3CA which is needed for nuclear localization of the protein nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATC1) in the bulge region. NFATC1 is activated by bone morphogenic protein (BMP) signaling which results in transcriptional repression of cyclin-dependent kinase 4 (*Cdk4*) and stem cell quiescence (54).

Oliver (55) studied the effect of orally administered CsA (10 mg/kg; 5 days/week for 7 weeks) on established and extensive areas of AA lesions on DEBR rats. New hairs appeared after 10 days of treatment and there was simultaneous regrowth of hair over the whole body with restoration of a full pelt by 5 weeks. Histological examination of CsA treated DEBR rats confirmed the transition from short, dystrophic anagen follicles to long, hair-producing anagen follicles. Concomitantly, renewed hair growth following systemic CsA treatment coincided with depletion of the mononuclear cell infiltrate around lesional follicles as seen in human AA patients (50). Because oral CsA has serious side effects, such as nephrotoxicity, topical CsA potentially could be an effective way to reduce side effects if skin barrier penetration problems are overcome. Verma et al. (48) evaluated the efficacy of topical 0.5% CsA in a liposomal formulation to treat AA in the DEBR model. 15 Rats in all the groups exhibited visible hair regrowth on the CsA treated site after drug application. Histological examination revealed a reduced inflammatory infiltrate and improved hair follicle morphology within the topically treated CsA skin area as compared to the contralateral vehicle treated skin. The results of this proof of concept preliminary study suggested that CsA in lipid vesicle formulations, with and without penetration enhancers, showed some promise as a topical treatment for human AA.

Tacrolimus (FK506)

Recently, a number of publications reported topical Tacrolimus was very efficacious in treating experimental bald animal models, such as mice, rats, and hamsters. Freyschmidt-Paul, et al. (56) reported treating C3H/HeJ mice with AA using topical 0.1% Tacrolimus ointment. Four of six of these Tacrolimus-treated mice showed complete hair regrowth, whereas 1/4 vehicle-treated mice regrew hair. The mice with AA who responded to Tacrolimus had reduced perifollicular infiltrates of CD4⁺ and CD8⁺ cells and a decreased expression of MHC class I and II and ICAM1 on hair follicle epithelium as compared to control mice (56). These results suggested that topical Tacrolimus was able to induce hair regrowth in the C3H/HeJ AA model, most likely by suppressing the T cell mediated immune responses. Another study (11) examined the efficacy of the topically applied Tacrolimus in the DEBR rat model. All Tacrolimus-treated DEBR rats regrew hair at the site of drug application within 14-21 days. Growth continued for 3 weeks beyond termination of

treatment after which gradual hair loss was observed. No hair growth was seen as a result of vehicle application and hair loss continued on untreated areas and in the untreated control group. Even though Tacrolimus proved effective in inducing hair regrowth in rodent AA models, it has not yet been shown to induce cosmetically acceptable hair regrowth in human AA (57). It is assumed that there is insufficient penetration into human skin due to thickness and barrier differences between human skin and the rodent models' skin.

Anthralin

Anthralin is a widely used topical anti-psoriatic drug that may have an immunomodulating effect on human AA as it does in psoriasis (58). Anthralin remains one of the most effective and most widely used therapeutic agents for psoriasis (59). At the molecular level, anthralin inhibits pro-inflammatory cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF), interferon- γ (IFN γ), interleukin-6 (IL6), interleukin-8 (IL8), and tumor necrosis factor- α (TNF α) produced by activated monocytes and epidermal keratinocytes (59,60). The mechanism of anthralin's potential beneficial effect in AA is speculative at this time. AA is believed to be an cell mediated disease and a number of pro-inflammatory cytokines have been indicated in the pathogenesis of AA including IL1 β , TNF α , and IFN γ (22,61,62). The inhibitory effects of anthralin on the production of the proinflammatory cytokines might be the mechanism responsible for hair regrowth in AA which was identified by using the DEBR rats (58). When AA affected C3H/HeJ mice were treated daily for 10 weeks on half of the dorsal skin with 0.2% anthralin hair regrowth was observed in 9/14 mice on the treated sides only. Similarly, AA-affected DEBR rats were treated on the dorsal surface using 0.1% anthralin ointment. After six to eight weeks of treatment, follicular activity was reversed with clear regrowth over nearly all of the treated side in all rats whereas the control side remained bald (63). The results indicated that anthralin may be an effective therapy for AA-affected C3H/HeJ mice or DEBR rats promoting a hair growth response similar to that seen with anthralin treatment of human AA. The results also imply that certain cytokines may be involved in the therapeutic effects of anthralin on restoring hair regrowth in these two animal models.

Corticosteroids

Corticosteroids are most often used to treat human AA and intralesional glucocorticosteroids are consistently effective in many studies (64-67). Using the mouse model, 11 AA mice received 0.5 mg of triamcinolone acetonide intralesionally. These mice developed a short, fine hair coat. Microscopically, mice showed either normal, uninvolved telogen follicles or biopsies contained anagen follicles with few inflammatory cell infiltrates (6).

Interferons

It was postulated that upregulation of MHC I in hair follicles was one of the initiating factors in the pathogenesis of AA (68). IFN γ can cause increased expression of Major Histocompatibility Complex I (MHC I) and it has been proposed that this could induce AA. To test this hypothesis, two independent labs used recombinant IFN γ in unmanipulated mice. One group, using a variety of strains, found alopecia developed in C3H/HeJ but not C3H/HeN mice or other strains evaluated (69). These results were surprising since several C3H substrains, including both C3H/HeJ and C3H/HeN, develop AA with similar frequency (7). This study was repeated independently using much larger study groups of C3H/HeJ (AA susceptible) and C57BL/6J (AA resistant) mice. The second study found that AA occurred with a frequency well within what was considered to be normal background levels for the C3H/HeJ mouse strain and concluded that injection of exogenous IFN γ into normal haired mice had no significant AA promoting effect (70). Although induction of AA in mice using various forms of recombinant IFN γ yield inconsistent results (69,71), this remains a reasonable target. Injections of rat anti-mouse IFN γ improved the hair growth index more

efficiently than control rat IgG in C3H/HeJ mice (18) suggesting this may be a useful approach but more importantly supports IFNG as a target for future drug therapy.

Monoclonal antibodies to block lymphocyte co-stimulatory pathways

Monoclonal antibodies (MoAbs) were used in rodents to test hypotheses not specifically as therapeutic approaches. While they offer interesting new options, MoAb therapy can have complications. These studies summarized below define potential drug targets for future therapeutic developments.

Anti-CD44v 10

The splice variant v10 (CD44v10) is expressed on leukocytes in several cell mediated skin diseases. It is thought to be involved in leukocyte homing into the skin. CD44v10 is also expressed on T cells in AA lesional skin (72). A mouse CD44v10-neutralizing antibody (anti-CD44v10) was reported to impair the delayed-type hypersensitivity (DTH) reaction (73). Using this antibody, Freyschmidt-Paul et al. (74) demonstrated that anti-CD44v10 monoclonal antibody (MoAb) was able to inhibit the onset of AA in the C3H/HeJ AA model. Mice were injected intraperitoneally twice a week for 11 weeks with anti-CD44v10. These data showed that anti-CD44v10 inhibited the onset of AA in AA graft induced C3H/HeJ mice. Also the AA graft induced mice treated with anti-CD44v10 had a marked reduction of perifollicular CD8⁺ lymphocytes and, to a lesser degree, CD4⁺ cells, as well as a decreased expression of MHC I on hair follicle epithelium as compared to untreated control mice. It is presumed that the anti-CD44v10 antibody impaired immune cell homing (e.g., CD8⁺ T cells), thereby decreasing the immunocytes in the target tissues.

Anti-B7.1/B7.2 (CD80/CD86); mCTLA-4-mIgG2am; 53±6.72 MoAb/ GK1.5 MoAb

To examine the kinetics of cellular and molecular events leading to hair loss in rodent models of AA, the spontaneous and skin graft induced AA mouse models were used (12). Agents specific for antagonizing lymphocyte co-stimulation pathways were employed in the mouse skin graft model to determine whether interfering with these pathways was sufficient to prevent disease onset. Carroll et al. (17) reported three types of monoclonal antibodies blocked T cell activation through co-stimulatory pathways which prevented onset of AA in mice.

Anti-B7.1/B7.2 (CD80/CD86)

One pathway of T cell activation requires a costimulatory signal via CD28 on the T cell surface interacting with B7 family members on the antigen presenting cell (APC) surface (17). Monoclonal antibodies directed against the antigen-presenting cell (APC) surface markers B7.1 (CD80) and B7.2 (CD86) were predicted to prevent onset of AA in mice. In this study, C3H/HeJ mice that received skin grafts from AA affected mice of the same strain were injected with B7.1-/B7.2-specific monoclonal antibodies intraperitoneally (100 mg per mouse). These antibodies effectively inhibited AA onset in all five skin-grafted mice (17).

mCTLA-4-mIgG2am

Similarly, this study confirmed that mCTLA-4-mIgG2am delayed AA onset. 15 mice that received AA skin grafts and 15 that received normal skin grafts were all injected intraperitoneally with 100 mg of mCTLA-4-mIgG2am per mouse. All mice were injected 1 day prior to receiving the full thickness graft and then three times a week for 1 week after surgery. Because mCTLA-4-mIgG2am effectively competed with CD28 to interact with B7.2, the onset of AA was prevented in 13 of 15 and 14 of 15 mice receiving AA grafts when administered for 1 or 4 weeks, respectively (17).

53±6.72 MoAb/ GK1.5 MoAb

Rat IgG2b isotype MoAb from clones 53±6.72 and GK1.5 target and deplete mouse CD8⁺ or CD4⁺ cells, respectively (17). Of 21 spontaneous AA-affected mice, seven received 53±6.72 MoAb, seven received GK1.5 MoAb, and seven received rat IgG. Each mouse received 100 mg of antibody per injection on days 0, 3, 7, and 10 injected intraperitoneally. Mice were permitted to regenerate their CD4⁺ and CD8⁺ cell numbers. Of seven AA affected mice depleted of CD4⁺ cells, three showed significant hair regrowth with the first observation for one mouse on day 27. All AA affected mice responding to CD4⁺ cell depletion showed hair re-growth by day 34 after initiation of the study. Pelage regeneration reached a maximum by day 41. For seven AA affected mice depleted of CD8⁺ cells, significant hair regeneration was noted in one and sparse hair growth in three. Three mice showed no re-growth. First hair regrowth was noted by day 31 and all responding mice showed re-growth by day 41. These results suggest that both CD4⁺ and CD8⁺ cells are involved in AA.

All the inhibition or modification of AA onset studies described above confirmed the crucial role of T cell activation via APC co-stimulation, blockade of CD28±B7 interactions and CTLA-4-mIgG2am competition with CD28 delayed AA onset. These data are consistent with earlier studies where blockade of T cell migration using an anti-CD44v10 MoAb prevented the onset of AA in the mouse graft model (74). Blockade of parts of the lymphocyte co-stimulatory cascade prevents onset of AA in the mouse AA graft-induced model suggesting that similar approaches in human patients may prevent the development of new AA lesions (17).

Alkylating agents

Mechlorethamine

Mechlorethamine was the first alkylating agent to be tested to treat human malignancies and is still being used to treat a variety of human cancers including leukemia, lymphoma, and other solid tumors (75,76). The topical form is widely used in the treatment of cutaneous T cell lymphoma (77) and Langerhans cell histiocytosis (78). The biochemical basis of a differential response to mechlorethamine in different cell types is currently unknown. The therapeutic effects of mechlorethamine is primarily due to apoptotic cell death caused by DNA damage through production of free oxygen radicals (79). Tang (80) used 0.02% mechlorethamine to fully restore regrowth of cell mediated-arrested follicles in the C3H/HeJ AA model. Mechlorethamine appears to specifically target activated T lymphocytes in AA affected skin and may down-regulate interleukin-12 (IL12), TNFA, TNFB, and IFNG. It was proposed that these effects may be responsible for mechlorethamine's therapeutic effects in C3H/HeJ mice with AA-like disease. Mechlorethamine may be a promising therapeutic reagent for human AA except for concerns over the long-term effects of DNA damage potentially inducing neoplasia.

Hair growth promotion drugs

Minoxidil

Minoxidil was introduced in the early 1970s as a treatment for hypertension. Hypertrichosis was a common side-effect in those taking minoxidil tablets (81,82) and included the re-growth of hair in male pattern baldness (83). How minoxidil, a pyrimidine derivative (2, 4-diamino-6-piperidinopyrimidine-3-oxide), promotes hair growth in AA and protects against radiation induced alopecia is currently unknown, but may be due to stimulation of prostaglandin PGE2 or an indirect effect as proposed for AGA (84,85). In one study, topical minoxidil solutions supplemented with tocopheryl polyethylene glycol succinate (TPGS) in

cosolvent systems of various compositions, were designed. These compounds were used to evaluate the efficacy of promoting hair growth after topical application, and the safety in terms of the amount of minoxidil absorbed through the skin into the circulation, using C57BL/6J mice as a model. The results revealed that the addition of 0.5% TPGS was able to enhance hair growth, but an increase in the amount of TPGS to 2% led to deterioration of this effect (86). Although small pilot studies using minoxidil to treat human AA showed possible efficacy, no definitive trials of minoxidil in the rodent models of AA have been reported and should be tested as minoxidil is widely used off-label to treat human AA.

Gonadal steroid hormone regulator agents

Circumstantial observations suggest changes in sex hormone levels may be a modifying factor for susceptibility to mouse AA (6). Studies show that gonadal steroid hormones may have a profound impact on autoimmune disease. Estrogens can either increase or decrease immune system sensitivity depending on hormone levels and the observed immune mechanism (87,88). McElwee et al. reported that gonadectomy conferred complete resistance to AA for a subset of both females and males and delayed the onset of induced AA by up to 4 weeks in 70% of females and 55% of males (89). The experiment was repeated with gonadectomized female and male mice plus non-gonadectomized mice subcutaneously implanted with capsules containing estradiol or dihydrotestosterone. Their study suggested that gonadal steroid hormones could modulate C3H/HeJ mouse AA with estradiol promoting rapid progression of AA while dihydrotestosterone increased resistance to AA onset. The accelerated AA development in this study may reflect an overwhelming promotion of immune system activation by estradiol (E2). It may also be possible that hair follicles in estrogen supplemented rodents are more likely to enter a prolonged telogen state under adverse conditions such as when challenged by an inflammatory infiltrate. These studies suggest the estrogen receptor pathway regulates both transition of hair follicles from anagen to telogen and modifies immune system activity. Both of these mechanisms may be significant in rodent susceptibility to AA. McElwee et al. also reported that adding phytoestrogens (soy oil) in the diet reduced the numbers of C3H/HeJ mice developing AA after they received AA skin grafts (90).

Chanda et al. (91) found that 17 β -Estradiol and ICI-182780 regulated the hair follicle cycle in mice through an estrogen receptor pathway. Estradiol (E2) applied topically twice weekly to mouse skin at doses as low as 1 nmol inhibited hair growth by blocking the transition of the hair follicle from the resting phase (telogen) to the growth phase (anagen). By contrast, topical treatment with the estrogen receptor (ER) antagonist ICI-182780 reversed the effects of E2, and caused a telogen-to-anagen transition and initiate hair growth (91). Furthermore, two other similar studies reported an estrogen receptor antagonist induced onset of anagen in a dose dependent manner (92,93).

Alternatively, Kim et al. (94) suggested that the central hypothalamic-pituitary-adrenal axis is involved in alopecia areata. Psychological stress may play a role in AA (95,96). Kim et al. (94) found AA human patients had higher corticotropin releasing hormone and alpha melanocyte stimulating hormone expression in their epidermis and pilosebaceous units and adrenocorticotrophic hormone strongly expressed in the upper epidermis compared to controls. They hypothesized that 1) defects of corticosterone production in AA patients may lead to compensatory increases in corticotrophin releasing hormone, adrenocorticotrophic hormone, and alpha melanocyte stimulating hormone, or 2) AA patients may over-react psychological stress that controls. These studies suggest that regulation of the hair follicle cycle in both mice and humans involves an complicated hormonal pathway but also suggests new approaches or drug targets to treat human AA in the future.

Approaches to using rodent models for testing new treatments for alopecia areata

C3H/HeJ Mouse model of AA

Sources—Alopecia areata occurs naturally in up to 20% of C3H/HeJ females by 18 months of age and to a lesser extent in males (6). Inbred C3H/HeJ mice and other C3H substrains can be obtained from many of the commercial vendors. While it has been confirmed as a low penetrance, strain-specific disease in the C3H/HeJ strain (The Jackson Laboratory, Bar Harbor, ME) (6), AA appears to occur spontaneously in several other C3H substrains including the relatively common C3H/HeN strain (7).

Skin graft—Alopecia areata can be reproducibly induced in C3H/HeJ mice by transferring full thickness skin grafts from affected C3H/HeJ mice to 10 week old female recipients (12,90) These mice develop patchy alopecia consistently within 10 weeks of engraftment with large areas of AA by 20 weeks after surgery. C3H/HeJ mice with AA induced by the skin graft method are available from The Jackson Laboratory (West Sacramento, CA; http://jaxmice.jax.org/services/alopecia_areata.html, <http://jaxmice.jax.org/library/notes/504/504b.html>).

DEBR rat model

Sources—Approximately 70% of female DEBR develop spontaneous AA lesions covering 50% or more of the skin surface by 18 months of age. The frequency of AA in males is much less, though the hair loss pattern and extent of AA in both male and female rats with AA is similar (97). The unusually high disease phenotype presence in female DEBR makes the spontaneous model a viable option for pharmacologic research and consequently an induced AA rat model has not been developed from this strain.

Attempts have been made to find a commercial vendor or public repository willing to accept and breed the DEBR, but so far without success. Breeding pairs of DEBR rats are available from surviving research colonies (contact: KJ. McElwee for current colony locations).

Routes of Administration—There are various ways to administer drugs to rodents to test for efficacy. These are summarized below with references to provide more specific details on how to conduct these types of studies.

Oral Routes: Mice can be dosed using a gavage tube (98). It is also possible to mix the drug with jelly and let the rodents eat measured portions, a method that was used to treat DEBR rats orally with CsA.

Injectable Routes: Several routes of administration are available. Frequency will depend on route, volume, and tolerance of the compound all depending upon Institutional Animal Care and Use Committee (IACUC) approval. Compounds can be administered intravenously (via the tail vein), intramuscularly, subcutaneously, intradermally, or intraperitoneally. However, due to the extremely thin skin in mice, intradermal injections are difficult to conduct successfully and the result is often subcutaneous injection. This latter method can be done if the mouse is anesthetized and it is done into anagen stage skin where there is just enough thickness to do it. Success can be determined by the degree of resistance to injection and the nature of the epithelial “blebbing” that results. These approaches require relatively minimal restraint and are not necessarily labor intensive. Details on how to perform these methods are provided elsewhere (98).

Topical Routes: Although topical applications are the most practical for human patients, it can be problematic for mice depending upon the vehicle. As such, a variety of approaches may be needed. Compounds in volatile vehicles (such as acetone), represent minor issues. The compound can be applied with a micropipettor, allowed to spread over a marked area (the site can be tattooed to ensure the compound is repeatedly applied to the same site and spread over the same unit area of skin) and allowed to dry. Small amounts can be applied repeatedly allowing for evaporation between applications to maintain volume in a defined area. Aqueous or ointment vehicles are more problematic. We have tried a variety of stick on bubble chambers, compression bandages, and “Elizabethan collars” for mice that are available from various vendors with disastrous results. These should be avoided. Bandaging the graft surgical site has proven to be very effective. A nonstick (Telfa) type pad is placed over the site and a small custom cut vest-shaped elastic bandage is used to hold this in place. The compound is applied under the bandage daily and the bandages are changed weekly or if untoward effects are noted (ulcers, swelling, etc.) (90).

Discussion

Two rodent AA models, the C3H/HeJ mouse (6) and DEBR rat (99), are useful for testing drug efficacy and safety screening. A variety of drugs have been tested to date in both the mouse and rat models, most of which cause some degree of hair growth response in human AA patients. The positive results, although sometimes limited, correlate closely with human trials and serve as a proof of concept that these rodent strains can serve as useful models in drug efficacy trials. Since both mice and rats are used extensively in toxicology studies, they will also be useful for preclinical safety evaluation when using totally novel compounds in the future. Furthermore, the availability and small size of these two rodent models makes them practical tools for screening drugs for efficacy. Lastly, since AA is a T cell mediated cell disease, these models are potentially very useful to screen for effective agents used to treat other T cell mediated diseases. It is very easy to screen a therapeutic response to a specific agent by observing regrowth of hair clinically. Histopathology, immunocytochemistry, and gene expression analysis are but a few of the many additional assays available to further validate results.

Compounds based upon promotion of immunoregulatory mechanisms used to treat human AA are also effective in the rodent AA models as described above. Many prior studies indicate that AA is a polygenic disease with certain genes correlated to AA susceptibility while other genes modify disease severity (1,16). Loss of hair during active disease is associated with an infiltrate of activated CD4⁺ cells around the hair follicles, along with a CD8⁺ peri- and intra-follicular infiltrate (100). The interaction of host antigen presenting cells with injected, AA antigen-specific CD4⁺ T cells may stimulate naive, endogenous CD8⁺ cytotoxic T cells, which may then function as specific effector cells and target hair follicles (101,102). Transfer of CD4⁺/CD25⁺ cells has been shown to prevent autoimmune disease in several models (103,104). The depressed frequency of CD4⁺/CD25⁺ cells in AA-affected mice significantly limited the availability of cells for use in AA transfer studies (105). However, McElwee et al. (106) reported an increased resistance to AA development in mice injected with CD4⁺/CD25⁺ cells while mice injected with CD4⁺/CD25⁻ cells developed AA. This study strongly suggested that the prevention of AA development after the transfer/co-transfer of CD4⁺/CD25⁺ cells at least partly relied on the high-level of regulatory IL10 cytokine expression.

Two other studies provided evidence that follicular melanocytes may be the targets for activated T cells in AA (107,108). Control of certain melanocytic peptides is linked to immune response MHC loci, providing another possible point of control of AA by the MHC (109). However, since AA can develop in other substrains of mice with no obvious

melanization of hair (white hair) (2), and in regions of white hair induced by dry ice branding in the AA graft model (89), it is unclear if targeting melanin related antigens is critical to the pathogenesis of all types of AA.

There are several potential targets within the mechanism of an autoimmune disease pathogenesis at which new treatment development could be directed. New and improved AA therapies might be based upon down-regulation of autoreactive cells in the lymphocytic infiltrate using an immunosuppressive or immunomodulatory agent. Another approach would be to promote anergy or depletion of autoreactive T cell clones. Protecting hair follicles from injurious effects due to inflammation or assisting hair follicles to block or attenuate the adverse inflammatory activity could be used. Blocking antigen recognition by masking target antigens, specific autoreactive T cell receptors, down-regulating co-stimulatory signalling pathways associated with antigen presentation, or modifying target antigen(s) or their expression to become non-stimulatory to autoreactive T cells are also approaches (110). Finding new drug targets for AA and other human cell mediated diseases by using rodent and other animal models has recently become more feasible with the rapid progress in developing gene array technology to define the genetic basis of signalling networks. This summary of rodent models of AA provides baseline data and approaches to speed up the search for new drugs for human AA.

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Abbreviations

AA	alopecia areata
AT	alopecia totalis
AU	alopecia universalis
B7.1/B7.2	currently CD80/CD86
C3H/HeJ, C3H/HeN, and C57BL/6J	3 inbred mouse strains
CD28	CD28 antigen
CD80	CD80 antigen formerly B7-1, B7.1, CD28l, or Ly53
CD86	CD86 antigen formerly B7-2, B7.2, B70, CD28l2, LY58, or MB7-2
CsA	cyclosporin A
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DEBR	Dundee experimental bald rat
DPCP	diphenylcyclopropenone
E2	estradiol
ER	estrogen receptor
FK506	Tacrolimus
GM-CSF	granulocyte macrophage colony stimulating factor

IC-182780	estrogen receptor antagonist
IL1, 6, 8, 10, and 12	interleukin-1, 6, 8, 10, and 12
IFNG	interferon gamma
MHC I	major histocompatibility complex I
SADBE	squaric acid dibutyl ester
DNCB	dinitrochlorobenzene
Tbx21	T-box 21
TNFA	tumor necrosis factor alpha
TPGS	tocopheryl polyethylene glycol succinate

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Figure 1. C3H/HeJ mice with full thickness skin graft-induced alopecia areata compared to the haired control that received normal skin from a normal C3H/HeJ mouse 20 weeks after skin grafts. Here are 3 views of the same mice (A-C) to illustrate the extent of the hair loss. The pale patch on the dorsal thorax is the healed full thickness skin graft from an affected C3H/HeJ mouse.

Figure 2. Histologic features of graft induced alopecia areata in C3H/HeJ mice. A late anagen hair follicle is surrounded and invaded by lymphocytes (A). Disruption of the root sheaths is seen in the higher magnification of A (B) and in cross sections of other affected follicles (C). Earlier stages of anagen and catagen can be affected (not shown).

Figure 3.

Bulbs of late anagen hair follicles in normal C3H/HeJ mice form a well organized, pigmented hair fiber (A). By contrast, there are various degrees of follicular dystrophy in the bulb of mice with AA associated with lymphocyte infiltration (B, C).

Figure 4. Normal DEBR rats have a pigmented “hood” around their head and neck (A). By contrast, rats with alopecia areata have a scant hair coat. The pigmented “hood” is still visible as a consequence of melanin incontinence in the dystrophic hair follicles (B). Histologically, late anagen hair follicles in non-AA affected rats are similar to those in mice (C) while those affected with AA have lymphocytes in and around the follicles with various degrees of follicular dystrophy (D).

Table 1

Comparison of human alopecia areata with similar diseases in the mouse and rat (alopecia areata, AA; alopecia totalis, AT; alopecia universalis, AU).

Features	Human AA	C3H/HeJ Mouse AA	DEBR Rat AA
Clinical forms	AA, AT, AU	AA, AT, AU	AA, AT, AU
Wax and wane	Yes	Yes	Yes
Spontaneous hair regrowth	Yes	Yes	Yes
Hair fiber defects	Yes	Yes	Yes
Nail defects	≤ 10% of patients	Not C3H but 1/10 strains w/AA	Under investigation
Sequella: white hair	Yes, variable	Yes, variable	Yes, variable
Hair follicle autoantibodies	Yes, many types	Yes, many types	Yes, many types
Dermal/hair follicle lymphocytes	CD4 ⁺ >CD8 ⁺	CD4 ⁺ <CD8 ⁺	CD4 ⁺ >CD8 ⁺
Location of lymphocytes	Bulb to sebaceous duct	Suprabulbar to sebaceous duct	Bulb to sebaceous duct
Sexual dichotomy	Yes, age dependent	Yes	Yes
Response to treatment	Variable	See Table 2	See Table 2
Research cost	Very expensive	Relatively cheap	More expensive than mice
Number of types of AA	4+	1 (slightly different forms in other inbred strains and congenics)	1
Inheritance	Yes - Unknown	Yes – Polygenic dominant with incomplete penetrance	Yes – Polygenic dominant with incomplete penetrance

Table 2

Drugs tested in alopecia areata rodent models.

Agent/ Therapy		Route/dose/ Time	Response – Gross Analysis (immunophenotypic analysis)	Ref.
DPCP	Mouse	Topical unilaterally 16 wks	9/14 mice regrew hair	(37)
	Rat	Topical unilaterally 16 wks	10/10 rats regrew hair, no shifts of lymphocytes	(37)
SADBE Mouse		1. Topical 0.1, 0.5, 1% SADBE for 7~ 21wks 2. Topical 0.1-1.0% SADBE weekly for 22 wks	1. 9/12 (75%) mice successfully treated 2. 9/11 (81.8%) mice regrew hair	(31) (32)
FK506	Mouse	Topical 0.1% FK506 (Tacrolimus)	4/6 (66.67%) of mice had complete hair regrowth	(56)
	Rat	Topical 0.25, 0.1% FK506 for 8 wks	All treated rats regrew hair within 14-21 days	(11)
CsA (Cyclosporin A) Rat		1. Oral 10mg/kg days/week for 7 wks 2. Topical 0.5% CsA twice a day for 6 wks	1. Regrowth began after 10 days and whole body and full pelt by 5 wks 2. Regrowth is visible in 2 wks with maximum density at 6 wks	(55) (48)
Corticosteroids Mouse		Intralesional 0.5 mg of triamcinolone acetone	11 experimental mice developed a short, fine hair coat	(6)
Anti-B7.1/ B7.2 (CD80/CD86) Mouse		IP Injection anti-B7.1/ B 7.2, 100µg/ mouse/time/ week/4 wks	AA onset inhibited in skin-grafted mice by anti-B7.1- and B7.2- specific monoclonal antibodies	(17)
Anti-CD44v10 Mouse		IP injection, 150µg -200µg CD44v10 in PBS-buffered saline, 2Xweek for 11 wks	Anti-CD44v10 inhibited AA onset	(74)
Mechlorethamine Mouse		Topical 0.02% mechloretha-mine 5 consecutive days/week for 10wks.	All 24 experimental mice regrew a full pelage	(80)
Anthralin	Mouse	Topical 0.2% anthralin daily for 5 days/ week for 10 wks	On treated side hair regrew in 9/14 (64.3%) mice with 4 mice had near normal hair density and length	(111)
	Rat	Topical 0.1% anthralin for 10 wks	Nearly all rats regrew hair	(58)
Dietary soy oil/ genistein Mouse		1/3 diets containing 1%, 5% or 20% soy oil 1mg IP genistein 3x week/10 wks	Of mice on 1%, 5%, and 20% soy oil diets, 43/50 (86%), 11/28 (39%), and 2/11 (18%) developed AA respectively 4/10 (40%) mice injected with genistein and 9/10 (90%) controls developed AA	(90)
T-box21 (<i>Tbx21</i>) Mouse		1. Antisense <i>Tbx21</i> , 0.5 mg/kg/day, 0.2 ml subcutaneously every 3 days into 6 alopecic regions 2. Non-sense oligonucleotides (same as above) 3. <i>Tbx21</i> siRNA, 10 ug subcutaneously every 7 days for 3 times into 8 alopecic lesions 4. Non-sense siRNA 5. Cationized gelatine conjugated <i>Tbx21</i> siRNA	1/2. Significantly more effective than non-sense oligonucleotide 3-5. Cationized gelatine-conjugated <i>Tbx21</i> siRNA injections more effective than naked <i>Tbx21</i> siRNA or non-sense siRNA conjugated with cationized gelatin	(18)
Rat anti-mouse IFNG		0.4 mg/kg in 0.1 ml PBS subcutaneously into 10 alopecic lesions daily for one week	Improved the hair growth index more efficiently than the negative control	(18)
Recombinant mouse IL4		0.1 ug/0.2 ml subcutaneously daily for 21 days into 10 alopecic lesions intralesional daily, 3 weeks	Significant and prolonged response compared to control	(18)