Alopecia areata is a common disfiguring hair loss disorder that primarily affects the hair follicle as it enters the prolonged growth phase called anagen. The last few years have yielded an explosion of more rigorously obtained data on the etiology and pathogenesis of this disorder. While a consensus is rapidly building in support of an autoimmune pathogenesis, there are still several enigmatic issues to be resolved. These include the possibility that alopecia areata is really a multientity disorder with causes that are multifactorial. This will have important implications for the research scientist’s search for the jigsaw puzzle’s largest missing piece—the identification of the target autoantigen(s). There is now much evidence that autoimmune diseases with both T and B cell components have shared target autoantigens/epitopes. It is likely that alopecia areata is similar, as there is now very strong evidence for the generation of autoantibodies as well as autoreactive T cells to hair follicles in the pathogenesis of this disease. The following brief review outlines the progress we have made over the last five to ten years in the characterization of hair follicle antigens targeted by antibodies in alopecia areata. Results of these studies now show that the elicitation of antibodies to hair follicle-specific proteins is a highly conserved phenomenon in all affected species studied to date. Candidate autoantigens that have been identified include the 44/46 kDa hair-specific keratin (expressed in the pre cortical zone of anagen hair follicles) and trichohyalin (an important intermediate filament–associated protein) expressed in the inner root sheath of the growing hair follicle. Moreover, there is evidence that anti-hair follicle antibodies are modulated during the disease process, and may be reduced in titer during successful treatment. Preliminary data from passive transfer experiments suggest that in some species these antibodies may disrupt hair cycling. We are currently applying a more molecular approach (e.g., cDNA library screening) to identify hair follicle antigens truly associated with the onset of the disorder. Keywords: autoimmunity immunoglobulin G/trichohyalin keratins/immunofluorescence.


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Abbreviations: AA, alopecia areata; HF, hair follicle; MHC, major histocompatibility; ORS, outer root sheath; IRS, inner root sheath; CTS, connective tissue sheath.
confusion may be due to the likelihood that AA is a multitenity disorder, with causes that are multifactorial. Furthermore, there was until very recently (Olsen et al, 1999) no universally accepted criteria to describe the clinical manifestations for AA research. Understanding the cause(s) of AA is important to discovering effective treatments and is of wider biological interest given that AA may be part of a still poorly understood form of auto-aggression seen in other putative autoimmune skin diseases.

Others in this issue will discuss the immunobiology of the HF and whether this skin appendage truly represents a rare example of immune privilege. Also covered elsewhere in these workshop proceedings is whether AA meets all the criteria for autoimmune designation. For my part, I will review our current knowledge of the abnormal antibody response to HF that occurs in humans and in nonhuman mammals with AA. Special focus will be directed to the remarkably conserved nature of the cellular and molecular HF targets in AA of all species studied to date. Although there is as yet no unequivocal evidence that autoantibodies to HF play an active role in the onset, maintenance, or potentiation of AA, there is every likelihood that autoantigen targets will be similar for both the antibodies and the autoreactive T cells involved.

THE “AUTOIMMUNE” BASIS OF ALOPECIA AREATA: SOME BACKGROUND

Requirements for autoimmune designation for a disorder include the demonstration of (i) unique antigen(s) in the affected organ, 
(ii) an autoimmune response (either antibody or T cell) to that antigen(s), (iii) an autoimmune response specifically associated with the disease, (iv) an autoimmune response that produces rather than follows the disease process, and (v) the passive transfer of the disease by autoantibody or T cells. In the absence of some of these, we may look for indirect clues for autoimmunity. These may include the association of the disease with a particular HLA haplotype, other autoimmune diseases, and a responsiveness to immunosuppression therapies. It is important to stress, however, that these are only clues and should not be taken alone as evidence of an autoimmune etiology. For example, many inflammatory nonautoimmune diseases benefit from immunosuppression.

The genetics of AA is still poorly understood. Frequency and family histories in this disorder suggest, however, a polygenic mode of inheritance (reviewed in Duvic et al, 2001; McDonagh and Tazi-Ahnini, 2002). As with many autoimmune diseases with predisposing HLA haplotypes, there have also been attempts to find associations with a particular HLA haplotype in AA. These studies have yielded conflicting results in the case of class I antigens, but increasingly convincing data are now being reported for associations with certain class II haplotypes. So far, factors that are associated with susceptibility and/or severity include particular HLA alleles (e.g., DQB and DR alleles (Welsh et al, 1994; Colombe et al, 1995)). Moreover, interleukin-1 (IL-1) cluster genes (e.g., IL-1 receptor antagonist) appear strongly associated with disease severity (Tazi-Ahnini et al, 2002). Finally, there appears to be some association with chromosome 21, as indicated by the link with Down’s syndrome and autoimmune polyendocrine syndrome type I. The mutations implicated here include the autoimmune regulator (AIRE) gene on chromosome 21q22.3 and MX1 (Tazi-Ahnini et al, 2000).

The issue of disease associations between AA and other conditions is an important one, not least because it may reveal common target antigens. Despite a plethora of data, however, the only AA-associated conditions for which there appear sufficiently firm data are atopy (De Weert et al, 1984) and Down’s syndrome (Du Vivier and Munro, 1975). AA, when associated with atopy, is reported to occur earlier in life, have a more severe course and prognosis, and respond less well to treatment. The incidence of AA does appear to be increased in patients with Down’s syndrome, a condition associated with functional deficiencies in T cell–mediated immune response and abnormalities in serum IgG levels. Despite textbook assertions to the contrary, careful review of the literature indicates that incidence of clinically evident thyroid abnormalities, such as Hashimoto’s chronic lymphocytic thyroiditis, thyrotoxicosis, exophthalmic goiter, and myxedema, does not differ significantly in AA patients from that in historical controls (Korkij et al, 1984). Thus, it is difficult to conclude that there is an association between thyroid disease and AA. By contrast, there is growing evidence of an increase in the prevalence of diabetes mellitus, especially type I insulin-dependent diabetes, in relatives of patients with AA but not in the patients themselves (Wang et al, 1994). These findings suggest a genetic association between the two diseases whereby the expression of AA protects against the development of diabetes mellitus.

Several studies have concluded that the incidence of vitiligo is increased in AA (Thompson et al, 1974). Here again, however, large-scale studies have found no significant association between AA and vitiligo or a family history of vitiligo (Schallreuter et al, 1994; Tan et al, 2002). Still, vitiligo (also referred to as “autoimmune”) may yield potential lessons for future directions in AA research, as it becomes increasingly clear that it exhibits intrinsic biochemical defects in the entire epidermal melanin unit in skin (Schallreuter et al, 1994; Schallreuter and Blau, 1997). Like vitiligo, AA is a systemic disease because there is frequent involvement of tissues other than the HF, including the nails and eyes (Horn and Odom, 1980; Tosti et al, 1985). Thus, the defect may be extrinsic to HF or, if local, it may target antigens shared with other distant tissues. It needs to be emphasized nonetheless that the majority of patients with AA are in good health; it has been estimated that only 3%–5% have another autoimmune or endocrine disease. Consequently, the association between AA and other autoimmune diseases is more the exception than the rule.

Involvement of a humoral immune response to hair follicles in alopecia areata

Although it has been known for over a hundred years that dystrophic HF in AA are associated with a leukocytic infiltrate; in 1958 the possibility was formally raised that AA is an autoimmune disorder (Van Scott, 1958). Only relatively recently, however, has there been widespread acceptance of AA’s immune-mediated basis. AA is associated with a number of immune abnormalities, some of which are nonselective while others are specific and point to an immune abnormality selectively directed to a component of the anagen HF. One example of a specific abnormality is the abnormal antibody response directed specifically to HF antigens.

Involvement of the humoral immune response in AA was first suggested by the observation that abnormal deposits of immunoglobulin and complement may occur along the basement membrane zone of the lower half of the HF (Bystryn et al, 1979). The deposits occasionally extend intercellularly to the adjacent ORS and are most common at the edge or active border of lesions. Interestingly, they are also present in uninvolved scalp. The most frequent immunoreactant deposited is C3 (Igarashi et al, 1980; Igarashi et al, 1981). It is important to note that deposits are rarely present along the basement membrane zone of epidermis overlying involved HF, suggesting that the immune response is directed to an antigen localized to the HF’s lower half. That they are present also in uninvolved scalp suggest that these humoral changes may precede (and thus be involved in the pathogenesis) rather than merely result from HF injury. Caution is required in the interpretation of these data, however, as immunoreactants may also be seen in latent HF of normal individuals, where they are associated with thickened and corrugated hyaline membrane. We recently observed by direct immunofluorescence that most dogs with AA also reveal deposition of IgG in the HF (Tobin et al, in press 2003). We also saw deposits of IgG on the lower HF glassy membrane/basal lamina in a majority of subjects and, less frequently, within the follicular papilla (FP), medulla, ORS, and hair matrix. Importantly, perifollicular IgG was detected in the cytoplasm of dermal plasma cells in all but one of the dogs tested.
It has been reported that some patients with AA have antibodies to endothelial cells (Nunzi et al., 1980) or pigment cells (Galbraith et al., 1988). The latter are discussed below in terms of clues to the identity of the cells targeted by immune injury in AA.

TARGETING OF HAIR FOLLICLES BY ANTIBODIES IN HUMAN ALOPECIA AREATA

Various organ-specific autoantibodies are said to occur with increased frequency in AA (Friedmann, 1981). These associations remain for the most part controversial. With the exception of thyroid autoantibodies, there are as many studies failing to show an association as there are showing one. Given that the HF is the central target tissue in AA, it may be expected that there would be evidence of specific immune responses to this skin appendage. Thus, about five years ago we reported for the first time that antibodies specifically directed to HF do indeed occur in AA (Tobin et al., 1994a; Tobin et al., 1997c). This observation provided a basis for rethinking the immune system’s ability to induce an immune response to HF proteins expressed on or in normal HF. Our contemporaneous report of antibodies to HF also in the sera of normal healthy individuals (Tobin et al., 1994b) was unfortunately overinterpreted by several in the field. Specifically, the message is as follows: Anti-HF antibodies in normal individuals are invariably found to be low titer, mostly IgM, and detectable using the highly sensitive immunoblot assay. Thus, these reactivities are likely to represent part of the natural autoantibody pool (Cohen and Cooke, 1986). By contrast, AA-associated anti-HF antibodies are high-titer IgG and can be detected additionally with the relatively low-sensitivity indirect immunofluorescence method. This observation suggests the occurrence of a switch in immunoglobulin isotype from IgM to IgG as a function of the disease process, indicative of the activation of the antibody response to HF antigens. It may be of note that pathogenic antibodies in autoimmune diseases tend to be IgG (Cohen and Cooke, 1986). These AA-associated reactivities are not part of this natural autoantibody pool, but instead are related in some way to pathology. Thus, a convincing strand of evidence supporting the view that AA has an autoimmune basis is the observation that antibodies to HF-specific antigens are much more common and present in higher levels in patients with AA than in control individuals, including those with other types of hair pathology (scarring alopecia, demodicosis, etc.).

Hair follicle components targeted by antibodies Many “AA” antibodies are directed specifically to the HF and do not react with adjacent epidermis or dermis. By contrast, control sera that react weakly with the HF epithelium also tend to react with the epidermis and the epithelium of other skin adnexa. The “AA” antibodies are directed to multiple structures within anagen HF and also to multiple antigens within each structure. While this level of heterogeneity may appear somewhat confusing, it is important to appreciate that, as in other immune-reactive conditions (e.g., multiple sclerosis), pathology may involve a shifting of autoactivity from primary initiating self-determinants to defined cascades of secondary determinants that sustain the inflammatory self-recognition process during disease progression (Tuohy et al., 1998). Moreover, molecular mimicry of such determinants by exogenous agents might readily facilitate spreading of an autoimmune response in genetically susceptible individuals.

The most common HF structure targeted in acute human AA is the supra-Auber’s matrix/precortex followed by ORS and IRS; all are keratinocyte-derived structures. Some of these targets are located in or near proliferating and differentiating areas of the anagen HF, and thus antibodies to them may have the potential to disrupt hair differentiation and growth (Tobin et al., 1997b). In some sera, IgG antibody binding to HF components exhibits sharp demarcation patterns of reactivity—for example, reactivity is associated only with the onset of cortex differentiation or to keratinocytes that will later form the IRS (Tobin et al., 1997b). By immunoblotting analysis, AA antibodies react strongly with multiple antigens of 44–60 kDa and 200–220 kDa solubilized from anagen HF using 6 M urea (Tobin et al., 1994a). The former molecular weight region contains many keratins, including HF-specific keratins, whereas the latter equates to the trichohyalin doublet.

HF antigen targets are highly conserved across species The last few years have seen significant progress in the development and characterization of potentially relevant animal models of AA. Foremost among these are the aging C3H/HeJ mouse (Sundberg et al., 1994; Tobin et al., 1997c; McElwee et al., 1998b) and the DEBR model (McElwee et al., 1996a). Additional progress continues to be made with the characterization of outbred models (reviewed in McElwee et al., 1998a), including dogs (Tobin et al., 1998b) and horses (Tobin et al., 1998a). In all species that we have examined to date (humans, mice, rats, horses and dogs), antibody reactivity to HF-specific targets has been detected. Particularly notable was the observation that similar target components are bound by IgG antibodies in these species and include the supra-Auber’s matrix/precortex, ORS, and IRS. In many dogs with AA, for example, we have detected highly specific reactivities to antigens expressed by IRS (Tobin et al., 1998b). Moreover, in canine AA, antibodies can target IRS antigens that are expressed during specific stages in the differentiation of this anagen-specific HF cell type, such that only the IRS of the proximal IRS exhibits reactivity. These findings strongly suggest that autoantigens in AA will include keratinocyte differentiation–associated proteins.

Use of alopecia areata–associated antibodies to identify hair follicle antigen targets One of the major hindrances to progress in AA research is the dearth of knowledge regarding the identity of the precipitating HF autoantigen. Nevertheless, immunoblotting studies conducted to date in human, murine, canine, and equine AA have revealed some intriguing clues. One of the more common bands of immunoreactive protein expressed in human HF extracts separates at 44/46 kDa in SDS-PAGE. When antibody bound to this doublet is eluted from human AA immunoblots and reprobed with fresh frozen sections of human haired scalp, antibody reactivity may be detected in the anagen hair bulb (Tobin and Bystryn, 1996a). This pattern of reactivity colocalized in the anagen hair bulb (i.e., was restricted to the precortex) with the 44/46 kDa hair-specific keratin detected with AE1 monoclonal antibody (Lynch et al., 1986). More important than this was the subsequent observation that all AA sera tested, but no control sera, contained IgG that could immunoprecipitate the 46 kDa hair-specific keratin. By contrast, AA and control sera equally immunoprecipitated the AE1 reactive soft keratins (Tobin & Bystryn, 1996a). Furthermore, the occurrence of high-titer IgG that strongly binds the IRS and reacts with a 200/220 kDa doublet by immunoblotting in all four species studied, suggested to us that the IRS-rich protein trichohyalin may be another target autoantigen. Indeed, we have now shown that whole canine and equine AA sera, but not control sera, can immunoprecipitate proteins from a crude HF protein extract that reacts specifically with AE1, a monoclonal antitrichohyalin antibody. The involvement of trichohyalin as a target autoantigen in many cases of AA is of particular note. Trichohyalin is a member of the intermediate filament-associated protein family and in the HF is believed to be necessary for correct alignment of keratin filaments of the IRS and medulla (O’Guin et al., 1992). It is likely that defects of IRS differentiation (an anagen-specific event) may result in defective hair shaft formation.

In further studies, designed to assess the extent of cross-reactivity of serum IgG in various AA-affected species, we observed that C3H/HeJ serum IgG reacted with HF-specific
proteins in human HF extract that were of a similar molecular weight to those detected when human AA serum was reacted against murine HF extracts (Tobin et al, 1997c). These findings again support the view that AA targets similar HF components and antigen in all species affected by this disorder.

### Cellular targets of the immune response in alopecia areata

The classic bulbitis in AA suggests that an antigen(s) expressed/located on/in the early-anagen hair bulb is the most likely immune response target. Three different cell types in this structure have been implicated as target cell populations. These include cortical keratinocytes (Messenger and Bleehen, 1984; Messenger et al, 1986; Tobin, 1997a), melanocytes (Messenger and Bleehen, 1984; Messenger et al, 1986; Tobin et al, 1990a; Bystryn and Tobin, 1994; Paus et al, 1993; Tobin, 1997a), and endothelial cells (Nunzi et al, 1980).

**Keratinocytes** Presently, there is insufficient information to determine which of these HF cell subpopulations is the primary target in AA, although clinical, histologic, immunohistochemical, and electron microscopic (EM) evidence indicates that pre cortical keratinocytes are damaged. By transmission electron microscopy, the changes consist of cytoplasmic swelling and vacuolization, frank cellular necrosis, and increased apoptosis (Messenger and Bleehen, 1984; Tobin et al, 1991a). Deposits of granular necrotic debris (commonly with an amorphous staining pattern) may be present particularly above the basal Lamina over the upper pole of the follicular papilla. HF keratinocyte involvement in the pathogenesis of AA, at least at some stage of the HF pathology, is strongly implicated by the strong reactivity of AA sera to HF-specific hard acidic keratins (44/46 kDa). These antigens are expressed preferentially in the supra-Auber’s region of the anagen hair bulb, the site of active keratinocyte differentiation into the hair cortex and the IRS. This view is further supported by the striking reactivity to trichohyalin (especially in canine and equine AA) and by the focal weakness in the hair cortex of “exclamation-mark” hair shafts commonly seen in acute disease (Tobin et al, 1990b).

**Melanocytes** AA is associated with several clinical pigmentary anomalies that suggest melanocyte involvement as a target of the (auto)immune response. There is, for example, relative sparing of white, depigmented hairs in AA, which may be clinically evidenced by the phenomenon of rapid “whitening” or “graying” of the hair of affected individuals (Guin et al, 1980). This phenomenon indicates that HF that are melanocyte containing and pigment producing are preferentially attacked in AA. Hair bulb melanocytes are indeed damaged in AA, as evidenced by the presence of melanophages around the involved hair bulb and within the cortical matrix (Tobin et al, 1990a; Tobin et al, 1991). Bulbar melanocytes may be the first cells in the hair bulb to be attacked by infiltrating immune cells (Peereboom-Wynia et al, 1986). We need to be cautious with this interpretation, however, as focal melanocyte death may occur in HF with little evidence of immune cell infiltration (Tobin et al, 1991). Further evidence for selective damage to melanocytes is that regrowing hairs are often depigmented and that, by transmission electron microscopy, there is a decreased number of and decreased functional activity of melanocytes at a stage at which HF have regained their capacity to make hair (Messenger and Bleehen, 1984). Of critical consideration here is the positive correlation between melanocyte activity in the hair bulb (which peaks during an early phase of anagen III/IV) and the stage at which anagen HF are most vulnerable to the disease insult (Paus et al, 1993).

Although AA is clearly associated with pigmentary anomalies, there is no evidence that epidermal melanocytes are affected. In this regard, AA presents us with further evidence that follicular melanocytes are antigenically distinct from those in the epidermis (Tobin and Bystryn, 1996b). Melanocytes cultured from human HF express antigens located cytoplasmically and occasionally on the cell surface (Bystryn and Tobin, 1996). Clearly these observations, which suggest that some AA patients have antibodies to distinctive pigment cell antigens, need to be explored further. Melanocytes in locations other than hair (e.g., in the eye) are also damaged in some patients with AA (Tosti et al, 1985). It has also been reported that antibodies to melanoma cells could be detected in AA patients by indirect immunofluorescence and western blotting Galbraith et al, 1988).

### ROLE OF ANTIBODIES TO HAIR FOLLICLE COMPONENTS IN ALOPECIA AREATA

The pattern and heterogeneity of anti-HF antibodies and their conserved nature in several affected species are impressive; however, a basic question remains as to whether anti-HF antibodies participate in the etiology or pathogenesis of AA. Several observations suggest that they are not the result of the disease process. One is that high-titer antibodies are not observed in normal individuals, despite the release of HF antigens during the normal HF cycle, or even in scarring alopecias. Another is that in the C3H/HeJ mouse model of AA the abnormal autoantibody response to HF is present both in affected mice and, to a lesser degree, in their as yet clinically unaffected littersmates. This suggests that the presence of antibodies to HF appears before the onset of hair loss and so may not be produced as a secondary response to HF damage in AA.

Recently we examined the anti-HF antibody reactivity profiles of sera taken each month from birth to one year in more than 100 C3H/HeJ mice (unpublished data). Of these mice, 23 developed AA by month 12 of age. Immunoblot analysis of sera taken from these AA-affected mice revealed that antibody expression patterns and titers were modulated during the 12 months and for several mice increased in expression and intensity before the onset of clinically detectable hair loss.

Modulation of antibody profile and titer was also seen during hair regrowth that was induced by topical immunotherapy using the contact sensitizer diphencyprone (DCP) (Tobin et al, 2002). A striking reduction was detected in both the titer and the range of HF components/antigens targeted by anti-HF IgG antibodies in patients who exhibited complete and sustained hair regrowth after DCP treatment. As this immunotherapy is associated with a reduction in the titer/pattern of anti-HF antibodies, this may hold a key to the identity of the hair follicle antigen targets in AA. Moreover, the presence/titer of anti-HF antibodies may be a marker for clinical disease activity and/or an opportunity for spontaneous regrowth.

Finally, in a recent preliminary study we showed that purified IgG from an AA-affected horse could adversely affect hair regrowth when passively transferred to normal mice (Tobin et al, 1998a). Interestingly, the passively transferred equine “AA” IgG antibodies localized to the HF but did not induce hair loss per se. Rather, HF in treated skin were retained in the resting, or telogen, stage of the hair cycle—although hair shafts were shed—around the site of antibody administration. In contrast, distant HF apparently cycled normally. This study, which needs to be confirmed, should be interpreted in light of an earlier study that reported the failure of passive transfer of whole serum from human patients with AA to inhibit hair growth in human scalp skin grafted onto nude mice (Gilhar et al, 1992).

### CONCLUSION

AA may indeed result from an autoimmune response, involving both T cells and antibodies directed to self-antigens in keratino-
cytes and/or melanocytes in hair bulbs, nail, eyes, and so forth. Inherent in this view is that antigenic properties of keratinocytes or melanocytes in hair differ qualitatively and/or quantitatively from similar cells in other locations in the epidermis and so may account for their selective destruction. This is supported by the expression of unique antigens in HF not detectable in similarly prepared extracts of epidermis or dermis (Tobin et al, 1994a). AA preferentially affects hair bulb cells during the surge of mitotic activity associated with anagen III/IV, phases associated with both the beginning of differentiation of cortical keratinocytes and the functional activation of melanocytes. Therefore, it is possible that the relevant antigens are differentiation antigens (Tobin et al, 1996a) of keratinocytes or melanocytes. It is becoming increasingly clear that AA occurs in all hair mammals and that these affected animals demonstrate an abnormal HF-specific immune response. The presence of tissue-specific autoantigens in HF, and of an abnormal antibody response to HF antigens expressed by several species with AA, provides the underlying framework necessary to explain the selective damage to HF that occurs in AA. Whether anti-HF antibodies have pathogenic potential still needs to be proved. Nevertheless, even if antibodies to HF are not a primary agent in the disease, their subsequent production could further damage or maintain HF pathology. In any case, perhaps the most valuable contribution that studies of anti-HF antibody reactivity can make is their help in guiding us to the relevant target antigens (Girone et al, 2001).

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