Antibodies to Hair Follicles in Alopecia Areata

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Although alopecia areata is suspected to be an autoimmune disease, no direct evidence of an altered immune response to components of the hair follicle has been reported. We studied whether antibodies to normal human anagen scalp hair follicles are present in individuals with alopecia areata. Thirty-nine alopecia areata sera and 27 control sera were tested by Western immunoblotting for antibodies to 6 M urea-extractable proteins of normal anagen scalp hair follicles. At serum diluted 1:80, all alopecia areata subjects (100%), but only 44% of control individuals, had antibodies directed to one or more antigens of approximately 57, 52, 50, 47, or 44 kD. The incidence of antibodies to individual hair follicle antigens in alopecia areata was up to seven times more frequent than in control sera and their level up to 13 times greater and was statistically significant for all five antigens. Tissue specificity analysis indicated that these antigens were selectively expressed in hair follicles.

These findings indicate that individuals with alopecia areata have abnormal antibodies directed to hair follicle antigens, and support the hypothesis that alopecia areata is an autoimmune disease. Key words: SDS-PAGE/immunoblotting/protein extraction. J Invest Dermatol 102:721–724, 1994

Alopecia areata (AA) is a common disorder that produces sudden, often patchy, hair loss. The etiology of AA is unknown, but an immune pathogenesis is suspected [1]. This is based on evidence that AA is a systemic disorder [1] associated with the presence of a lymphocytic infiltrate around or in hair bulbs [2], increased numbers of Langerhans cells in affected hair follicles (HF) [3], deposits of immune reactants around HF [4], abnormal thymulin levels in AA individuals,† an increased expression of class I and class II MHC antigens in the hair bulb [5], and the fact that effective therapies for AA have, as a common denominator, an effect on immune cells in skin [1]. Reports of increased organ-specific serum antibodies in AA are so contradictory, with the exception of anti-thyroid antibodies, that it is not possible to assess their significance [1]. There are single reports of circulating antibodies to endothelial cells and pigment cells in AA [6,7], but these studies need to be confirmed.

In a previous study, we demonstrated the presence of low-titer autoantibodies to HF antigens in normal individuals [8]. These antibodies are directed, in part, to antigens that are selectively expressed in anagen HF. These observations indicate that unique antigens are expressed in normal HF and that autoimmune responses selectively directed to these antigens can occur in humans. These findings provide the underlying framework necessary to explain the selective damage to HF that occurs in AA [9]. The current study was conducted to examine whether there are abnormalities in antibodies to HF in patients with AA.

**MATERIALS AND METHODS**

**Serum** Serum was collected, from 39 AA patients during periods of active loss (10 male and 29 female; age 11–60 yrs, mean 30 yrs), and from 27 control individuals without hair abnormality (12 male and 15 female; age 2–75 years, mean 35 years), and stored at −20°C until used.

**Tissues**

*Hair Follicles:* Two procedures were used to obtain HF antigens.

Method A: *plucked hair follicles* For the majority of experiments, pigmented HF lacking follicular papillae were individually plucked from the parietal regions of the scalp of five normal healthy individuals (four male and one female, mean 27 years) using fine forceps. Anagen HF were identified microscopically by the presence of prominent outer root sheath and well-pigmented bulb. HF collected by this method rarely contained follicular papillae and those that did were excluded from study. One hundred anagen HF were collected and placed immediately into 1 ml of 6 M urea in distilled water (pH 7.8) with 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml of leupeptin, pepstatin, antipain, and chymostatin (Sigma, St. Louis, MO) and then incubated overnight at 4°C. The supernatant was collected by centrifugation at 8800 X g for 15 min, and stored at −20°C until used.

Method B: *Intact HF* In some experiments, whole anagen HF, containing follicular papillae, were obtained from scalp cosmetic surgery procedures. The skin specimens were cut into small (1 cm²) pieces and the subcutaneous fat cut away. The epidermis was separated from the dermis by incubation with 1 M NaBr containing 1 mM phenylmethylsulfonyl fluoride for 2 h at 37°C. The dermis was bisected with a scalpel approximately 2 mm above the level of the pigmented terminal anagen hair bulbs. The lower portion of the dermis, which in all instances contained the lower half of terminal anagen HF (i.e., below the level of the arrector pili muscle and below the level of telogen HF as confirmed by light microscopy), was incubated with 0.25% collagenase (Sigma) at 37°C for 30 min and gently vortexed to release the anagen HF. The HF, containing both connective tissue sheath and follicular papillae (confirmed by light microscopy), was collected by centrifugation at 250 X g for 5 min, and washed repeatedly with phosphate-buffered saline (PBS) to remove contaminating, non-follicular, dermal tissue; then the follicles were extracted with 6 M urea overnight at 4°C and the supernatant collected by centrifugation at 8800 X g for 15 min, and stored at −20°C until used. The HF proteins obtained using methods A and B were never combined.

**Control Tissue and Cell Extracts:** Specificity analysis was conducted using similarly prepared 6 M urea extracts of a panel of fresh, normal, and malignant tissues and cells. Normal follicular papilla cells were obtained from HF isolated using method B and further treated with 0.05% trypsin and 0.53 mM ethylenediamine tetracetic acid (Gibco BRL, Grand Island, NY)
for 5 min at 37°C. The resultant cell suspension contained follicular papillae that remained intact and floated at the liquid air interface. The follicular papillae were removed intact by gentle aspiration using a Pasteur pipette, placed into plastic petri dishes, and cultured in keratinocyte-serum-free basal medium (Gibco BRL) containing 10% fetal bovine serum. Normal tissues consisted of scalp epidermis and dermis (derived from method B above), and human colon, kidney, and spleen (provided by Tisch Hospital, Department of Pathology, NYU School of Medicine, New York, NY). Fresh human melanoma tissue was provided by Cooperative Human Tissue Network (Ohio State University, Columbus, OH). Lung carcinoma (A-549) and rhabdomyosarcoma (RD) cells (obtained from American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (BioWhittaker, Walkersville, MA) containing fetal bovine serum and antibiotics [10]. Confluent cells were washed three times in Hank’s balanced salt solution without calcium chloride (CaCl₂), magnesium chloride (MgCl₂), and magnesium sulphate (MgSO₄) (Gibco BRL) and incubated at 37°C until the cells lifted off the substratum. The cells were collected by centrifugation at 250 X g for 5 min and resuspended in 6 M urea lysis buffer as described above.

**Protein Analysis** 6 M urea extracts, both reduced and unreduced (i.e., without 2-mercaptoethanol and boiling), were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [11] on a mini gel of an 8% polyacrylamide resolving gel and a 4% stacking porous membranes (Millipore, Bedford, MA) and stained with Fast Green FCF (Bio-Rad, Richmond, CA). Protein concentrations were determined by a dye-binding protein assay (Bio-Rad).

**Dopa Oxidase Staining** Unreduced 6 M urea extracts of plucked pigmented anagen HF were resolved by SDS – 8% PAGE. The gels were stained with L-3,4-dihydroxyphenylalanine (L-dopa) (Nutritional Biochemical Corp, Cleveland, OH) as previously described [12].

**Assay of Hair Follicle Antibodies** These were measured by Western immunoblotting [13]. Tissue extracts (15 μg protein per lane) were run on SDS – 8% PAGE, under reducing and non-reducing conditions, and electroblotted onto polyvinylidene difluoride microporous membranes. The membranes were blocked with 5% non-fat milk in PBS (pH 7.4) for 2 h at 25°C and reacted with individual sera, diluted 1:80 (unless otherwise indicated) in blocking buffer, overnight at 4°C. The membranes were then incubated with biotinylated goat anti-human IgG or IgM monospecific antisera (Organon Technika, Westchester, PA) diluted 1:100 for 2 h and then with avidin-peroxidase (Organon Technika), diluted 1:100, for 1 h and developed with 4-chloro-1-naphthol with intervening washes in PBS-Tween. Antibody level was assessed by quantitative densitometry performed by the Image 1 system gel scanning function to measure band intensity (Universal Imaging Corporation, Media, PA) [14], and analyzed for statistical significance using the Student t test. A p value of less than 0.05 was considered statistically significant.

**Results**

**Presence of Antibodies to Hair Follicles in Individuals with Alopecia Areata** The presence of antibodies to pigmented HF in the sera of 39 AA patients and 27 control individuals was measured by Western immunoblotting using as antigen source reduced 6 M urea extracts of freshly plucked, anagen scalp HF (method A). The studies were conducted at 1:80 sera dilution to minimize interference by low levels of HF antibodies that are present in most normal individuals [8]. The results are illustrated in Fig 1, and summarized in Table I. HF antibodies were detected in all (100%) patients with AA but in only 44% of normal individuals. These were predominantly directed to one or more antigens of approximately 57, 52, 50, 47, or 44 kD. The most common responses were to the 47 kD and 57 kD antigens, antibodies to which were present in 67% of AA patients but only in 11% of control individuals. Occasional sera also reacted to antigens of 220, 205, and 130 kD, but these responses were uncommon and occurred with equal frequency in both AA and control individuals.

**Level of Anti-Hair Follicle Antibodies in Alopecia Areata** Because some normal individuals have antibodies to HF antigens that co-migrate with antigens defined by AA sera, comparative studies were conducted to measure the levels of these antibodies in both groups of individuals. Antibody level was estimated from the density of bands on Western immunoblots, which was quantitated by computer-assisted densitometry [13]. The results are summarized in Table II. The analysis was restricted to patients who were antibody positive. The average level of antibody in patients with an antibody response to HF antigens was in all cases much higher in AA patients than in normal control individuals; up to 11 times greater in the case of antibodies to the 47-kD and 44-kD hair follicle antigens.

**Isotype of HF Antibody Response** The nature of the HF antibody response in individuals with AA was further examined by analyzing the immunoglobulin isotype usage in 15 HF antibody-positive patients with AA and in 15 HF antibody-positive control individuals. The studies were done at 1:20 serum dilution, to detect more easily low-titer HF antibody present in normal individuals. Representative results are illustrated in Fig 2. All control individuals had both IgG and IgM hair follicle antibodies, and the incidence and level of HF antibodies were similar in the two classes. In AA, by contrast, the HF antibodies were predominantly IgG.

**Specificity of Hair Follicle Antibodies** To determine the tissue distribution of the HF antigens defined by antibodies in AA, a panel of HF antibody-positive AA sera was reacted with 6 M urea extracts of fresh scalp pigmented anagen HF (method B), adjacent epidermis, and dermis obtained from surgical procedures in the same individual; with allogeneic fresh normal human colon, kidney, spleen; with cultured normal allogeneic follicular papilla cells; and with fresh human melanoma tissues, and cultured colon carcinoma and rhabdomyosarcoma cells, all adjusted to the same protein concentration. The results obtained with a representative serum are

<table>
<thead>
<tr>
<th>Table I. Incidence of Antibodies to Hair Follicles in AA*</th>
<th>Serum Dilution 1:80</th>
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<tbody>
<tr>
<td>HF Antigen</td>
<td>AA Number of pts (%) (n = 39)</td>
</tr>
<tr>
<td>57 kD</td>
<td>26 (67%)</td>
</tr>
<tr>
<td>52 kD</td>
<td>18 (46%)</td>
</tr>
<tr>
<td>50 kD</td>
<td>18 (46%)</td>
</tr>
<tr>
<td>47 kD</td>
<td>26 (67%)</td>
</tr>
<tr>
<td>44 kD</td>
<td>24 (62%)</td>
</tr>
<tr>
<td>Any antigen</td>
<td>39 (100%)</td>
</tr>
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</table>

* Comparison of anti-HF antibody incidence in AA and control sera. HF antibodies were extracted in 6 M urea, separated by SDS – 8% PAGE, and immunoblotted using serum diluted 1:80. Incidence was determined by the presence of a band on developed immunoblots. AA, alopecia areata.
The level of the antibody response to these 44-57-kD antigens was seven times more frequent in individuals with AA than in controls. Of antigens recognized in different AA individuals, as well as the ent HF antigens. Also much higher in AA than in the control group, up to 13 times on the individual serum tested, the source of the HF, the method of individu al patients, indicating that different patients have antibodies to different HF antigens. Overall antibody responses in AA were most common to HF antigens with molecular weights of 44 kD (62%), 47 kD (67%), 50 kD (46%), 52 kD (46%), and 57 kD (67%). Antibody responses to the 47 kD and 57 kD HF antigens were up to seven times more frequent in individuals with AA than in controls.

The level of the antibody response to these 44-57-kD antigens was also much higher in AA than in the control group, up to 13 times greater for the response to the 44 kD and 47 kD HF antigens. These findings indicate an abnormally increased production of antibodies to normal anagen HF in AA.

**DISCUSSION**

The results of this study show that abnormalities in circulating antibodies to hair follicle antigens are present in individuals with AA. This provides direct evidence of an abnormal immune response to the hair follicle in AA.

We have previously shown that HF express unique antigens that are not detectable in adjacent scalp epidermis or dermis, and that low titer of antibodies to these antigens are common in normal individuals [8]. Antibodies to HF in normal individuals were detected only at low serum dilutions and were usually not detectable at serum dilutions of 1:80 or greater. The role of these low titer antibodies, which are directed to antigens specifically expressed on HF in the physiology of hair growth, is unknown.

In this study, high titers of antibodies to anagen HF were present in 100% of AA individuals studied but in only 44% of control individuals. The antibody response was complex and directed to multiple HF antigens. The pattern of antibodies detected depended on the individual serum tested, the source of the HF, the method of protein extraction, and the antibody isotype examined. The pattern of antigens recognized in different AA individuals, as well as the intensity of reactions to different antigens, varied between different patients, indicating that different patients have antibodies to different HF antigens. Overall antibody responses in AA were most common to HF antigens with molecular weights of 44 kD (62%), 47 kD (67%), 50 kD (46%), 52 kD (46%), and 57 kD (67%). Antibody responses to the 47 kD and 57 kD HF antigens were up to seven times more frequent in individuals with AA than in controls.

The level of the antibody response to these 44-57-kD antigens was also much higher in AA than in the control group, up to 13 times greater for the response to the 44 kD and 47 kD HF antigens. These findings indicate an abnormally increased production of antibodies to normal anagen HF in AA.

**Table II. Level of Antibodies to Hair Follicles in AA* (Serum Dilution 1:80)**

<table>
<thead>
<tr>
<th>HF Antigen</th>
<th>Number of Ab- Positive Sera</th>
<th>Antibody Level* (Mean ± SEM)</th>
<th>Significance (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>57 kD</td>
<td>26</td>
<td>20 ± 5.7</td>
<td>3 ± 1.3 p &lt; 0.0005</td>
</tr>
<tr>
<td>52 kD</td>
<td>18</td>
<td>19 ± 7.3</td>
<td>5 ± 3.1 p &lt; 0.05</td>
</tr>
<tr>
<td>50 kD</td>
<td>18</td>
<td>24 ± 7.5</td>
<td>5 ± 6.4 p &lt; 0.005</td>
</tr>
<tr>
<td>47 kD</td>
<td>26</td>
<td>46 ± 9.5</td>
<td>3 ± 0.6 p &lt; 0.005</td>
</tr>
<tr>
<td>44 kD</td>
<td>24</td>
<td>33 ± 8.9</td>
<td>3 ± 0.4 p &lt; 0.05</td>
</tr>
</tbody>
</table>

*Comparison of anti-HF antibody level in AA and control sera. HF antigens were extracted in 6 M urea, separated by SDS-8% PAGE and immunoblotted sera diluted 1:80. Antibody level was assessed by quantitative densitometry performed with an Image 1 system gel scanning function to measure band intensity and analyzed for statistical significance using the Student t test. A p value of less than 0.05 was considered statistically significant.

*Analysis restricted to antibody-positive sera.

shown in Fig 3. The 44-57-kD antigens defined by AA-associated antibodies were all expressed in HF extracts, but were not detected in the other extracts except for the 57-kD antigens, which was also detected in epidermis, dermis, and several other control tissues. Occasional AA sera also contained antibodies to HF antigens of 105 kD and 130 kD (Fig 3, lane A) that were not expressed in epidermis, dermis, or other control tissues, and to a 205-kD antigen that was expressed in HF and epidermis but not in other control tissues. Control experiments to determine whether the 57-kD antigen present in HF and control tissues are the same or different have not been done.

**Figure 2. Isotype of HF antibody response in AA.** (a) Immunoblot analysis of plucked pigmented HF antigens defined by normal IgG and IgM antibodies at low serum dilution (1:30). Reduced 6 M urea-extractable proteins of plucked anagen scalp HF were separated by SDS-8% PAGE and immunoblotted using control sera diluted 1:20 and then incubated with goat anti-human IgG or IgM monospecific antisera. (b) Immunoblot analysis of plucked pigmented HF antigens defined by AA IgG and IgM antibodies. Reduced 6 M urea-extractable proteins derived from plucked anagen scalp HF were separated by SDS-8% PAGE and immunoblotted using AA sera diluted 1:20 and then incubated with goat anti-human IgG or IgM monospecific antisera. Each lane contained 15 μg protein.
Alopecia areata serum

Figure 3. Tissue-specificity analysis of intact pigmented HF antigens defined by AA anti-HF antibodies. Reduced 6 M urea-extractable proteins of pigmented HF (A), epidermis (B), and dermis (C) from the same scalp specimen, follicular papillae cells (D), normal fresh kidney (E), colon (F), spleen (G), melanoma (H), and rhadomyosarcoma (I) and lung carcinoma (J) cells were separated by SDS–8% PAGE and immunoblotted using a representative AA sera diluted 1:30 and then incubated with goat anti-human IgG antiserum. Each lane contained 15 μg protein.

In addition to the increased HF antibody titers in AA compared with control individuals, the HF antibodies in AA were predominantly IgG. By contrast, the low titer HF antibodies present in normal sera were both IgM and IgG. The reason for this is unclear. However, autoantibodies associated with disease tend to be IgG [15] whereas “natural” background autoantibodies are predominantly IgM [16,17], and a switch in isotype from IgM to IgG may occur at the onset of overt immune complex disease [18]. These findings support the hypothesis that the IgG anti-HF antibodies in AA may be pathogenic.

The hair follicle antibodies in AA were directed, in part, to antigens that were specific. When AA sera was examined against scalp hair follicle, scalp epidermis and dermis, and other control tissues AA serum reactivity to antigens of 44, 47, 50, 52, and 105 kD were found to be expressed predominantly in HF tissue. This finding may explain why the HF and not other skin components are selectively damaged in AA.

The individual HF cell(s) that are the target of HF antibodies in AA remain to be identified. Possible target cells include keratinocytes [19], HF melanocytes [20], and perifollicular endothelial cells [6]. However, we found that AA serum is unreactive with 6 M urea extracts of cultured follicular papillae cells, suggesting that this cell population is not the target of antibodies in AA. The HF melanocyte has been the focus of particular interest as the target of immune attack in AA [1], as AA preferentially affects pigmented hair and spares white hair, and regrowing hair in the disease is often initially white [21]. In addition, melanocytes may be selectively damaged both in affected hair bulbs [19] and in other locations, e.g., the eye [22] of patients with AA. There have been unconfirmed single reports of antibody reactivity to melanoma [7] and endothelial cells [6] in AA. However, there have been no reports investigating the presence of antibodies to melanocytes derived from the HF. Thus, the cell population in HF that is targeted by autoantibodies associated with HF remain to be identified.

The biologic relevance of anti-HF antibodies in AA remains to be determined. They could interrupt normal keratinocyte differentiation, resulting in the production of dystrophic anagen hairs in mild cases or, in more severe cases, the premature entry of early anagen HF into catagen, where the early anagen-associated keratin-related antigens may be down-regulated or absent.

In conclusion, we have demonstrated the presence of high-titer anti-HF antibodies in AA. These antibodies are most commonly directed to 6 M urea extractable antigens of approximate molecular weights of 57, 52, 50, 47, and 44 kD. These AA antibody-defined antigens appear to be HF-specific as they are not detectable in adjacent epidermis and dermis or other control tissues. These findings provide direct evidence of an abnormal immune response to the HF in AA and supports the hypothesis that AA is an autoimmune disorder.

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REFERENCES