Alopecia Areata Susceptibility in Rodent Models

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With our current view of alopecia areata as an autoimmune disease, it is probable that disease development in an individual is dependent on multiple genetic and environmental factors interacting in a complex system. Rodent models afford the opportunity to investigate alopecia areata development and to define the significance of the different factors involved. Recently, rodent model characterization has been conducted using flow cytometry, microarray analysis, and functional studies. From these a pattern of events in alopecia areata development has emerged. Although the preliminary activation events for the onset of alopecia areata remain unknown, the response of the immune system is characterized by antigen presentation and co-stimulation of lymphocytes in the lymph nodes and skin, a deficiency of CD4+//CD25+ regulatory cells, and an action of activated lymphocytes on hair follicles via Fas/FasL signaling and cytokines. Thus, onset of disease may require appropriate (or inappropriate) expression of stimulatory antigens within the hair follicle, the breakdown of the putative hair follicle immune privilege, the presentation of antigens to the immune system, a failure of immune system regulation, and the ability of the activated immune system to disrupt anagen-stage hair follicles. Once the sequence of events is initiated, it may become a self-perpetuating cycle, with epitope spreading leading to a wider range of targets in chronic alopecia areata. Rodent model studies have provided significant insight into alopecia areata, but much more remains to be explained about the mechanisms of disease development. Keywords: inflammation/autoimmune disease/mouse model. JID Symposium Proceedings 8:182–187, 2003

Human alopecia areata (AA) is clinically, pathologically, and genetically a heterogeneous disease defined by a number of criteria (Hoffmann and Happle, 1999). It most likely comprises several disease subsets that may be caused by multiple pathogenic mechanisms. There are now several candidate AA models available, including SCID/human xenograft, spontaneous disease development in a variety of domesticated species, and spontaneous and induced disease in inbred rodent strains (Gilhar et al., 1998; McElwee et al., 1998a; McElwee et al., 1998b; McElwee et al., 1999a; Smyth and McNeil, 1999). Each of these models has the potential to provide significant insight into the mechanisms of human AA. As with research on other autoimmune diseases (Griffiths and Remmers, 2001), each model may not reflect the full range of highly variable clinical and pathological presentations observed in humans; however, collectively they may define common, fundamental parameters and individually yield information on particular subforms of the disease. The most extensively characterized model to date is the C3H/HeJ mouse (Sundberg et al., 1994). This model meets the basic criteria of AA: The disease is tissue specific, affecting anagen-stage hair follicles; hair follicle morphology is disrupted, but no scar tissue is formed in response to the inflammatory infiltrate; no persisting infectious agents have been found that could explain inflammation; and the disease is chronic but potentially reversible with immunomodulatory treatments or by spontaneous remission. Most recently, this model was extensively examined using flow cytometry and microarray analysis and subsequent functional studies. From these studies an understanding of mouse AA development has emerged (McElwee and Hoffmann, 2002).

Currently, AA is generally regarded as an autoimmune disease, although the antigenic target(s) of attack has not yet been proved (McElwee et al., 1999b). Classically, autoimmune diseases are controlled through a complex interaction between multiple genetic and epigenetic factors. The activity of these components working synergistically and antagonistically likely determines the degree of disease susceptibility, actual disease onset, severity, duration, and potential spontaneous or treatment-induced resolution of the condition. Genes and environmental modulation of autoimmunity can operate within the individual at three primary levels: the overall reactivity of the immune system, the specific antigen and its presentation, and the target tissue itself (Marrack et al., 2001). For an understanding of AA as an autoimmune disease, each of these areas requires investigation.

**IMMUNE SYSTEM REACTIVITY**

**Environmental stressors** The events that lead to autoimmune disease in humans remain unresolved. It has been postulated that antigenic determinants of pathogens that cross-react with target organ–expressed antigens may trigger an autoimmune response by molecular mimicry and that this ultimately leads to
autoimmune disease (Oldstone, 1998; Hiemstra et al., 2001). Alternatively, an infectious agent could induce cellular injury and release self antigens, generating cytokine-mediated bystander activation of self-reactive lymphocytes that cross-react with additional, but genetically distinct, self antigens (Horwitz et al., 1998; Regner and Lambert, 2001). Similar events have been postulated and discounted for AA over many years, and research on C3H/HeJ mice has failed to define a candidate infectious agent (Sabouraud, 1896; Ikeda, 1967; Skinner et al., 1995; Jackow et al., 1998; McElwee et al., 1998c). It is possible, however, that the inciting agent is only transiently expressed and that analysis of individuals or models downstream of the activating event yields a negative result. The viral or microbial infection that initiates the autoimmune phenomenon may not be present by the time overt disease develops (Oldstone, 1998). Thus, the possibility of exogenous agent involvement in AA onset cannot be ruled out. It is also possible that the general viral and vaccine load on the immune system may raise sensitivity and reactivity and increase the probability of an inappropriate immune response in an immune system with genetically determined instability.

Other exogenous immune system stimuli may be involved in AA susceptibility. In the mouse model, diet, specifically soy oil content, has been identified as one factor capable of increasing resistance to AA development. Mice fed a diet high in soy oil were less likely to develop AA after skin grafting, a known method of transferring AA between affected and unaffected animals (McElwee et al., 1998b). In part, this resistance may be due to soy isoflavones such as genistein. The challenge from the skin graft and the subsequent failure of overt AA development lead to an alteration in the immune system with a possible increase in activation-induced cell death in the spleen (McElwee et al., 2002). Gonadal hormones have been identified as a further AA susceptibility-modifying factor in the mouse model. While estrogen supplementation may increase disease severity, testosterone supplementation can inhibit disease onset in the skin grafted induced model (McElwee et al., 2000). These and other studies demonstrate that, while AA susceptibility in rodent models and humans is genetically determined, significant input from the environment may modulate disease onset, severity, persistence, and remission, in part because of modulating general immune system reactivity.

AA frequency and severity are apparently associated with a greater frequency of allergies as compared to the general population ( Muller and Winkelman, 1963; Ikeda, 1965; Penders, 1968; De Waard-van der Spek et al., 1989; Weise et al., 1996). It remains to be seen whether there is a direct relationship whereby an allergic response is capable of increasing immune system sensitivity and instability such that AA susceptibility increases, or whether the increased frequency of allergies in AA patients is merely a reflection of genetically determined immune system sensitivity. In rodent models, the incidence of allergies has not been investigated, although the DEBRR model has a high degree of sensitivity to anesthesia (McElwee, in press). Stress has been suggested as a potential instigator of autoimmune diseases, possibly through glucocorticoid modulation of inflammatory cytokine expression (Elenkov and Chrousos, 2002). Stress has also been postulated as a potential influence on AA development, but, although the circumstantial evidence is significant, little direct evidence is available in support of the claim (Mehlman and Griesemer, 1968; Colon et al., 1991; Liakopoulos et al., 1997; Kavak et al., 2002). Other promoters of the immune system in autoimmune diseases may include drugs and toxins (Bigazzi, 1994; Elkayam et al., 1999; Holsapple, 2002; Lawrence and McCabe, 2002). In principle, one or more of these factors may modulate the immune system in AA, but in practice little is known about the effects of environmental stimuli on rodent models or humans.

**Autoreactive leukocytes** For hair follicles to be targeted and overt hair loss to occur, relevant autoreactive cell subsets must be present in an activated state and in significant numbers such that the follicles are adversely affected. The primary cell types found around and within human and rodent AA-affected hair follicles are CD4+ and CD8+. (Ranki et al., 1984; Zhang and Oliver, 1994). Recent flow cytometry studies defined a significant skin infiltrate of these cells, whereas skin-draining lymph nodes contain an increased percentage and total number of antigen-presenting cells (Zoller et al., 2002; McElwee et al., 2002). The high numbers of CD4+ and CD8+ lymphocytes consistently suggest their involvement in hair follicle dystrophy. CD4+ lymphocytes of the Th1 subset are responsible for transfer of experimental autoimmune encephalomyelitis in rodent models ( McDonald and Swanborg, 1988; Karpus and Swanborg, 1989; Mustafa et al., 1991). In experimental autoimmune encephalomyelitis, activated pathogenic T cells cross the blood–brain barrier and secrete proinflammatory cytokines and chemokines that attract other leukocytes to the local environment. Similarly in mouse AA, the putative immune privilege of the anagen-stage hair follicle is quickly broached by skin-draining lymph node cells derived from AA-affected mice transferred to non-AA-affected littermates (Carroll et al., 2002). Investigations to define the relevant subsets of immune cells capable of transferring AA are in progress. However, preliminary studies in which CD4+ or CD8+ cells were targeted in rodent models demonstrated that removal of one or the other population from AA-affected individuals is associated with hair regrowth. With recovery of the cell population, there is a reinitiation of hair loss (McElwee et al., 1996; McElwee et al., 1999c; Carroll et al., 2002). The SCID/human xenograft model has also provided data supporting a collaboration between CD4+ and CD8+ cells to promote AA (Gilhar et al., 2002). These and other studies suggest that CD4+ and CD8+ cell subsets are capable of perpetuating the AA lesion. In mouse and rat models, there is a clear division in distribution between the CD4+ and CD8+ cell populations. CD4+ cells are almost exclusively perifollicular, whereas CD8+ cells readily penetrate the hair follicle to take up intrafollicular residency. As such, these cells are likely to be the population with the greatest pathogenic potential, leaving CD4+ cells possibly providing CD8+ cell support and directly acting on the follicles through intermediaries, including hair growth–inhibiting cytokines, secretory Fas, and granymes (Carroll et al., 2002; Zoller et al., 2002; Frensches-Paul et al., 2003).

**Immune system regulation** Subsequent to immune system activation, other mechanisms of regulation must fail for AA to develop and be perpetuated. CD4+/CD25+ cells have been identified as one of probably several lymphocyte regulatory cell subsets that can restrain pathogenic cell activity. They are essential for maintaining homeostasis, they are able to suppress the induction of autoimmune disease and suppress CD4+ / CD25+ cells, and they inhibit the effector function of autoreactive CD8+ cells (Suri-Payer et al., 1998; Gao et al., 1999; Annacker et al., 2001). Little is currently known about immune regulatory mechanisms in human AA. Recent studies in the skin graft–induced AA mouse model revealed that CD4+ / CD25+ cell levels drop significantly on activation of AA and prior to the onset of overt hair loss (Zoller et al., 2002). In contrast, sham-grafted mice are able to maintain CD4+ / CD25+ cell numbers and quickly recover normal inflammatory cell numbers after injury. This apparent active depression of regulatory cells in AA-challenged mice may be a key factor in AA susceptibility. This and other regulatory methods may be defective in AA development, and their investigation may yield further insight into the mechanisms of the disease.

**ANTIGEN AND PRESENTATION**

**MHC restriction** For autoimmune disease to occur, a sustained activation of autoreactive lymphocytes is required. The amount
and localization of the antigen presented, along with the background responsiveness and regulation of the immune system and the availability of high-avidity autoreactive lymphocytes, are defining parameters in successful disease induction (Ludewig et al, 2001). For the most part, lymphocytes recognize antigenic peptides only in association with self MHC molecules and are initially activated exclusively in lymphoid organs (Karrer et al, 1997). Retrieval, processing, and presentation of antigenic peptides are conducted by professional antigen-presenting cells. The number of these cells, the duration of presentation, and the quality of presentation help determine whether autoimmunity occurs. Antigen-presenting cells are equipped with MHC class II for presentation of antigens to T helper, CD4\(^+\) cells (Inaba et al, 1998) and particular MHC alleles are known to be associated with autoimmune disease susceptibility (Nepom and Erlich, 1991). The structural nature of the MHC molecule can permit preferential binding of specific antigenic peptides and may consequently increase the level of antigen presentation to the degree that autoimmunity may develop (Wucherpfennig et al, 1995; Wucherpfennig, 2001). Studies with humans have defined several HLA gene alleles in association with the AA phenotype (de Andrade et al, 1999; Colombé et al, 1999). In a genome-wide analysis of the C3H/HeJ mouse model, a susceptibility locus for AA was identified within the region of H2, the mouse equivalent of the human HLA region (Sundberg et al, 2003). More recently, dendritic cells have been characterized as expressing MHC class I and have the capacity to present antigens derived from apoptotic cells via MHC I direct to CD8\(^+\) cells (Albert et al, 1998; Larsson et al, 2001; Fonteneau et al, 2002). Although attempts to associate MHC class I gene alleles with the AA phenotype have so far met with little success, CD8\(^+\) cells are fundamentally involved in the development of AA in rodent models (McElwee et al, 1996; Carroll et al, 2002). Thus, in addition to MHC class II, MHC I restriction may be important, and the presentation of antigens in association with MHC I on dendritic cells may play a role in the onset of AA.

Costimulation For AA to develop, the immune system must be receptive to activation. Assuming that AA is an autoimmune disease, this requires the presence of antigen-presenting cells providing excitatory stimuli for lymphocytes within the individual. For successful lymphocyte stimulation to occur, antigen-presenting cells must express costimulatory molecules, primarily B7.1, B7.2, ICAM-1, and LFA3. These bind ligands CD28, CTLA4, LFA1, and CD2 on naive lymphocytes, and this, in association with MHC- restricted antigen presentation, induces full lymphocyte activation. Recent flow cytometry analysis revealed significant changes in expression of costimulatory molecule expression in both skin-infiltrating lymphocytes and skin-draining lymph node-derived cells (Zöller et al, 2002). Other studies have shown that monoclonal antibodies against the costimulatory antigens B7.1 and B7.2 can inhibit the onset of AA in the skin graft-induced mouse model, demonstrating the importance of antigen-presenting cells in AA induction (Carroll et al, 2002). Use of the same monoclonal antibodies in mice that already overtly express AA has little effect, however, indicating that chronic AA involves multiple redundant pathogenic mechanisms. Removal of one part of the pathogenic system is not enough to block the perpetuation of the disease. This may have implications for future treatment development and suggests that a multitarget therapeutic approach may be superior to targeting a single aspect of the immune system’s activity.

TARGET TISSUE

Loss of immune privilege The hair follicle is regarded as an immune-privileged site (Westgate et al, 1991; Paus et al, 1999). In human and rodent model AA, disease onset is associated with increased expression of MHC class I and class II in the lower, transient portion of anagen-stage hair follicles (Messinger and Blehan, 1985; Bröcker et al, 1987; Zhang and Oliver, 1994). Previously, it was postulated that a loss of immune privilege through an abnormal increase in hair follicle MHC expression might be the inductive factor for AA (Paus et al, 1993). However, no evidence of constitutive MHC expression has been defined in rodent models in the transient portions of anagen-stage hair follicles prior to inflammatory cell infiltration of AA-affected hair follicles. Sham-graft injury that induces a localized expression of MHC class II in hair follicles does not activate AA (Zöller et al, 2002; McElwee, personal observations), and AA can be readily transferred from affected mice to normal-haired recipient mice by skin grafting or by lymph node–derived cell transfer (McElwee et al, 1998b; Carroll et al, 2002). This suggests that hair follicle MHC expression is a consequence of inflammation, rather than a cause, probably because of the high levels of IFN-\(\gamma\) and TNF-\(\alpha\) known to be expressed by inflammatory cells (Zöller et al, 2002). The expression of MHC molecules by hair follicle keratinocytes is nonetheless likely to be very important for the inhibition and disruption of hair follicles by autoreactive lymphocytes.

Hair follicle immunoprotection is likely transient due to the nature of the hair follicle cycle. Research suggests that the onset of catagen is associated with an infiltration of immune cells; candidate antigen-presenting cells (Parakkal, 1969; Westgate et al, 1991; Paus, 1996; Eichmüller et al, 1998). Regression of the hair follicle in catagen involves high levels of apoptosis and significant remodeling of the lower, transient portion of the hair follicle (Weedon and Strutton, 1981; Lindner et al, 1997). It is thus possible that the immune system is constantly exposed to low levels of hair follicle–derived antigens as the follicles cycle through catagen and given the ability of dendritic cells to present apoptosis-derived antigens. Autoimmune disease is not regarded as an all-or-nothing event. Rather, there are degrees of autoimmune disease, and the threshold level above which autoimmune disease is induced (Ludewig et al, 2001; McElwee et al, 1999b). A reflection of this may be the low level of hair follicle–specific antibodies found in some humans and rodents in the absence of overt AA (Tobin et al, 1994a; Tobin et al, 1994b; Tobin et al, 1997). If, however, catagen regression became disordered and the immune cell infiltrate associated with catagen inappropriately presented antigenic peptides in association with expression of costimulatory molecules, antigen presentation to the immune system might breach the threshold for stimulation of autoreactive cells. In a genetically susceptible individual, resident in a permissible environment, AA might follow. In the C3H/HeJ mouse model, AA is rapidly induced in a normal-haired mouse by transplantation of AA-affected skin, indicating that what hair follicle immune privilege there may be is little defense against the aggression of an activated immune system (McElwee et al, 1998b).
MHC molecules or alter T cell receptor (TCR) recognition and so increase the degree and nature of antigen presentation (Wucherpfennig, 2001). Thus, hair follicle defects in themselves may not promote the clinical AA phenotype, but it is possible that they may increase the chance of peptide recognition by autoreactive cells and maintain antigen presentation at a high level once autoimmune disease begins.

Hair follicle disruption While high cytokine expression in isolation is not known as a trigger factor for autoimmune disease development, high expression around and within the target tissue in conjunction with other stimuli can enhance disease pathogenesis (von Herrath et al., 1995). The inflammatory infiltrate in rodent models expresses high levels of both Th1- and Th2-type cytokines (Zöller et al., 2002), some of which may act directly on the hair follicle. TNF-α binding to its receptor is required to promote apoptosis in experimental autoimmune encephalomyelitis and inflammatory bowel disease (Piguet et al., 1998; Kassiotis and Kollias, 2001). Similarly, TNF-α activity in AA may have a negative impact on the survival of hair follicle keratinocytes in AA. While the Th2-type cytokine IL-4 is commonly regarded as a potential regulator of autoimmunity, recent studies identified its pathogenic potential in the NOD mouse model for autoimmune diabetes (Balasa et al., 2000). One study using the mouse model and histocompatible mice deficient in IL-10 suggests that IL-10 may also contribute to AA susceptibility (Freysschmidt-Paul et al., 2002). IFN-γ may promote keratinocyte expression of MHC molecules that aid other inflammatory mechanisms. Other cytokines may negatively regulate keratinocyte cell proliferation and encourage hair follicles to truncate their growth cycle and enter a telogen resting state (Randall, 2001). Overall, cytokines may be significant modulators of hair follicle disruption.

Cytotoxicity may also be mediated through Fas/Fas Ligand (FasL) interaction. Expressed on activated lymphocytes, the binding of FasL expressed on target cells by FasL on activated lymphocytes, or released in a secretory form, results in apoptosis for the target cell. This mechanism of tissue destruction is not MHC restricted and has the potential to damage innocent bystander cells not directly targeted by the pathogenic lymphocyte clones. Fas/FasL signaling is also believed to promote antigen presentation. Thus, Fas has multiple roles (Siegel et al., 2000). In the C3H/HeJ mouse model for AA, Fas and FasL are significantly differentially expressed in skin-infiltrating lymphocytes, and Fas is highly expressed on dystrophic hair follicle keratinocytes (McElwee et al., 2002; Freyschmidt-Paul et al., 2003). Histocompatible mice deficient in Fas or Fasl are comparatively resistant to the induction of AA, a result similar to that defined for insulin-dependent diabetes mellitus models (Itoh et al., 1997; Su et al., 2000). This resistance was in part localized to the skin as Fas- and FasL-deficient skin transplanted to AA-affected mice resisted immune attachment and continued to grow hair (Freysschmidt-Paul et al., 2003). The possibility of an autocrine and paracrine action of Fas and FasL within and between hair follicle keratinocytes may add to the action of the inflammatory infiltrate and may explain why FasL-deficient hair follicles were also relatively resistant to AA.

Lymphocytes may also effect tissue damage through direct cell-specific mechanisms. Perforin, produced by cytotoxic cells, is a potent mediator of cell lysis (Russell and Ley, 2002). For perforin-induced lysis of target cells to occur, the TCR of an autoreactive cell must ligand with the relevant antigenic peptide presented in conjunction with MHC on the target cell. Whether perforin-mediated cell destruction occurs in AA is unknown, but the presence of highly activated CD8+ cells within dystrophic hair follicles and the high expression of MHC molecules on hair follicle keratinocytes circumstantially suggest that MHC-restricted, perforin-mediated tissue damage can occur in AA. Other cytotoxic cell mechanisms may also be involved. Granzymes, which may gain access to target cells via perforin or induce apoptosis independent of perforin through binding cell surface receptors (Motyka et al., 2000), have been identified by microarray analysis as highly expressed in human AA (Carroll et al., 2002). Inducible nitric oxide synthase expression is apparent during mouse AA development and may suggest a role for nitric oxide in disease pathogenesis (McElwee, personal observation).

Resistance to pathogenic mechanisms Hair follicles may have some methods of resistance against these effector mechanisms of tissue damage. They may avoid total destruction by entering a telogen resting state, where antigen expression is much more limited. There is some argument as to whether there is a significantly increased frequency of telogen-stage hair follicles associated with human AA. In rodent models, AA progression is typically characterized by an initial increase in dystrophic anagen follicles, but, as individuals reach the chronic stable phase of AA, most follicles are in a telogen state (McElwee, personal observation). The natural properties of hair follicles and their regenerative ability may also provide resistance to the destructive capacity of cytotoxic lymphocytes. As long as dermal papilla cells survive and remain in close communication with keratinocytes, hair follicles may be reformed if inflammation is removed.

In this way, despite the aggressive nature of the peri- and intrafollicular inflammation and the severe disruption of the hair...
folicule unit, the possibility of spontaneous or treatment-elicted recovery from AA is possible.

CONCLUSIONS

In our current understanding of rodent models, the development of AA fundamentally involves a general genetic susceptibility with possible supplementation by a variety of minor disease severity-modifying genes. However, the actual onset of the disease along with its extent, persistence, and resistance to treatment in a given individual rodent or human may be modulated by epigenetic factors, including diet, gonadal hormones, pathogens, and other environmental influences. Both genetic and environmental factors will modulate AA at three levels. The responsiveness of the immune system may be influenced through pathogen, allergen, stress, and toxin load, with sensitivity to each defined by genetics. The specific inciting antigens and how they are presented to the immune system may be defined by environment-derived cross-reactive antigens or by inappropriate antigen exposure in the hair follicle cycle, while genes may dictate the threshold level required for stimulating autoreactive cells through the nature of the inciting or cross-reactive self antigens, MHC, TCR, costimulatory molecules, and regulatory cells. Within the skin, the failure of anagen-stage hair follicle immune privilege may be modulated by local resident pathogens or by physical trauma, and the subsequent adhesion molecule expression, cell infiltration, cytokine milieu, and hair follicle disruption may involve a complex gene interaction.

Onset of AA is dependent on a sequence of events (Fig 1) involving the presence of autoreactive lymphocytes with high affinity and avidity for hair follicle antigenic epitopes; antigen retrieval, processing, and carriage to lymphoid organs; appropriate antigen presentation in association with costimulation; deficiency of lymphocyte regulation; breakdown of immune privilege in hair follicles and perhaps other hair follicle-specific defects; and failure of the hair follicle unit to resist the onslaught of focal inflammation. The primary mechanism of hair follicle disruption in rodent AA is effected by CD8+ lymphocytes in a manner dependent on Th1 CD4+ lymphocytes. Although a Th1-mediated mechanism is fundamental to AA development, Th2 cell activity is also involved, particularly in the chronic stages of disease. Without knowing the source of the specific antigen epitopes that trigger AA onset, it is not possible to conclusively define AA as fundamentally autoimmune in nature. However, rodent model research has produced significant evidence consistent with this hypothesis.

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