Increased Frequencies of Cochlin-Specific T Cells in Patients with Autoimmune Sensorineural Hearing Loss


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Autoimmune sensorineural hearing loss (ASNHL) is the most common cause of sudden hearing loss in adults. Although autoimmune etiopathogenic events have long been suspected in ASNHL, inner ear-specific Ags capable of targeting T cell autoreactivity have not been identified in ASNHL. In this study, we show by ELISPOT analysis that compared with normal hearing age- and sex-matched control subjects, ASNHL patients have significantly higher frequencies of circulating T cells producing either IFN-γ (p = 0.0001) or IL-5 (p = 0.03) in response to recombinant human cochlin, the most abundant inner ear protein. In some patients, cochlin responsiveness involved both CD4+ and CD8+ T cells whereas other patients showed cochlin responsiveness confined to CD8+ T cells. ASNHL patients also showed significantly elevated cochlin-specific serum Ab titers compared with both normal hearing age- and sex-matched control subjects and patients with noise- and/or age-related hearing loss (p < 0.05 at all dilutions tested through 1/2048). Our study is the first to show T cell responsiveness to an inner ear-specific protein in ASNHL patients, and implicates cochlin as a prominent target Ag for mediating autoimmune inner ear inflammation and hearing loss. The Journal of Immunology, 2006, 177: 4203–4210.

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utoimmune sensorineural hearing loss (ASNHL) is characterized as a bilateral rapidly progressive adult hearing impairment that responds therapeutically to corticosteroids (Ref. 1 and Health Information on Sudden Deafness, National Institute on Deafness and Other Communication Disorders, (www.nidcd.nih.gov/health/hearing/sudden.asp)). This treatment response distinguishes ASNHL from most other hearing disorders and implies a great potential for further contemporary medical intervention. Despite its name, there is a limited understanding of possible inner ear-specific Ags that may target autoimmunity in ASNHL.

Although sera from ASNHL patients contain Abs capable of immunostaining human inner ear tissues (2–4), the Ag specificity of the autoantibodies is currently unclear. Heat shock protein 70 (HSP70) has been implicated as a possible target Ag in ASNHL (5–7) but its ability to target inner ear-specific pathology is questionable in light of its expression in a variety of noninner ear tissues and its failure to target hearing loss in immunized mice (8). Collagen II has also been proposed as a target Ag in ASNHL (9, 10). However, the ubiquitous expression of collagen II in numerous organs makes it an improbable candidate for targeting the inner ear-specific autoimmune features prevalent in patients with “primary” ASNHL who show no signs of additional inflammatory disorders or systemic autoimmunity.

Most other putative autoimmune diseases are linked to targeted recognition of major differentiation proteins. For example, proinflammatory T cell responses to myelin proteolipid protein, myelin basic protein, and myelin oligodendrocyte glycoprotein have been associated extensively with multiple sclerosis (11), and high serum titers specific for the acetylcholine receptor have been prominently linked to the symptoms of myasthenia gravis (12). Thus, it is reasonable to consider that ASNHL may be due to autoimmune responses targeted against inner ear-specific differentiation proteins.

One prominent candidate protein for targeting inner ear-specific autoimmunity in ASNHL is cochlin, primarily because it is the most abundant protein expressed in inner ear tissues (13) and because its expression in adult humans is substantially confined to the cochlear and vestibular labyrinth (14, 15). Cochlin is a product of the COCH gene mapped in humans to chromosome 14q12-q13 (16), and its mutations cause DFNA9, an autosomal dominant, nonsyndromic, progressive sensorineural hearing loss with vestibular pathology (14–18). Cochlin is an integral part of the inner ear extracellular matrix and its expression is substantially confined to the fibrocyte regions of the spiral limbus and spiral ligament, inner ear regions showing histologic abnormalities in humans with COCH mutations (14, 15).

Cochlin immunogenicity has already been implicated in hearing loss. Cochlin coprecipitates with the choline transporter-like target...
Ag recognized by the KHRI-3 IgG1 mAb that induces hearing loss in animals (19, 20). Moreover, our prior work has shown that immunization of SW/Jm mice with the p131–150 peptide derived from mouse cochlin results in a significant loss of hearing that may be passively transferred into naive recipients with peptide-activated CD4 T cells (21, 22). Cochlin antigenicity has also been shown in ASNHL since many patients have elevated serum levels of cochlin Abs (23).

Despite the ability of cochlin to target hearing loss in animals and the presence of serum cochlin Abs in ASNHL patients, there is currently no evidence implicating cochlin-specific T cell responsiveness in ASNHL. In this study, we show by ELISPOT analysis that ASNHL patients have significantly increased frequencies of circulating cochlin-specific IFN-γ-producing CD4 and/or CD8 T cells. In addition, our data indicate that ASNHL patients also have significantly higher frequencies of regulatory IL-5-producing T cells that may be targeted for expansion and immune-deviating treatment of ASNHL. The higher frequencies of cochlin-specific T cells in ASNHL were accompanied by significantly higher cochlin serum Ab titers in ASNHL patients compared with both normal hearing age- and sex-matched control subjects and patients with noise- and/or age-related hearing loss. Thus, our study implicates inner ear-specific autoreactivity in ASNHL and provides a rational basis for developing contemporary immunomodulatory strategies for treating ASNHL, a hearing disorder proven to be responsive to therapeutic intervention.

Materials and Methods

Production of recombinant human cochlin

Human cochlin cDNA generated as previously described (17), was inserted into pQE82L (Qiagen) for producing a His-tagged fused protein. XLI-Blue Escherichia coli (Stratagene) were transformed and screened for expression with HRP-conjugated His Ab (Qiagen). High level expression colonies were selected and the plasmid was maxiprepped and sequenced for verifying proper orientation and alignment. His-tagged cochlin was purified under denaturing conditions on a Ni-NTA agarose column (Qiagen). A total of 10 μl of samples in denaturing SDS-PAGE buffer were loaded on a 15% Tris-HCl gel (Bio-Rad) and blotted onto Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) and stained with HRP-conjugated His Ab (Qiagen). Detection was performed with the ECL Western Blotting Analysis System (Amer sham Biosciences) and exposure to Biomax MR film (Kodak). Molecular weight was determined by Kaledi scope Prestained Standards (Bio-Rad). Cochlin purity was optimized by HPLC purification of the Na-NTA product using a Beckman System Gold 126 solvent module (Beckman Coulter) with a Vydac C4 semipreparative column (Grace Vydac). Initial acetonitrile concentration was 5% and was ramped up in a linear fashion to 95% over 45 min. Detection was performed with a Beckman System Gold 166 detection module reading at 280 nm. Peak cochlin elution occurred at an acetonitrile concentration of 61%. Purified cochlin fractions were collected and lyophilized overnight on a Savant ModulyoD-115 system (Thermo Electron). After purification by HPLC, the purified recombinant human cochlin was resuspended in double-distilled deionized H2O, and the final concentration was determined using a Bio-Rad Protein Assay.

Selection of ASNHL study subjects

All protocols were reviewed and approved by the institutional review board of the Cleveland Clinic Foundation, and all study subjects provided informed consent before inclusion in the study. Main entry criteria for selection of ASNHL study subjects were those defined recently by Harris et al. (1). Inclusion criteria were defined as patients with bilateral sensorineural hearing loss of at least 30 decibels (dB), and progression of loss in at least one ear within 3 mo as measured by a 10 dB pure tone worsening over three consecutive test frequencies and/or a 16% decrease in word discrimination score (WDS) using a 25-word list. Exclusion criteria were defined as patients with congenital or genetic disease or patients with acquired otologic disease other than idiopathic or sudden hearing loss as well as patients with any additional autoimmune abnormalities as determined by clinical and medical history. Thus, our ASNHL study subjects represent a patient population believed to have an organ-specific ‘primary’ autoimmune disorder involving only inner ear abnormalities (Table I). Although our study and a previously published national study (1) did not restrict participation to any ethnic group(s), all patients in our study and 90% in the national study were Caucasian. The basis for this high Caucasian frequency is currently unclear. Normal hearing age- and sex-matched control study subjects were selected based on no prior clinical or medicinal history of either hearing loss or other immune abnormalities (Table I).

Selection of control study subjects with other hearing loss (OHL) abnormalities

In addition to ASNHL subjects, we evaluated cochlin serum Ab titers in patients with bilateral sensorineural hearing loss not associated with rapidly progressive inner ear disease or with any immune or autoimmune disorder (Table II). These OHL control study subjects included patients with noise-induced hearing loss and patients with presbycusis (age-related hearing loss). Inclusion criteria were defined as patients with normal hearing in both ears early in life, recent gradual progressive hearing loss equally in both ears, >60 years of age for presbycusis, >40 years of age for noise-induced hearing loss, normal otoscopic examination with audiogram showing bilateral symmetric sensorineural hearing loss unless mild asymmetry is due to noise exposure greater to one ear. Exclusion criteria were defined as patients with a family history of genetic hearing loss, ototoxic medication, corticosteroids taken in recent 3 mo for any reason or for hearing loss at any time previously, rapid progression of hearing loss occurring within 3 mo or less, history of systemic immune disease, prior or concomitant diagnosis of other ear disease, abnormal otoscopy, or audiogram showing conductive or mixed hearing loss in

### Table I. ASNHL patients and age- and sex-matched normal hearing control study subjects

<table>
<thead>
<tr>
<th>ASNHL Patients</th>
<th>Sex/Age</th>
<th>Hearing Lossb (dB PTA)</th>
<th>Hearing Drop Withinc</th>
<th>Disease Durationd</th>
<th>Steroid Responsee</th>
<th>Control Subjects</th>
<th>Sex/Age</th>
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<tr>
<td>P1f</td>
<td>F/73</td>
<td>48.8 33.8</td>
<td>3 mo 1 mo</td>
<td>4 yr</td>
<td>2 mo Negatived</td>
<td>C1 F/73</td>
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<tr>
<td>P2</td>
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<td>52.5 65</td>
<td>SP 1 wk</td>
<td>19 yr</td>
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<td>C2 F/69</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>F/53</td>
<td>48.8 45</td>
<td>1 mo 3 mo</td>
<td>1 yr</td>
<td>2 yr +</td>
<td>C3 F/52</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>F/54</td>
<td>25 81</td>
<td>1 wk 1 wk</td>
<td>1 mo</td>
<td>5 yr + + + + +</td>
<td>C4 F/54</td>
<td></td>
</tr>
<tr>
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<td>F/51</td>
<td>43.8 26.3</td>
<td>1 mo 1 mo</td>
<td>5 mo</td>
<td>1 yr + + + + + + +</td>
<td>C5 F/51</td>
<td></td>
</tr>
<tr>
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<td>M/41</td>
<td>38 26.3</td>
<td>2 mo SP</td>
<td>6 yr</td>
<td>39 yr + + + + + +</td>
<td>C6 M/41</td>
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<tr>
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<td>60 22</td>
<td>3 mo 1 mo</td>
<td>15 yr</td>
<td>1 yr Negative</td>
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<td></td>
</tr>
<tr>
<td>P8</td>
<td>F/49</td>
<td>60 68</td>
<td>1 mo SP</td>
<td>12 yr</td>
<td>22 yr + + + +</td>
<td>C8 F/49</td>
<td></td>
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</tbody>
</table>

* A 68-kDa Ab represents anti-HSP70.

b Hearing loss indicates the degree of each patient’s dB hearing level determined as the PTA of 500, 1000, and 2000 Hz at the time of ELISPOT testing. NR indicates no response.

c Time over which hearing loss occurred in weeks, months, or slowly progressive (SP).

d Disease duration indicates the period between onset of hearing loss and ELISPOT testing in weeks, months, or years.

e Degree of hearing improvement measured by dB PTA after steroid therapy: + none; + + 10–20 dB increment; + + + 20–30 dB; + + + + + + restored normal hearing.

Two (25%) of eight of our ASNHL patients failed to show corticosteroid responsiveness similar to the 20% failure rate observed by Harris et al. (1).
POT plates (Polyfiltronics) in wells precoated with IFN-γ. Recombinant human cochlin was added to wells at a final concentration of 50 ng/ml (no. M701; Endogen) or IL-5 capture Ab (no. 18551D; BD Biosciences). Cells were removed, and wells were treated with secondary Ab binding. All cochlin and control assays were performed in triplicate wells. After 24 h incubation and washing, spots were visualized by sequential treatment with biotinylated mouse anti-human IFN-γ Ab (no. M-700A; Endogen) or mouse IgG1 control Ab (no. 18551D; BD Biosciences), a 1/1000 dilution of 3,3′-diaminobenzidine-tetrahydrochloride substrate, and a 1/2048 dilution of 30% H2O2. The reaction was halted after 10 min by repeated washing with double-distilled deionized H2O.

**ELISPOT analysis**

ELISPOTs were detected using an automated Series-1 Immunospot Satellite Analyzer (Cellular Technology) with proprietary software designed to distinguish real spots from artifact. Digitized images of the wells were analyzed for detecting concentrated red color spots in which the spot density exceeds background by a factor individually calculated on the basis of hybridization patterns using a program provided by the kit manufacturer. Higher resolution analysis to arrays of motif-specific beads was determined by flow cytometry using CD4+FITC and CD8+PerCP-conjugated Abs (BD Biosciences). Purification of human CD4+ and CD8+ T cells

PBMC from ASNHL patients were enriched to ~90% CD4+ T cells by negative selection following treatment with anti-CD8 microbeads and double passage through a MidiMACS magnetic column (Miltenyi Biotec). CD8+ T cells were similarly enriched by double passage negative selection of anti-CD4 microbead-treated PBMC. Purity of the negatively selected T cell subpopulations was determined by flow cytometry analysis using CD4+FITC and CD8+PerCP-conjugated Abs (BD Biosciences). The negatively selected enriched populations were tested in ELISPOT assays as described above.

**Cochlin Ab titers**

Sera from ASNHL and control subjects were tested by direct ELISA for cochlin Ab titers. Recombinant human cochlin was plated at 10 μg/ml on 96-well Nunc-immuno plates, MaxiSorp (Nalge Nunc International), and sera were added in duplicate wells at dilutions ranging from 1/4 through 1/2048. Presence of bound Ab was determined using a peroxidase-goat anti-human IgG and L chain (Zymed Laboratories) followed by sequential treatment with ABTS substrate and H2O2 (Sigma-Aldrich). PBS was used as a control substitute for the secondary detection Ab. The reaction was stopped after 30 min by adding SDS/dimethylformamide, and absorbance at 405 nm was measured using a Wallac 1420 VICTOR2 Multilabel ELISA reader (PerkinElmer).

**HLA typing**

Low- to intermediate-level HLA class I and II typing was accomplished by sequence-specific oligonucleotide probing using commercial kits (LABType, One lambda). Briefly, purified DNA was amplified using locus-specific biotin-coupled primers followed by hybridization with motif-specific oligonucleotides coupled to flow cytometry beads. Specific hybridization to arrays of motif-specific beads was determined by flow cytometry (Luminex 100; Luminex). HLA assignment was made on the basis of hybridization patterns using a program provided by the kit manufacturer. Higher resolution HLA-DRB1* typing was accomplished by direct DNA sequencing of PCR-amplified products using primers and conditions as previously described (25, 26). Sequencing of PCR products was performed using DNA polymerase and base-specific fluorescence-labeled dideoxynucleotide termination reagents (dye terminators; Applied Biosystems). Analysis was conducted by capillary gel electrophoresis (AB 3730 DNA Analyzer; Applied Biosystems) and obtained sequences were compared with known alleles for HLA assignment (Table III).

### Table II. Patients with OHL abnormalities

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>History of Noise</th>
<th>Hearing Lossa (dB PTA)</th>
<th>WDS (%)b</th>
<th>Most Likely Cause of Hearing Lossc</th>
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<tbody>
<tr>
<td>OHL1</td>
<td>46/M</td>
<td>Yes</td>
<td>12</td>
<td>96</td>
<td>Noise</td>
</tr>
<tr>
<td>OHL2</td>
<td>53/F</td>
<td>Yes</td>
<td>12</td>
<td>96</td>
<td>Noise</td>
</tr>
<tr>
<td>OHL3</td>
<td>55/F</td>
<td>Yes</td>
<td>20</td>
<td>100</td>
<td>Noise</td>
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<tr>
<td>OHL4</td>
<td>56/M</td>
<td>Yes</td>
<td>10</td>
<td>96</td>
<td>Noise</td>
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<tr>
<td>OHL5</td>
<td>57/M</td>
<td>Yes</td>
<td>20</td>
<td>96</td>
<td>Noise</td>
</tr>
<tr>
<td>OHL6</td>
<td>62M</td>
<td>Yes</td>
<td>46</td>
<td>84</td>
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<tr>
<td>OHL7</td>
<td>67M</td>
<td>Yes</td>
<td>NAd</td>
<td>NAd</td>
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<tr>
<td>OHL8</td>
<td>65M</td>
<td>No</td>
<td>32</td>
<td>90</td>
<td>Agec</td>
</tr>
</tbody>
</table>

a Hearing loss indicates the degree of each patient’s dB hearing level determined as the PTA of 500, 1000, and 2000 Hz at the time of phlebotomy.

b The WDS measures the percentage of words that can be correctly identified when presented at a comfortable loudness level. One PTA and one WDS score is given for each patient because symmetric hearing loss equal in both ears was required for each control, i.e., the hearing impairment is the same for both ears.

c Usually a PTA > 20 is considered impaired in an adult. The reason three of our OHL controls have PTA < 20 dB is because both noise and age affect primarily the higher frequencies above 2000 Hz; the subjective impairment would not be evident in the PTA measurement but might still bother the patient enough to come in for testing. There is no reporting convention that averages test results in only the higher frequencies above the speech range. The PTA is the only reporting convention although it sometimes includes 3000 Hz as well. Generally, a WDS better than 80% is considered normal. Very often WDS is preserved despite high frequency hearing loss from noise, age, or both. This explains the apparent normal WDS scores as well. Once the audiogram review shows the high-tone loss objectively, the diagnosis of noise-induced loss is based solely on the patient history (i.e., military service, 7000 Hz cochlear noise, or work-related).

d NA means audiogram not available. A retrospective review of patient charts did not identify any record of audiogram and the diagnosis was based on written records of the audiologist and clinician.

e Age-related loss was based on a history that did not suggest an alternative cause of hearing loss and the patient’s age was 60 or more.

### ELISPOT assays

ELISPOT assays were performed as previously described (24). Briefly, PBMC were separated by centrifugation on Ficoll-Paque (Amersham Biosciences) and the leukocyte fraction was washed and resuspended at 3 × 10^6 cells/ml HL-1 medium (Cambrex) supplemented with l-glutamine, penicillin, streptomycin, HEPES buffer (Invitrogen Life Technologies), and 5% autologous serum. Cells were added at 3 × 10^5 cells/well in ELISpot plates (Polybiotinics) in wells precoated with IFN-γ capture Ab (no. M-700A; Endogen) or IL-5 capture Ab (no. 18551D; BD Biosciences). Recombinant human cochlin was added to wells at a final concentration of 100 μg/ml in a final volume of 200 μl. Positive control wells contained 10 μg/ml human CD3 mAb (BD Biosciences), a dose that has reliably provided us with distinguishable nonconfluent spots. Negative control wells contained no Ag. Ag-specificity control wells contained a 1/1000 dilution of tuberculin-purified protein derivative (PPD; Evans Medical). All cochlin and control assays were performed in triplicate wells. After 24 h incubation and washing, spots were visualized by sequential treatment with peroxidase-goat anti-human IFN-γ Ab (no. M701; Endogen) or mouse anti-human IL-5 (no. 18522D; BD Biosciences). After overnight incubation and washing, spots were visualized by sequential treatment with peroxidase-conjugated streptavidin (DakoCytomation), filtered 3-aminopropyltriethoxysilane (APS), and a 1/2000 dilution of 30% H2O2. The reaction was halted after 10 min by repeated washing with double-distilled deionized H2O.

### Purification of human CD4+ and CD8+ T cells

PBMC from ASNHL patients were enriched to ~90% CD4+ T cells by negative selection following treatment with anti-CD8 microbeads and double passage through a MidiMACS magnetic column (Miltenyi Biotec). CD8+ T cells were similarly enriched by double passage negative selection of anti-CD4 microbead-treated PBMC. Purity of the negatively selected T cell subpopulations was determined by flow cytometry analysis using CD4+ FITC and CD8-PerCP-conjugated Abs (BD Biosciences). The negatively selected enriched populations were tested in ELISPOT assays as described above.

### ELISPOT analysis

ELISPOTs were detected using an automated Series-1 Immunospot Satellite Analyzer (Cellular Technology) with proprietary software designed to distinguish real spots from artifact. Digitized images of the wells were analyzed for detecting concentrated red color spots in which the spot density exceeds background by a factor individually calculated for each plate based on the appearance of spots in positive and negative control wells. Parameters for automated spot counting have been established to distinguish contiguous and overlapping spots, and spot size criteria and circularity were used to exclude noise caused by nonspecific Ab binding.
We next used our recombinant human cochlin to determine frequencies of IFN-γ-specific T cells that are not at all specific to the inner ear making interpretation of a variety of Ags, each at undefined and different concentrations. In addition, homogenates contain factors that are not at all specific to the inner ear making interpretation of tissue-specific responsiveness quite tenuous. To address these concerns directly, we generated E. coli derived recombinant human cochlin from human cDNA (Ref. 17; Fig. 1).

Increased frequencies of cochlin-responsive T cells in ASNHL

We next used our recombinant cochlin to determine frequencies of IFN-γ-producing T cells capable of responding to human inner ear homogenate (24). Despite its ubiquitous use in ASNHL studies, inner ear homogenate is essentially an uncharacterized assortment of a variety of Ags, each at undefined and different concentrations. In addition, homogenates contain factors that are not at all specific to the inner ear making interpretation of tissue-specific responsiveness quite tenuous. To address these concerns directly, we generated E. coli derived recombinant human cochlin from human cDNA (Ref. 17; Fig. 1).

Results

Production of recombinant human cochlin

We have previously shown that ASNHL subjects have increased frequencies of IFN-γ-producing T cells in response to activation with 10 μg/ml human anti-CD3 (Fig. 3, B and E) and by frequencies of IFN-γ-secreting T cells (p = 0.83) and IL-5-secreting T cells (p = 0.31) in response to activation with a 1/1000 dilution of the irrelevant Ag, tuberculin PPD (Fig. 3, C and F).

CD4+ and CD8+ T cells from ASNHL patients respond to cochlin

We next examined whether IFN-γ responsiveness to cochlin was mediated by CD4+ and/or CD8+ T cells. PBMC from ASNHL patients were incubated with either anti-CD4 or anti-CD4 microbeads (Miltenyi Biotec) and passed twice through a MACS LS magnetic column. Flow cytometry analysis showed that the negatively selected CD3+ T cell populations were consistently enriched to ~90% CD4+ T cells (in anti-CD8-depleted PBMC) or CD8+ T cells (in anti-CD4-depleted PBMC; Fig. 4A). ELISPOT analysis showed that cochlin-induced IFN-γ production occurred was not significantly different between ASNHL patients and normal control subjects as determined by frequencies of IFN-γ-secreting T cells (p = 0.14) and IL-5-secreting T cells (p = 0.48) in response to activation with 10 μg/ml human anti-CD3 (Fig. 3, B and E) and by frequencies of IFN-γ-secreting T cells (p = 0.83) and IL-5-secreting T cells (p = 0.31) in response to activation with a 1/1000 dilution of the irrelevant Ag, tuberculin PPD (Fig. 3, C and F).

Statistical analysis

The unpaired Student t test was used to analyze differences in ELISPOT frequencies and Ab titers between ASNHL and control study subjects.

Table III. Frequencies of HLA class I and class II types in ASNHL patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>A*</th>
<th>A*</th>
<th>B*</th>
<th>B*</th>
<th>Cw*</th>
<th>Cw*</th>
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</tbody>
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#Rare allele combinations of DQBI*0611,0614 not excluded.
#HLA-DRB1* typing for P3 was performed at low resolution due to sample limitation.
#Rare allele combinations of DPB1*0502,7801; *7201/9901; *2301,9201; *5601,8001 not excluded.
#Rare allele combinations of DPB1*7501,8901 not excluded.
#Rare allele combinations of DPB1*2301,9201 not excluded.

FIGURE 1. Production of purified recombinant human cochlin. His-tagged human cochlin from transformed E. coli was purified on a Ni-NTA agarose column, loaded onto a 15% Tris-HCL gel in SDS-PAGE buffer, blotted onto a PVDF membrane, and stained with HRP-conjugated His Ab. Lane 1 of Western blot shows crude culture sample, induced with isopropyl β-D-thiogalactoside and allowed to express for 4.5 h at 37°C with shaking. Lane 2 shows purified product corresponding to the predicted molecular mass of His-tagged human cochlin.
in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations in some patients (Fig. 4B; ASNHL no. P6) but was confined in other patients to the CD8<sup>+</sup> T cell population (Fig. 4B; ASNHL no. P7).

**ASNHL patients have elevated cochlin Ab titers**

Sera from ASNHL patients, from patients with noise- and/or age-related hearing loss (Table II), and from normal hearing age- and sex-matched control subjects were tested by direct ELISA for binding to recombinant human cochlin. At all serum dilutions from 1/32 through 1/2048, ASNHL patients showed significantly elevated titers to cochlin when compared with control OHL subjects with noise and/or age-related hearing loss or to normal hearing control subjects (Fig. 5). Surprisingly, the cochlin serum Ab titers of OHL subjects were also significantly higher than those of age- and sex-matched controls. Differences in titers between all three study groups were significant at all dilutions tested from 1/32 to 1/2048 with <i>p</i> values ranging from <i>p</i> = 0.05 to <i>p</i> < 0.0005.

**HLA typing of ASNHL patients**

Class I HLA-B*13 and CW*06 and class II DRB1*07 types were found, respectively, in two of eight (25%), three of eight (37.5%), and five of eight (62.5%) of our ASNHL patients (Table III), but are found, respectively, in only 5.4, 13.6, and 25.5% of the normal American Caucasian population (Review Population Study, USA Caucasian American Bethesda, www.allelefrequencies.net). These differences did not reach statistical significance and the study of additional

**FIGURE 2.** Increased frequencies of cochlin responsive IFN-γ- and IL-5-secreting T cells in ASNHL patients. A, PBMC from eight ASNHL patients were tested by ELISPOT for frequencies of IFN-γ-secreting T cells in response to 100 μg/ml recombinant human cochlin. ASNHL patients showed highly significant (<i>p</i> = 0.0001) increased frequencies compared with (B) age- and sex-matched control subjects. Frequencies of IL-5-producing T cells were also significantly higher (<i>p</i> = 0.03) in (C) ASNHL patients compared with (D) controls. As described previously (24), IFN-γ ELISPOTS were determined at 24 h whereas IL-5 spots were determined at 48 h. n.d., Not determined. Error bars indicate ±SE.

**FIGURE 3.** PBMC from ASNHL patients have significantly increased frequencies of cochlin-responsive T cells. A, The difference in frequencies of cochlin-specific IFN-γ-secreting T cells was highly significant (<i>p</i> = 0.0001) between ASNHL and control subjects. D. The difference in frequencies of cochlin-specific IL-5-producing T cells was also significant (<i>p</i> = 0.03). Differences in nonspecific responses were not significant as determined by measuring (B) IFN-γ (<i>p</i> = 0.14) or (E) IL-5 (<i>p</i> = 0.48) ELISPOT frequencies in response to anti-CD3 or by measuring (C) IFN-γ (<i>p</i> = 0.83) or (F) IL-5 (<i>p</i> = 0.31) T cell frequencies in recall responses to PPD. Error bars indicate ±SE.
patients is needed. Higher frequencies of these HLA types in ASNHL subjects were not observed in studies by other groups (27–33).

Discussion

Our data implicate cochlin-specific IFN-γ-producing T cells in the etiopathogenesis of ASNHL and support the view that cochlin, as the most abundant protein of the inner ear, serves as a prominent candidate Ag for targeting inner ear inflammation and autoimmune-mediated hearing loss. Our data also confirm the presence of cochlin-specific IgG Ab in ASNHL and suggest that both ELISPOT determination for IFN-γ-producing T cells and cochlin Ab titers may be useful as diagnostic support assays for ASNHL.

Current diagnostic support for ASNHL focuses on detection of Abs to the HSP70 heat shock protein. In light of the lack of inner ear specificity of HSP70 and the inability of HSP70 to mediate inner ear pathology in animals, the appearance of such elevated serum Abs may represent an epiphenomenon that accompanies ASNHL symptoms rather than a causative event that initiates or mediates ASNHL. In any case, the potential for using cochlin ELISPOT and Ab responses for ASNHL diagnostic support remains appealing particularly since all of the ASNHL patients in our study had higher frequencies of IFN-γ-producing T cells and higher Ab titers than the highest control subject tested. It remains to be determined whether cochlin autoreactivity assays may be useful in discerning ASNHL from other hearing disorders including age-related hearing deficiency and noise-induced hearing loss.

Our data also show that CD4+ and CD8+ T cells are involved in cochlin recognition and in some cases only CD8+ T cell recognition of cochlin was evident. This implies that CD8+ T cells may be sufficient to account for inner ear autoimmunity. Indeed, several studies have implicated higher frequencies of a variety of HLA class I alleles in ASNHL including A2, A25, B18, B35, B39(B16), B41, Bw16, Cw4, Cw5, and Cw7 (27–30). The increased frequencies of numerous different class I alleles in ASNHL implies that multiple proteins and their derived peptides may be involved in inner ear-specific self-recognition. Although none of the above-mentioned HLA class I Ags was overrepresented in our study, we did find that B*13 and Cw*06 were found, respectively, in two of eight (25%) and three of eight (37.5%) of our ASNHL patients, compared with 5.4 and 13.6%, respectively, in the American Caucasian population ([www.allelefrequencies.net]). These increases did not reach statistical significance and should be confirmed by the study of additional patient populations.

Overrepresentation of numerous class II alleles has also been implicated in ASNHL including increased frequencies of DRB1*03,
frequencies of cochlin-specific IFN-γ-producing T cells, but also have increased numbers of cochlin-responsive IL-5-producing T cells. Although the absolute numbers of these regulatory Th2-like cells are much lower than their proinflammatory counterparts, their increased presence in ASNHL provides a basis for enhancing or complementing this native regulatory response. Several contemporary treatment strategies for autoimmune disease may be useful in this regard including treatment with IFN-β, statins, as well as targeted inhibition of TNF, and Ag-based therapies including those involving altered variants of targeted self-peptides or TCR vaccination (35).

Our data indicating increased cochlin Ab titers in ASNHL patients confirm those of Boulassel et al. (23). However, we also found that OHL control subjects with noise- and/or age related hearing loss have significantly higher cochlin Ab titers compared with normal hearing control subjects. The observed intermediate cochlin autoantibody titers in the OHL cohort implies that an adaptive inner ear autoimmune response may be implicated in noise- and/or age-related hearing loss. Indeed, elevated serum Abs against other autoantigens, most notably HSP60 and HSP70, have been reported in patients with noise-induced hearing loss (36, 37). Although it is appealing to consider that noise-induced inflammation may trigger pathogenic inner ear self-recognition events that lead to ASNHL, a direct link between acoustic trauma and the development of ASNHL has not been shown. Thus, it remains unclear whether the intermediate cochlin autoantibody levels in OHL patients have any pathogenic autoimmune implications.

In summary, our study shows that cochlin autoreactivity is significantly enhanced in patients with ASNHL. This cochlin responsiveness involves IFN-γ-producing Cd4+ and Cd8+ T cells as well as elevated cochlin-specific serum Abs. Detection of cochlin autoreactivity may ultimately prove to be diagnostically supportive and the low level but significant production of cochlin-specific IL-5-producing regulatory T cells may serve as a platform for the development of contemporary immunomodulatory adjunct therapies for one of the few hearing disorders shown to be responsive to therapeutic intervention.

References


