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


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


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.

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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/J 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-10-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. XL1-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 (Amersham Biosciences) and exposure to Bi-jugated His Ab (Qiagen). Detection was performed with the ECL Western Blotting Analysis system (Amersham Biosciences) and exposure to Bi-jugated His Ab (Qiagen).

**Purification of the Ni-NTA product using a Beckman System Gold**

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 reconstituted in double-distilled deionized H2O, and the final concentration was determined using a Bio-Rad Protein Assay.

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

<table>
<thead>
<tr>
<th>ASNHL Patients</th>
<th>Hearing Lossb</th>
<th>Hearing Drop Withinc</th>
<th>Disease Durationd</th>
<th>Steroid Responset</th>
<th>Control Subjects</th>
<th>Sex/Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1f</td>
<td>F/73 (48.8 dB)</td>
<td>3 mo 1 mo</td>
<td>4 year 2 mo</td>
<td>Negative</td>
<td>C1 F/73</td>
<td></td>
</tr>
<tr>
<td>P2f</td>
<td>F/69 (52.5 dB)</td>
<td>1 wk 2 wk</td>
<td>19 year 2 mo</td>
<td>Negative</td>
<td>C2 F/69</td>
<td></td>
</tr>
<tr>
<td>P3f</td>
<td>F/53 (48.8 dB)</td>
<td>1 mo 3 mo</td>
<td>1 year 2 year</td>
<td>++</td>
<td>C3 F/52</td>
<td></td>
</tr>
<tr>
<td>P4f</td>
<td>F/54 (25.5 dB)</td>
<td>1 wk 1 wk</td>
<td>1 mo 5 year</td>
<td>++</td>
<td>C4 F/54</td>
<td></td>
</tr>
<tr>
<td>P5f</td>
<td>F/51 (43.8 dB)</td>
<td>1 mo 1 mo</td>
<td>5 mo 1 year</td>
<td>++ ++</td>
<td>C5 F/51</td>
<td></td>
</tr>
<tr>
<td>P6f</td>
<td>F/41 (38.9 dB)</td>
<td>2 mo 3 mo</td>
<td>5 year 1 year</td>
<td>++ ++ ++</td>
<td>C6 F/41</td>
<td></td>
</tr>
<tr>
<td>P7f</td>
<td>F/57 (60.2 dB)</td>
<td>1 mo 1 mo</td>
<td>1 year 1 year</td>
<td>++ ++ + +</td>
<td>C7 F/57</td>
<td></td>
</tr>
<tr>
<td>P8f</td>
<td>F/49 (60.8 dB)</td>
<td>1 mo 1 mo</td>
<td>15 year 1 year</td>
<td>++ + +</td>
<td>C8 F/49</td>
<td></td>
</tr>
</tbody>
</table>

a 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)
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 × 10^5 cells/ml HL-1 medium (Cambrex) supplemented with l-glutamine, penicillin, streptomycin, HEPEXS buffer (Invitrogen Life Technologies), and 5% autologous serum. Cells were added at 3 × 10^6 cells/well in ELISpot plates (Polyfilter, Microlab, Harpenden, England) selected enriched populations were tested in ELISPOT assays as described above.

ELISPOT analysis

ELISPOTs were detected using an automated Series-I Immunospot Satellite Analyzer (Cellular Technology) with proprietary software designed to distinguish real spots from artifacts. 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 to distinguish contiguous and overlapping spots, and spot size criteria and circularity were used to exclude noise caused by nonspecific Ab binding.

Table II. Patients with OHL abnormalities

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>History of Noise</th>
<th>Hearing Loss (dB PTA)</th>
<th>WDS (%)</th>
<th>Most Likely Cause of Hearing Loss</th>
</tr>
</thead>
<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>
</tr>
<tr>
<td>OHL4</td>
<td>56/M</td>
<td>Yes</td>
<td>10</td>
<td>96</td>
<td>Noise</td>
</tr>
<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>
<td>Noise and age</td>
</tr>
<tr>
<td>OHL7</td>
<td>67M</td>
<td>Yes</td>
<td>NA^d</td>
<td>NA^d</td>
<td>Noise and age</td>
</tr>
<tr>
<td>OHL8</td>
<td>65M</td>
<td>No</td>
<td>32</td>
<td>90</td>
<td>Age^e</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.

^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, work-related noise, or work-relevant).

^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.

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. 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 H 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 class II typing was accomplished by sequence-specific oligonucleotide probing using commercial kits (LABType, One λ). 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 deoxyoligonucleotide 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).
We next used our recombinant human 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).

**Increased frequencies of cochlin-responsive T cells in ASNHL**

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**Results**

**Production of recombinant human cochlin**

We have previously shown that ASNHL subjects have increased 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).

**Increased frequencies of cochlin-responsive T cells in ASNHL**

We next used our recombinant human cochlin to determine frequencies of IFN-γ-producing and IL-5-producing T cells in PBMC from several ASNHL patients and from age- and sex-matched control subjects with no history of hearing loss (Table I). We focused our analysis on these two cytokines because IFN-γ represents a proinflammatory Th1-type cytokine while IL-5 represents an anti-inflammatory or regulatory Th2-like cytokine produced predominantly by T cells. Thus, the relative frequencies of T cells expressing each of these cytokines indicate the predominance of inflammatory vs regulatory autoreactivity. We found that ASNHL patients had substantially higher frequencies of cochlin-specific IFN-γ-secreting T cells in their PBMC when compared with age- and sex-matched control subjects (Fig. 2, A and B). ASNHL patients showed a mean of $472 \pm 64 SE/1 \times 10^6$ PBMC cochlin-specific IFN-γ-producing T cells compared with $118 \pm 64 SE/1 \times 10^6$ PBMC in control subjects. The difference in frequencies of cochlin-specific IFN-γ-secreting T cells between eight ASNHL patients (mean age 55.88 $\pm$ 10.49 SD) and eight normal control subjects (mean age 55.75 years $\pm$ 10.54 SD) was highly significant ($p = 0.0001$; Fig. 3A), and all ASNHL patients showed IFN-γ T cell frequencies higher than the highest frequencies observed in any of the control subjects.

Frequencies of cochlin-specific IL-5-secreting T cells in ASNHL patients vs normal controls were also significantly higher ($p = 0.03$) but were substantially much lower than frequencies of IFN-γ-producing T cells (Figs. 2, C and D; Fig. 3D). ASNHL patients showed a mean of $69 \pm 25 SE/1 \times 10^6$ PBMC cochlin-specific IL-5-producing T cells compared with $8.4 \pm 2.3 SE/1 \times 10^6$ PBMC in control subjects. Nonspecific immune responsive-

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**Statistical analysis**

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

**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-CD8 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...
in both CD4⁺ and CD8⁺ T cell populations in some patients (Fig. 4B; ASNHL no. P6) but was confined in other patients to the CD8⁺ 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 \( p \) values ranging from \( p < 0.05 \) to \( p < 0.0005 \).

**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](http://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 (\( p = 0.0001 \)) increased frequencies compared with (B) age- and sex-matched control subjects. Frequencies of IL-5-secreting T cells were also significantly higher (\( p = 0.03 \)) 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 (\( p = 0.0001 \)) between ASNHL and control subjects. D, The difference in frequencies of cochlin-specific IL-5-producing T cells was also significant (\( p = 0.03 \)). Differences in nonspecific responses were not significant as determined by measuring (B) IFN-γ (\( p = 0.14 \)) or (E) IL-5 (\( p = 0.48 \)) ELISPOT frequencies in response to anti-CD3 or by measuring (C) IFN-γ (\( p = 0.83 \)) or (F) IL-5 (\( p = 0.31 \)) 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 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.

Disclosures
The authors have no financial conflict of interest.

References