How Close Are We to Solving the Puzzle? Review of the Alopecia Areata Research Workshop David Norris

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Speakers at the Fourth Research Workshop on Alopecia Areata clearly presented the state of current knowledge in three fundamental areas of alopecia areata (AA) research: genetics; immunology; and targets, triggers, and controls. The final speakers reviewed what we know about the treatment of AA and about the prospects for treatment modalities in the future. It is clear that research related to hair biology in general and AA in particular is now firmly established and expanding into important areas of fundamental biology, including stem cell research and sophisticated studies of epidermal differentiation and morphogenesis. That the mechanism of hair follicle dysfunction in AA is immunological, controlled by activated T cells, is evident. Whether this response is truly autoimmune remains to be firmly established, but there are strong indications that self antigens, including melanocyte antigens, are the key targets. Susceptibility to AA is likely to be genetically determined. Linkage to genes controlling the immune response is strongest in patients with severe alopecia totalist (AT) and alopecia universalis (AU), and it is probable that AA is a polygenic complex trait, with distinct genetic linkages determining specific gene subsets. Our understanding of the mechanisms through which the cycling hair follicle is damaged and dysfunctional in AA was thoroughly reviewed in this conference.

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In spite of the progress reported here, we have a long way to go before we completely understand the mechanisms of disease and can identify AA-specific targets for treatment. On the other hand, we may be very close to providing better treatment approaches. Treatments proved to be effective in other autoimmune skin diseases can be used in AA in "proof of concept" trials, which will both verify mechanisms of disease and provide practical approaches for treatment. Because AA is likely to be a complex genetic trait, an understanding of the complexities of polygenic interactions is necessary to grasp how genetic analysis should proceed. Fred Nijhout from Duke University presented an elegant perspective on complex genetic interactions, using models developed in *Drosophila* to study the nature of robustness and genetic mechanisms. Complex traits do not follow Mendel's laws, the correlations are often not linear, and there are complex networks of interaction that can be mathematically modeled. Even within these networks, however, there may be flat planes of response in which linearity exists. The effect of individual genes in such models depends entirely on context. The summation of properties law states that the sum of the squares of the effects of all genes in a pathway is one; thus, identification of a gene with a major effect means that the effects of other genes in the pathway may be low. If, for example, we identify an immune response gene that is necessary for the development of AA, then the other genes in the pathway may be of rather minor importance and only some of them may be necessary for expression of disease. Moreover, certain genes may determine which subsets of disease, such as AA, AU, or AT, may occur. The importance of context is clear in the hair follicle, where cross-talk among different cell populations controls follicle cycling. This point was made repeatedly in many of the presentations here, especially in the elegant presentation by Elaine Fuchs on the control of epidermal and follicular differentiation.

Richard Kalish presented a summary of data that he and Amos Gilhar have produced over the past ten years, demonstrating that AA is an autoimmune disease mediated by T lymphocytes in which autoantiogens are necessary to activate T cells that produce the disease. The model they used is a SCID mouse (deficient in the ability to respond to human skin grafts) engrafted with hair-bearing human skin. Leukocytes from AA patients were stimulated *in vitro* with hair follicle antigens and injected into these engrafted mice. The result was immunologic hair loss like that seen in AA. Similar SCID mouse models have been effectively used to study autoimmune skin disease in psoriasis and photosensitive lupus, but the Kalish/Gilhar model is particularly useful and robust. With it, Kalish and Gilhar showed that cells from AA patients, activated by hair follicle antigens, can transfer AA. In this adoptive transfer model, both $CD4^+$ and $CD8^+$ T cells are necessary for maximal effect, even though $CD8^+$ cells alone are sufficient to induce hair loss. More recently, the two researchers showed that hair follicle antigens as a requirement to stimulate T cells in this transfer model can be replaced by follicular melanocytes. This provides evidence that follicular melanocytes may be the targets for activated T cells in AA, as originally proposed by Tobin and Bystryn. It is interesting that control of certain melanocytic peptides is linked to immune response major histocompatibility complex (MHC) loci, providing another possible point of control of AA by the MHC.

Jerry Niederkorn provided some new perspectives on how certain locations in the body, such as the eye, might have very unique immunologic environments. This is a concept developed by his mentor, Wayne Streilein. The eye is a unique structure characterized by "immune privilege," that is, the resistance to induction of acquired immunity in that site. This resistance to immune response is based on a number of mechanisms: lack of lymphatic drainage, anterior chamber immune deviation, immunosuppressive effects of the aqueous humor itself, low MHC expression, low expression of Langerhans cells or antigen-presenting cells, and inhibition of natural killer cells. Niederkorn proposed the idea that immune privilege in the hair follicle also protects the key structures of the follicle, such as stem cells in the bulb, from immune destruction.

Ralf Paus is the father of the concept of immune privilege in the hair follicle. MHC class I molecules are not expressed in the hair matrix, in the dermal papilla, or in the outer root closest to

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the bulb. They are necessary in initiating immune responses in which endogenous antigens are presented to activate $CD8^+$ T lymphocytes.

INF-γ generated by activated T lymphocytes and natural killer cells can upregulate class I expression. In the hair follicle, such expression may be inhibited locally by growth factors, such as α-MSH, TGF-β, and IGF-1, acting in this instance as immunomodulating molecules. But Paus proposes that it is inhibited locally by a number of growth factors acting through the receptors, which in this sense act as immunomodulating chemicals.

It may be that the purpose of immune privilege in the hair follicle is indeed to protect the follicle's stem cells. I propose an alternative explanation: that the lack of MHC class I expression in the lower regions of the hair follicle is intended to protect the follicle from induction of autoimmunity during regression of the catagen follicle. In regression of the catagen follicle, the epithelial cells and melanocytes of the matrix, and the epithelial cells of the root sheaths nearest the hair bulb, all undergo apoptosis. Apoptotic fragments of the cells are available for ingestion by cells locally. If those cells had class I expression, could internalize those antigens, and present them on the surface in the context of class I that would induce an immune response against self antigens. The hair follicle is unique in that it repeatedly undergoes cycles of cell death and then regrowth, continually leaving apoptotic materials within the dermis. The differentiating epidermis, on the other hand, disposes of apoptotic corneocytes by desquamation.

In this context, Paus proposes an "immune privilege collapse model" to explain the development of autoimmunity in AA. In this model, infections, bacterial superantigens, or follicular damage trigger release of INF-γ, which induces expression of class I MHC on follicular cells leading to induction of $CD8^+$ cytotoxic cells and also of class II MHC molecules, leading to induction of $CD4^+$ help and then to downstream autoimmune phenomenon. Downstream from all of this is a spread of the immune response with antibody, macrophagus, expression of Fas Ligand, apoptosis, and damage to follicular cells. Is there a genetic predisposition to the breakdown of immune privilege in AA? I believe that is clearly one area of hair biology research that requires additional funding.

David Duggan provided a clear review of how gene arrays might be generally applied to the study of gene expression in cancer and inflammatory diseases. Several speakers presented their work on the application of microarray technology to the study of AA in human and animal models. I was impressed with Jerry Shapiro's studies using microarray and proteomic analysis to look at gene and protein expression patterns in response to therapy in the rat AA model. Another important approach is that reported by John Sundberg, using C3H/HeJ mice to study genes that are turned on in induction of AA. In both of these approaches, microarray changes are studied in the context of turning on or turning off AA. Initial studies by Sinha of microarray analysis in human AA have shown predictable changes in keratins and cytokines. David Duggan made a case for application of microarray technology to human AA, but we should be wary in the choice of experimental approach. I would favor using microarrays as they have been applied in animal models of AA, looking at experimental situations where AA is turned on or off—that is, at AA in transition, not just in established disease.

C3H/HeJ mice develop spontaneous AA-like disease in about 20% of cases and will predictably develop multiple sites of alopecia if involved skin is grafted onto uninvolved mice. This allows the development of large populations of alopecia mice with active progressing disease, which is an ideal model for gene and protein expression studies. Using microarray analysis, Sundberg found a decrease in genes controlling pigment and hair proteins early in the disease, which is just what we would expect. As the hair follicles are involuting, he found, IFN-γ, antigen-presenting cells, and, later, induction of antibodies of multiple specificities increase. The changes in antigen-presenting cells may be an important determinant of the development of autoimmunity. The C3H/HeJ animal model, which has the capacity for induction of disease by the grafting of AA-affected skin, may be perfect for the study of immune privilege breakdown in AA and for the study of T cell responses in the disease.

Epitope spreading is the expansion of the specificities of an immune response with time, characterized by the spreading of the immune response to recognize more epitopes on the same protein or epitopes similar to the one that initiated the immune response. An epitope is a small polypeptide fragment recognized by antibodies or T cell receptors. The C3H/HeJ mouse model is the place to begin looking for epitope spreading, for T cell receptor repertoire, and for specific receptors and what antigens they recognize, using the inducible aspect of the model to study early and later immune responses. The genetic associations in the model show strong linkage to chromosome 17: DQB1, DRB1, TNF, and LT, and to chromosome 9: CD3 NCAM. These are clearly regions that control the immune response and provide some idea of the genetic loci that will be identified in human AA.

Madeleine Duvic presented an update on the National AA Registry. This registry will provide the means for collection of data on disease subsets and natural history. In addition, it will focus on collection of multiplex families and sib pairs, which will be important in the study of genetic linkages in AA, AT, and AU.

The power of genetic approaches was addressed by Angela Christiano. She reported on the results of a study of five large multiplex families with AA, using some families not enrolled in the registry. Her initial results show significant linkages at four sites: chromosomes 10, 6, 16, and 18. So, we are starting down the road of finding the genes responsible for this disease.

Elaine Fuchs discussed the WNT signaling pathway and how it controls cell proliferation, transcription, and adhesion and attachment. WNT signaling is important in stem cell development, in hair follicle determination, and in the commitment of hair matrix cells to formation of hair keratins. The details of how the signaling pathways link cell attachment, proliferation, and differentiation will provide fundamental understanding of the molecular control of hair follicle development and cycling. In the context of AA, this knowledge may make it possible to maintain follicle growth even in the face of immune attacks.

Another crucial signaling pathway controlling hair follicle development is Sonic Hedgehog (SHH), discussed by Andrzej Dlugoz. SHH is required for hair follicle growth but not for differentiation, and mutations in this pathway produce basal cell carcinoma (BCC). Downstream mutations stop hair follicle regression and induce tumors like BCC. We are developing a fundamental understanding of control of the development and cycling of hair follicles involving multiple signaling elements and growth factors: WNT and Sonic Hedgehog, keratinocyte growth factor, fibroblast growth factor-V, and NOGGIN.

It is clear that neuropeptides are involved in controlling the immune response in psoriasis and atopic dermatitis. Vladimir Botchkarev proposed that they may control $CD8^+$ lymphocyte populations in AA as well. His evidence is compelling that neurotrophins are present in the hair follicles in AA lesions and that they can kill peripheral blood $CD8^+$ T cells from AA patients. Do neurotrophins eliminate $CD8^+$ suppressors intended to terminate the immune response? David Whiting showed us that T cells are eliminated from lesions of long-established AA. Are neurotrophins the mechanism for their disappearance? We need new studies to determine what stimulates T lymphocytes in AA and what makes them disappear in long-standing lesions.

Kevin McElwee presented additional work showing how valuable the C3H/HeJ mice model can be. Grafting involved skin from one mouse onto syngeneic mice without AA leads to progressive AA development. In these induced mice, there are complex immunologic changes: inflammatory infiltrates consisting of $CD4^+ > CD8^+$ lymphocytes, macrophage, and B cells; local increase in cytokines IL-12, IL-6 > IL-10, and IL-4; all T cell subsets, including T regulatory cells; and hair follicle changes requiring Fas and FasL. The specific role of T cell cytokines in

inducing apoptosis in the follicle in AA will be an area of active investigation.

Desmond Tobin has persisted in his quest to identify antibodies in different AA models—human, mouse, horse, chicken, and others. In human AA, there are high-titer antibodies to hair follicle antigen, sometimes as high as 1:10,000; in the rat model they precede hair loss. If melanocytes are grown from hair follicles, these antibodies bind to them and the binding can be nuclear, cytoplasmic, and membrane. In multiple animal models, there are also antibodies to keratin or trichohyalin. Effective treatment of AA decreases the antibody levels, which I think is further evidence for a complex immune response. These antibodies can also be used as tools to determine immunologic specificity as they can be easily sampled and antibody specificities can be more easily measured than with T cell specificities. I believe this is going to be a useful way to look at epitope spreading and maturation of the immune response in AA. It was recently shown that immunotherpy in melanoma treatment can induce regression of both melanoma and vitiligo. The melanocyte destruction in these areas of AA appears to be mediated by autoantibodies, reawakening our interest in antibody destruction of melanocytes.

Jerry Shapiro showed that modern gene expression techniques can be used in animal models to determine the mechanism of action of the established treatment approaches of topical anthralin and nitrogen mustard. Anthralin induced hair regrowth in the rat alopecia model and decreased interferon-γ and thymosin and JNK signaling. Topical nitrogen mustard also decreased INF-γ, and increased IGF-1. These studies validate INF-γ as an important mediate in AA and suggest that other signaling pathways may be necessary for re-emergence of new hair follicles.

Jonathan Vogel presented an interesting view of the future and how gene creams or solutions or the injection of DNA can be used to modify skin function and may be directed to treat hair disorders such as AA. Even better, hair follicles may be removed, transduced *ex vivo*, and then transplanted to AA patches. Will gene therapy make AA a surgical disease?

Amy McMichael and Vera Price presented their views on the new immunomodulators developed for the treatment of psoriasis and now available to test for AA. These therapies fall into four major categories: killing activated T cells, inhibiting T cell activation, inducing immune deviation, and inhibiting key cytokines. The prototype drug for killing activated T cells is Ontak, a fusion protein containing a toxin and an anti-IL-2R antibody. Drugs that block T cell activation interfere with macrophage–T cell interactions by binding the TCR or secondary adhesion and signaling pairs. Immune deviation drugs switch the T cell response from TH1 to TH2. Cytokine-inhibiting drugs interfere with cytokines, such as TNF-α, or their receptors (Enbrel and Remicaide). Another important approach in development is interference with leukocyte trafficking through the binding of important adhesion structures such as ICAM-1 and selectins.

The presentations at this Fourth International Alopecia Areata Workshop taught us about the current state of research on AA and hair biology and related topics in cutaneous biology. I think that it is also important that we learn from others who have studied cutaneous autoimmune diseases and that we discover how to apply some of the lessons from these diseases to a better understanding of AA.

Lupus erythematosus is the prototypical autoimmune disease, and there is much to learn from it. John Harley just presented, at the Montagna Symposium on the Biology of Skin (2002), the results of an 8-year study of the genetics of lupus, in which he found that many of the genetic associations could be identified only by study of carefully segregated subsets of disease based on organ involvement, autoantibody specificity, and clinical features (Harley 2002; Kelly, 2002). In implementing the Alopecia Areata Registry, we must take care to collect similar clinical data related to AA subsets.

Vicky Werth has studied the susceptibility to photosensitivity in cutaneous lupus, focusing on polymorphisms in genes associated with immune response and cell death, specifically TNF-α (Werth, 2000). This is a key mediator in inflammation in the skin and in control of cell death. It is believed that these TNF polymorphisms may be important in the UV-induced cell death that leads to autoantibody production in photosensitive lupus. Polymorphisms in TNF, IL-1, and other MHC-associated molecules will be important in studies of AA biology and genetics.

Landmark work done over the past six or seven years by Livia Casciola-Rosen and Anthony Rosen at Johns Hopkins has shown that many autoimmune diseases are produced by autoantibodies specific for fragments of self antigens produced during apoptosis and presented to a defective immune system (Rosen, 1999; Rosen, 2001). The prototype of this mechanism is photosensitive lupus, in which UVR-induced apoptosis induces fragments of ρ antigens (Casciola-Rosen, 1996). Apoptosis is the principle mechanism of cell death in the regressing catagen follicle, and it may be that these apoptotic cells—hair matrix melanocytes, matrix keratinocytes, and root sheath keratinocytes—are being presented to the immune response and that autoimmunity develops in AA because of genetically determined immune response defects. Is the immune response in AA directed against proteolytic fragments of autoantigens exposed during catagen? Is the principal genetic defect in AA related to how the cells of the hair follicle die during catagen?

Psoriasis provides many possible lessons for those studying AA. It is a T cell-dependent disease, often triggered by infection. What is most unique about psoriasis is that carefully planned clinical trials were used to understand the mechanisms of disease (Gottlieb, 1995, Krueger, 2002). The model was simple and direct: Employ a highly specific treatment modality that targets a single point in the mechanism you are evaluating. Jim Krueger and Alice Gottlieb used highly selective anti-T cell treatments to reverse intractable psoriasis. Those that killed activated T cells (Ontak, UVR) led to remission of disease, whereas those that merely inhibited T cell activation (cyclosporine) produced clinical response but no remission when treatment was discontinued.

In AA, we must determine if killing T cells will also allow return of normal hair follicle function. If AA is triggered by an infection, will the immune response persist after the infection is gone? If there is epitope spreading, will the disease persist if the activated T cells are killed? Studies using specific anti-T cell treatments are essential to finding answers to these questions. There are a number of different models that we could use: drugs that target pathogenic T cells, drugs that inhibit T cell activation, drugs that induce immune deviation toward TH2 cells, and drugs that inhibit cytokines such as TNF-α. Killing pathogenic T cells is probably the best treatment because it can be seen if a remission is achieved when treatment is discontinued. Such drugs need to be used in "proof of concept" experiments.

Vitiligo is another autoimmune disease from which we can learn many lessons. Also a complex autoimmune disease, it is characterized by both autoreactive T cells and autoantibodies. In vitiligo, repigmentation of affected skin is a separate process that involves more than just suppression of the immune response. Repigmentation is distinct from pigment cell destruction (Norris, 1994), which requires activation of a special population of melanocyte precursors in the outer root sheath (Horikawa, 1996). In AA, hair cycling returns when immunosuppressives are used, but not in every patient. In the patients that do not respond to the blocking of the immune response, does something else have to be added to directly stimulate the hair follicle? A better understanding of follicle cycling and control will provide us the tools to answer this question and perhaps to find stimuli for anagen growth that overcome the inhibition imposed by the immune response in AA.

This workshop summarized the significant accomplishments in understanding hair biology and AA achieved since the first NIAMS-sponsored workshop. We have established that AA is a T cell-mediated disease and that it appears to be autoimmune. Investigators have identified some of the antigens to which the immune response is directed in AA, an accomplishment not yet

achieved in psoriasis. We have had a tremendous growth in understanding of the biology of the follicle and its role as an immune-privileged site. Moreover, we have initial genotyping of immune response-associated genes, especially in AT and AU. We are also identifying other genes, and we will continue to do that as patient populations become larger. Through the National Alopecia Areata Registry, we are collecting the patient data necessary to understand the natural history and epidemiology of this disease, and we are beginning to stratify patients by disease subset, which is necessary for these studies and for the genetic analyses now under way. We have begun to correlate the different stages of pathology of the hair follicle with disease subset, with biochemical markers of immune disease, with hair follicle response, and with the complex genetic determinants of AA and its subsets.

What needs to be done? We must continue to support basic research in hair follicle biology as the foundation on which understanding of pathophysiology and treatment will be built, and we need to support our Alopecia Areata Registry as the basis for fundamental mechanism and treatment trials. We also need to strongly support genetic studies if we are to understand disease mechanism and disease subsets, and we need to fund opportunities to apply new research approaches such as gene and protein expression through microarray analysis and proteomics. I strongly believe that we need to aggressively organize proof of concept clinical trials to verify that activated T cells are essential for development of AA and that anti-T cell drugs cause its reversal. We have to study the return of hair growth after the immune response is blocked to see if additional stimuli are needed to "restart" cycling. I think it is time to take our animal models and develop the appropriate collaborations with fundamental T cell biologists to determine the T cell receptor repertoire in the lesions of AA. What are these cells recognizing? Is it the same patient to patient? Is there epitope spreading? Does the T cell response cease late in disease? Indeed, we are close to major breakthroughs in understanding the mechanism and treatment of AA.

I must express sincere appreciation to NIAMS for their support of this entire field over the past ten years, and I personally and specifically want to thank Alan Moshell, head of the Skin Program at NIAMS, who in so many ways has helped to support the investigators in their efforts to understand AA and hair biology through these meetings, through publication of these proceedings in the *Journal of Investigative Dermatology*, and through good advice on funding approaches. Of course, I also should thank Steve Katz, the director of NIAMS, for his support of the development of this area of research.

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