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# Induction of MHC Class II Antigens on Cells of the Inner Ear

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## Key Words

Antigen presentation · Labyrinthitis · Major histocompatibility complex class II · Fluorescence-activated cell sorter · Immunohistochemistry ·  $\gamma$ -Interferon

## Abstract

Growing evidence supports the concept that immune reactions occur in the cochlea, where they can function either in protection or as a source of inflammation. Since immunity is generally initiated by antigen presentation of foreign substances to T cells, antigen-presenting cells expressing major histocompatibility complex (MHC) class II molecules are required. Under resting conditions, cochlear cells usually express no MHC class II. However, we show that exposure to  $\gamma$ -interferon *in vitro* induces an increase in MHC class II expression in neonatal cochlear cells of mice. In addition, MHC class II immunoreactivity was observed in the inner ear of adult mice after induction of sterile labyrinthitis *in vivo*. It is concluded that the induction of MHC class II molecules by inflammation may render cochlear cells competent to initiate and participate in immune reactions and may therefore contribute to both immunoprotective and immunopathological responses of the inner ear.

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## Introduction

The inner ear was once thought to be a site from which immune responses were largely excluded, in order to protect the delicate cells of the inner ear from inflammatory damage. However, intensive evidence now supports the participation of immunity in the cochlea [Tomiyama and Harris, 1987; Takahashi and Harris, 1988; Gloddek et al., 1991]. Specific immunity has been shown to protect the inner ear from viral infection [Woolf et al., 1985]. In contrast, immune responses are thought to contribute to the etiology of a growing number of inner ear disorders [McCabe, 1979; Harris and Sharp, 1990; Arnold et al., 1987; McKenna, 1997]. Therefore, it is important to elucidate the basic immunological properties of the inner ear.

The major histocompatibility complex (MHC) class II molecules play a critical role in induction of an immune response. These molecules participate in the presentation of processed antigens to CD4<sup>+</sup> T helper cells [Cresswell, 1994]. The localization and identity of antigen-presenting cells (APCs) that contribute to inner ear immunity have received relatively little attention. Takahashi and Harris [1988] indirectly demonstrated the presence of MHC class II molecules by showing that macrophages invade the cochlea during an immune response. Altermatt et al.

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[1990] demonstrated the presence of MHC class II in the normal human endolymphatic sac directly, using anti-MHC-class-II antibodies. This is consistent with the observation that reaction to foreign substances and viruses occurs preferentially in the endolymphatic sac, inducing endolymphatic hydrops after repeated inflammation [Tomiyama and Harris, 1987; Gloddek and Harris, 1989; Tomiyama, 1992]. However, there is evidence from experimental and clinical data that immunological reactions of the inner ear are not always associated with endolymphatic sac involvement [Hughes et al., 1988; Veldman, 1997]. Even following endolymphatic sac destruction, specific immunity is observed in the cochlea in response to antigenic challenge at a reduced level [Tomiyama and Harris, 1987]. This suggests the presence of APCs in the cochlea itself.

$\gamma$ -Interferon (IFN- $\gamma$ ) is a potent inducer of MHC class II expression in other organs including the brain and blood vessels [Wong et al., 1984; Kreisel et al., 2001]. Although MHC class II molecules are normally expressed on professional APCs, such as macrophages and dendritic cells, expression on other cell types including astrocytes and endothelial cells can be induced during inflammation or by cytokines alone [Wong et al., 1984, Cresswell, 1994; Neumann et al., 1996; Kreisel et al., 2001].

The purpose of the present study was to determine whether MHC class II antigens can be induced on resident cells in the cochlea and whether this occurs during cochlear inflammatory reactions.

## Material and Methods

### *Experimental Design*

The baseline expression of MHC class II glycoproteins was evaluated in cultured cells from the cochlea using cell analysis with a fluorescence-activated cell sorter (FACS) and immunohistochemically in cochlear sections from mice. Two approaches were used to modify their levels: treatment with IFN- $\gamma$  in vitro and induction of sterile labyrinthitis in vivo.

### *Animals and Dissection*

All procedures were in accordance with the NIH guidelines regarding animal experimentation and were approved by an animal subject committee (VAMC, La Jolla, Calif., USA). Postnatal day 3–5 CBA mice were sacrificed, and tissue was dissected as described previously [Van de Water and Ruben, 1971; Aletsee et al., 2001]. After anesthesia, the skull was opened midsagittally under sterile conditions and the temporal bone identified after removal of the brain. Employing a dissection microscope (OPMI 1, Carl Zeiss, Germany), the membranous labyrinth was exposed after gentle removal of the cartilaginous cochlear capsule. The lateral wall and the spiral ligament, the organ of Corti as well as the modiolus including the spiral

ganglion and the eighth nerve were dissected and separated from each other.

### *Cell Culture*

For each tissue type, 8 explants were divided between wells of a 48-well tissue culture plate (Costar), 4 per well. Explants were placed in Dulbecco's phosphate-buffered saline (PBS; Gibco) immediately after dissection. In order to obtain single-cell suspensions, explants were digested with 1 mg/ml trypsin, 10 mg/ml collagenase and 1 mg/ml elastase (Sigma, St. Louis, Mo., USA) for 40 min at 37°C. After removing the supernatant, 500  $\mu$ l PBS containing 5% fetal calf serum with Hepes buffer 0.025 M and penicillin 30 U/ml was added per well. Cell dispersion was obtained by gentle trituration using a 1-ml syringe with a 25-gauge needle. Spleen and brain served as controls and were treated identically. One well of each tissue type received IFN- $\gamma$  stimulation, while the other well served as unstimulated control. Stimulation consisted of two doses of 100 IU/ml recombinant mouse IFN- $\gamma$  (R&D Systems Inc., USA) in the same media over a 48-hour period. An additional well of spleen cells served as a negative control for FACS analysis as described below. The entire procedure was replicated 5 times. In additional cultures, cell survival was evaluated by trypan blue exclusion.

### *Fluorescent Staining and Flow Cytometric Analysis*

After culture in the presence or absence of mouse IFN- $\gamma$  and removal of the supernatant, cells were washed and incubated for 30 min at 4°C with fluorescein-isothiocyanate-conjugated rat anti-mouse MHC class II (I-A/I-E) monoclonal antibody (Pharmingen, La Jolla, Calif., USA) diluted 1:100 in PBS. Cells were washed twice with cold PBS and gently detached with 400  $\mu$ l accutase (Innovative Cell Technologies, San Diego, Calif., USA). Flow cytometric acquisition of the samples was performed on a Becton-Dickinson FACScan (San Jose, Calif., USA) using standard Cell Quest acquisition/analysis software. Five thousand live events, which were gated on their forward/side scatter characteristics, were collected. Autofluorescence was evaluated in unstimulated spleen cells. The antibody was replaced by the same volume of PBS followed by FACS analysis. Gating for positivity of all samples was based on the response of the unstained spleen cells. The limits of positivity were set such that less than 1% of the negative control spleen cells were counted. The same settings were then used for all additional samples.

### *Induction of Sterile Labyrinthitis in Mice*

This procedure has been described in detail in a previous publication [Takahashi and Harris, 1988]. Mice were immunized subcutaneously with the antigen keyhole limpet hemocyanin (KLH; Pacific Biomarine Supply Co., Venice, Calif., USA) in complete Freund's adjuvant and boosted 10 days later with KLH in incomplete Freund's adjuvant. For the inner ear challenge, the right middle ear bulla was opened and the posterior half of the tympanic membrane removed. A microhole was drilled into the basal turn of the cochlea and 5  $\mu$ l KLH injected slowly into the cochlea. The hole was sealed with bone wax. Seven days after the inner ear challenge, the animals were deeply anesthetized, and intracardiac perfusion with periodate-lysine-paraformaldehyde fixative was performed. After removal and decalcification of the temporal bones with 4% EDTA at 4°C, the tissue was frozen in OCT compound (Tissue Tec, Naperville, Ill., USA).

*Immunohistochemistry*

The frozen temporal bone was serially sectioned (5–7 μm) parallel to the modiolus. After air-drying of the sections, an indirect immunoperoxidase technique was used with the following antibody: goat antimouse MHC class II (1:50, Santa Cruz Biotechnology, USA) incubated for 1 h at room temperature. Staining was carried out employing the test kit Vectastain ABC (Vector Laboratories, Burlingame, Calif., USA) using biotinylated rabbit anti-goat antibody and avidin-horseradish-peroxidase. Color reaction was performed with diaminobenzidine (Vector Laboratories). A light counterstaining

with hematoxylin was performed. As a negative control, the primary antibody was replaced by goat serum. Murine spleen and subcutaneous tissue around the antigen injection site were handled in the same way and served as positive control tissues.

*Statistical Analysis*

Statistical analysis, using a nonparametric Mann-Whitney U test (Statview 5.0), was performed. Differences associated with p values <0.05 were considered to be statistically significant. All data are presented as means ± standard error.

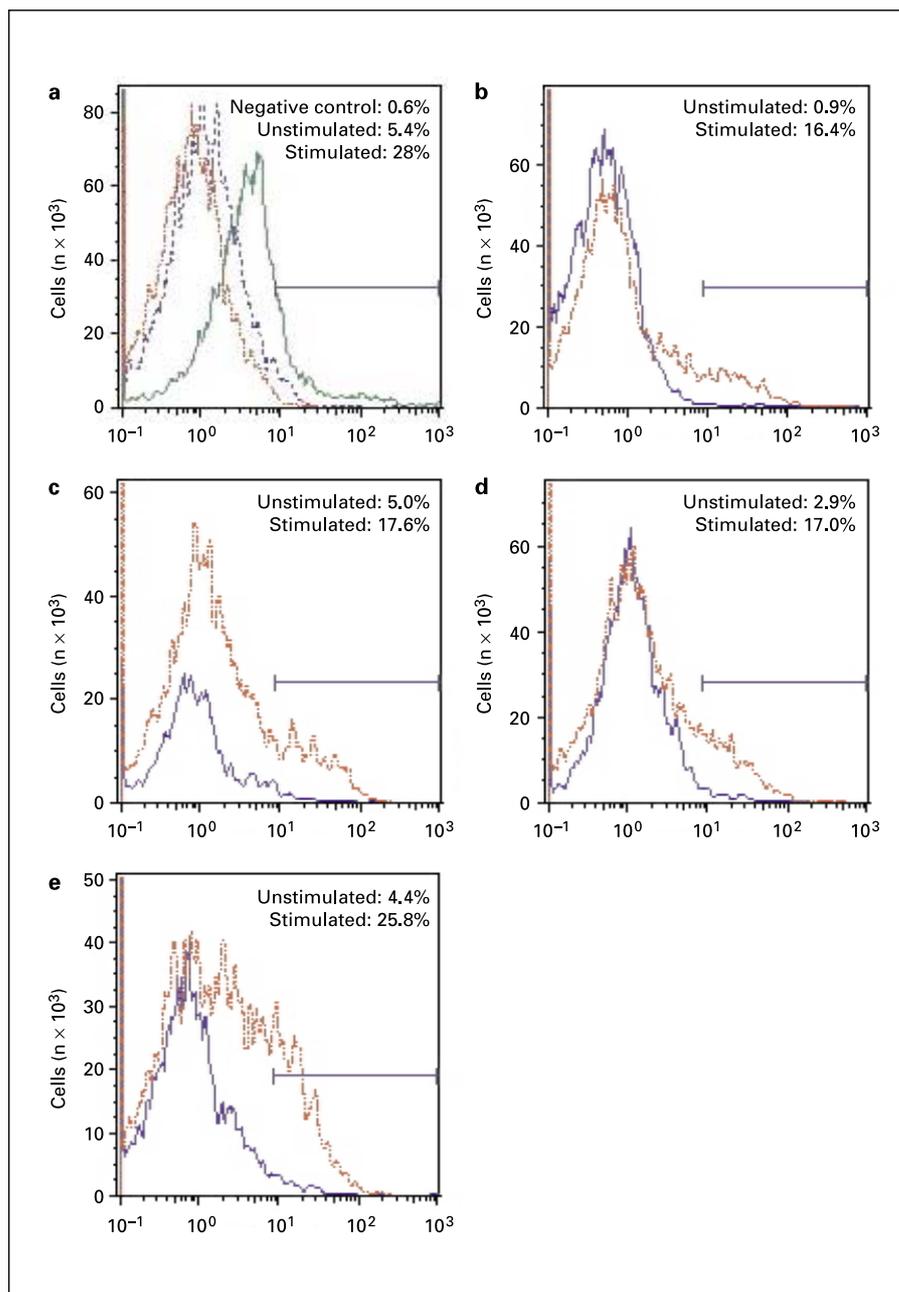


Fig. 1. Spleen (a), lateral wall (b), organ of Corti (c), modiolus (d) and spiral ganglion (e) were harvested and dissociated, and half of the cells were treated with IFN-γ. Both populations were then subjected to FACS analysis to measure MHC class II expression. A representative run is shown. The green line in a denotes sorting of spleen cells with the secondary antibody only as a negative control. The blue and red lines represent sorting of untreated or IFN-treated cells, respectively, using both the primary and secondary antibodies. The x axis depicts fluorescence intensity.

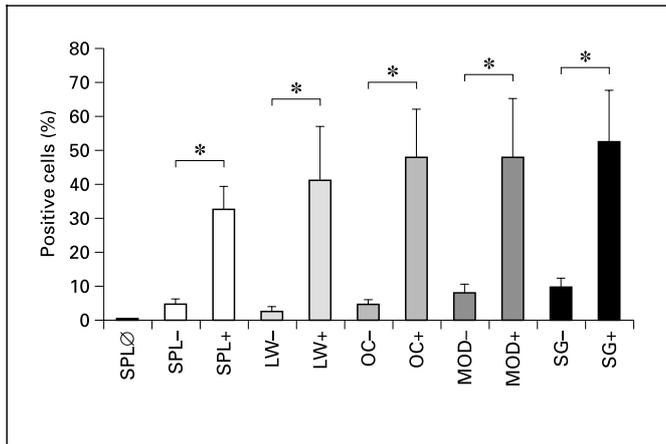


Fig. 2. Statistical analysis of 5 independent runs of the FACS as illustrated in figure 1. Statistical analysis was performed using the Mann-Whitney U test. Bars represent mean values, standard errors are indicated by lines. SPLØ = Negative control; SPL- = unstimulated spleen; SPL+ = stimulated spleen; LW- = unstimulated lateral wall; LW+ = stimulated lateral wall; OC- = unstimulated organ of Corti; OC+ = stimulated organ of Corti; MOD- = unstimulated modiolus; MOD+ = stimulated modiolus; SG- = unstimulated spiral ganglion; SG+ = stimulated spiral ganglion. \* $p < 0.01$ : in each group, the unstimulated value was compared to the stimulated value.

## Results

### Cell Culture

The initial number of inner ear cells ranged from 8000 to 12000 per well. The survival rate was about 65–75% after 48 h in culture, indicated by trypan blue exclusion, irrespective of the population and IFN- $\gamma$  stimulation.

### FACS Results, Phenotype after Activation

MHC class II molecules were expressed in unstimulated spleen culture, in a resting state, at a mean value of 5% with an increase to 33% after IFN- $\gamma$  treatment (fig. 1a, 2). On resting inner ear cells, these molecules were expressed at levels ranging from 3 to 8% (fig. 1b–e). After stimulation with IFN- $\gamma$ , expression was significantly upregulated to values between 33 and 53% (fig. 1b–e, 2;  $p < 0.01$ , Mann-Whitney U test). Figure 2 represents the summarized results of 5 independent runs of the FACS demonstrating the statistical significant increase in MHC-class-II-positive cells after stimulation in all tissues.

### Immunohistochemical Localization of MHC II

Inflamed subcutaneous tissue stained for MHC class II revealed intense labeling of scattered cells (fig. 3a). No MHC class II molecules were detected in the untreated

inner ear (fig. 3b). One week after induction of labyrinthitis, infiltration of a great number of inflammatory cells into the inner ear was observed (fig. 3c). Approximately 30% of these inflammatory cells were strongly positive for MHC class II immunoreactivity (fig. 3f). Cells lining the perilymphatic space and in the spiral ligament also expressed moderate levels of MHC II after stimulation (fig. 3d). Interestingly, a few supporting cells of the organ of Corti (in the region of Hensen's and Claudius' cells) expressed lower levels of MHC class II antigen, as did neurons of the spiral ganglion (fig. 3e). When the primary antibody was replaced with goat serum, background staining was found to be very low in all sections.

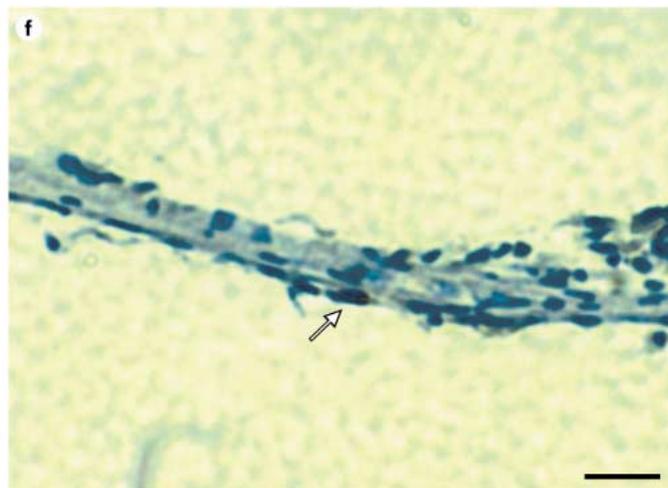
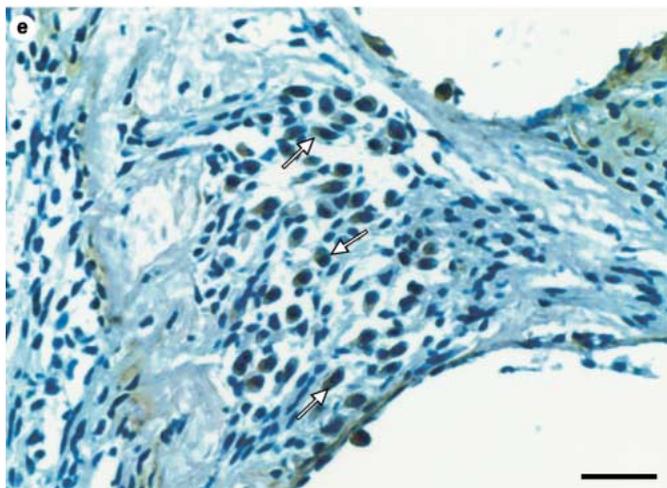
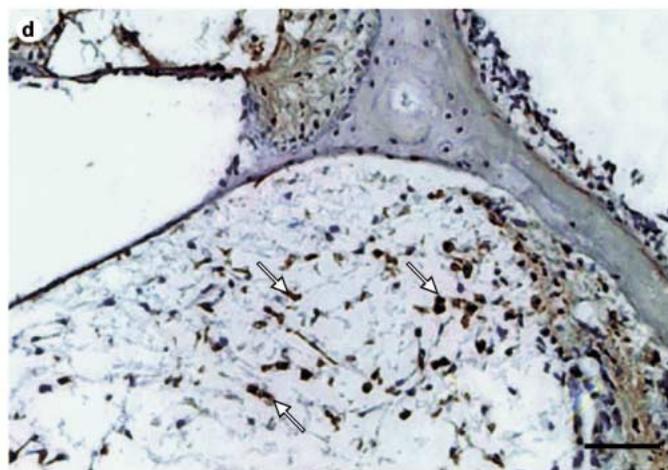
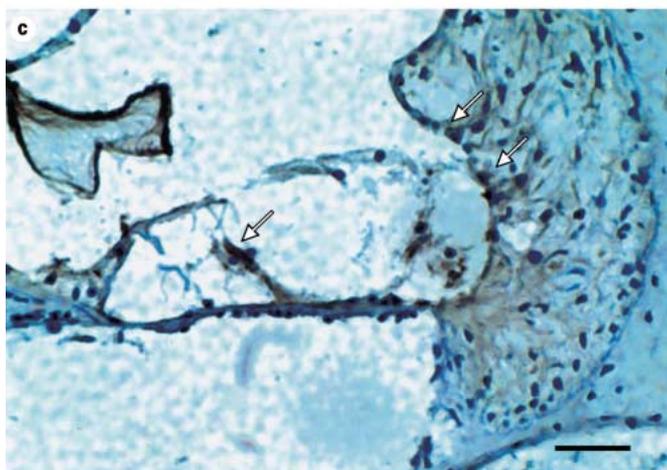
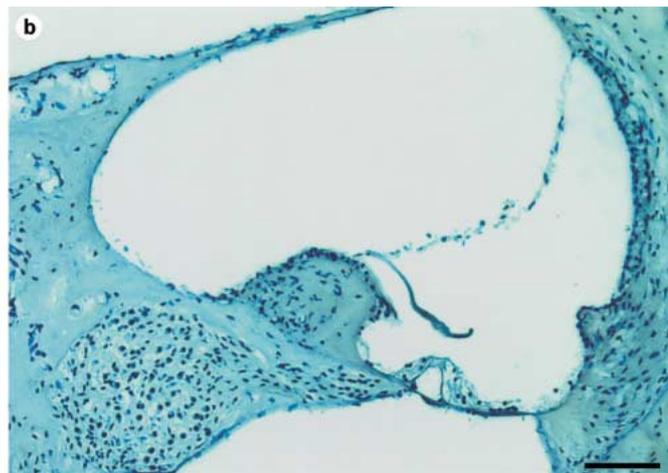
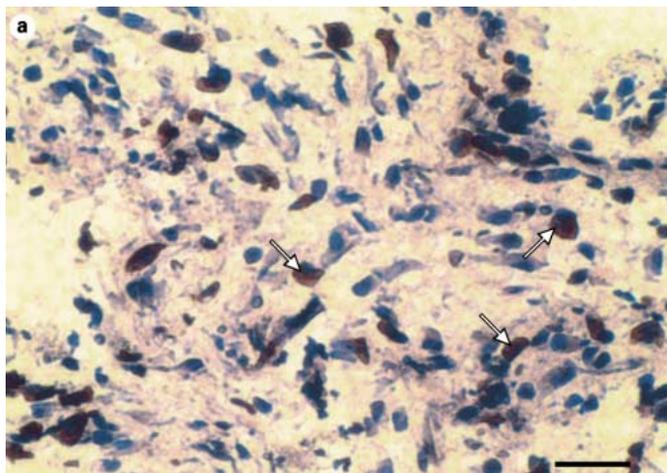
## Discussion

When dispersed, neonatal cochlear explants of the lateral wall, organ of Corti, modiolus or spiral ganglion were stimulated with repeated doses of IFN- $\gamma$  in culture, a significant increase in MHC class II molecules was detected by the FACS in contrast to untreated control cells.

While FACS analysis is an established method in other experimental fields such as brain or blood vessel research [Wong et al., 1984; Kreisel et al., 2001], its application for studies of the inner ear is new. This method has distinct advantages. These include preservation of antigenicity in unfixed tissue and the controlled and reproducible stimulation of cells in culture. Disadvantages include the necessity of neonatal tissue, the trauma of tissue dissociation, changes in cellular characteristics due to culture and cellular heterogeneity in each portion of the cochlea. For this reason, the FACS findings were augmented by in vivo data, employing immunohistochemistry.

Histological localization of MHC class II was achieved in an established model of inner ear inflammation [Takahashi and Harris, 1988]. No MHC class II was seen in the normal cochlea. However, after inflammation not only immigrating immunocompetent cells, but also resident cochlear cells, including cells lining the perilymph space, cells of the lateral wall, neurons of the spiral ganglion and supporting cells of the organ of Corti, expressed MHC

Fig. 3. Immunohistochemical localization of MHC class II molecules in inflamed subcutaneous tissue (a), normal inner ear (b) and during labyrinthitis (c–f). a Subcutaneous tissue around the KLH injection site revealing severe inflammation. Arrows depict MHC-



class-II-positive cells (brown). Scale bar = 25  $\mu$ m. b Normal cochlea demonstrating only background MHC class II labeling. Scale bar = 100  $\mu$ m. c Cochlear tissue 7 days after induction of labyrinthitis with labeling of supporting cells and cells of the spiral ligament (arrow) of the organ of Corti. Scale bar = 50  $\mu$ m. d

Strongly MHC-class-II-positive inflammatory cells in the infiltrate filling the scala tympani (arrows). Scale bar = 100  $\mu$ m. e Labeled neurons of the spiral ganglion (arrows) in labyrinthitis. Scale bar = 25  $\mu$ m. f MHC-class-II-positive mesothelial cell (arrow) lining the scala tympani. Scale bar = 100  $\mu$ m.

class II molecules. These observations were in agreement with the *in vitro* results.

The expression of MHC class II molecules on APCs is an important factor in the process of antigen recognition by T cells. MHC class II molecules are required for antigen presentation to helper T cells [Cresswell, 1994]. In this study, we observed a surprisingly high expression of MHC class II molecules by resident cochlear cells. This may be related to antigen presentation by these cells as observed in stimulated neurons of the brain [Wong et al., 1984; Hart and Fabry, 1995]. Antigen presentation by resident cells in the vicinity of blood vessels or lining the perilymph spaces seems to be a reasonable possibility given the route of lymphocytes into the inner ear during the immune response [Harris et al., 1990; Gloddek et al., 1991]. Lymphocytes enter the cochlea via the spiral modiolar vein and enter the perilymph in the scala tympani. Antigen presentation by spiral ganglion neurons, which are completely surrounded by Schwann cells, or by supporting cells in the avascular organ of Corti seems less likely. MHC class II expression in these cells may be related to alternative functions of these molecules. For example, in the spiral ganglion MHC class II molecules could be related to the newly discovered guidance function of MHC molecules for injured neurons, or to the elimination of synaptic connections in less active neurons [Di Prospero et al., 1997; Boulanger et al., 2001].

On the normal, *in vivo* cochlea and in unstimulated cochlear cells *in vitro*, we saw little MHC class II expres-

sion. The lack of MHC class II molecules under normal conditions may be a factor in limiting immune responses, which could lead to irreversible damage to cochlear cells. On the other hand, the lack of expression of MHC glycoproteins might contribute to the persistence of viruses in resting cochlear cells. Without MHC on the cell surface, for example, virally infected neurons of the brain escape immunological detection and lysis [Joly et al., 1991]. While favorable to the neurons, this allows viruses to persist in these cells. Viral persistence and reactivation has been suggested as a cause of some forms of sensorineural hearing loss [Kumagami, 1996; Arnold and Niedermeyer, 1997].

From clinical and experimental studies, it is known that systemic application of IFN can cause hearing loss. This has been hypothesized to be caused by metabolic and biochemical changes in the cochlea [Kanda et al., 1995; Akyol et al., 2001]. Our results indicate that many cell types in the cochlea respond to IFN- $\gamma$ , supporting the idea that this cytokine has robust biological effects in cochlear cells.

In conclusion, inflammation leads to an increase in MHC class II molecule expression in the cochlea. This could play a role in immune response to infectious agents or in autoimmune reactions to cochlear proteins. MHC expression may also participate in other responses of cochlear cells, such as the reorganization of tissue during damage [Wierzba-Brobowicz et al., 2000].

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