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A Rat T-Cell Line That Mediates Autoimmune Disease of the Inner Ear in the Lewis Rat

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Key Words
Inner ear · Autoimmune disease · Cell transfer · Rat

Abstract
In various patterns of sensorineural hearing loss including Ménière's disease, which may show improvement in auditory function following immunosuppressive therapy, an isolated autoimmune disease of the inner ear has been postulated. Because of the lack of well-defined diagnostic criteria to identify autoimmune processes within the inner ear and the fact that the human inner ear is one of the few organs of the body not amenable to diagnostic biopsy, there has been great interest in developing animal models that mimic these clinical entities. Previous studies have found evidence that this process might be cell mediated and that the endolymphatic sac functions as an immunodefensive organ for the inner ear. By heterologous immunization of inbred Lewis rats with inner ear tissue, an autoreactive inner-ear-specific T helper cell line was established. After passive transfer of these cells a labyrinthitis was induced in recipient animals. Immunohistochemically, T helper cells were first identified in the cochlea suggesting that this cell type might carry the autoantigenic epitope. Autoantibodies against inner ear tissue were demonstrated in animals with histologically evident labyrinthitis. We conclude that this experimental design can serve as an animal model for cell-mediated autoimmune disease of the inner ear and could be used to explain the etiology of certain types of sensorineural hearing loss such as Ménière's disease. With this approach the identification of the causative autoantigen should be possible and will lead to the development of appropriate clinical tests to diagnose autoimmune diseases of the inner ear in humans.

Introduction

Otolaryngologists have long sought to identify causes of sensorineural hearing loss which might be reversible with medical treatment. More and more clinical, experimental and histopathological evidence indicates that some sensorineural hearing loss is related to autoimmune inner ear disease [1–3]. The improvement in auditory function in these patients following immunosuppressive therapy supports this hypothesis [4]. Because of the lack of well-defined detection methods to identify autoimmune processes within the inner ear and the fact that the human inner ear is one of the few organs of the body not

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amenable to diagnostic biopsy, there has been great interest in developing animal models that mimic these clinical entities. Previous studies have found evidence that the inner ear may be a target organ of autoimmune processes in animal experiments and that this process is cell mediated [5, 6]. In the inner ear the endolymphatic sac (ES), a blind-ending, fluid-filled pocket lying in the posterior cranial fossa with fluid-filled connections to the endolymphatic spaces of the cochlea and vestibular organ, fulfills all the critera of an immunodefensive organ [7–10]. Several studies have demonstrated that whereas the ES immune response serves to protect the inner ear from potential pathogens, repeated inflammatory responses may be deleterious to the function of the ES in maintaining fluid homeostasis [10, 11]. Repeated inflammation of the ES causes fibrosis or ossification, a situation which can lead to permanent dysfunction and to a disease state such as Ménière's disease [10, 12, 13]. Despite these observations, the cochlear autoantigen which could induce an autoimmune inner ear disease must still be identified. Until now animal models for autoimmune disease of the inner ear have used the injection of crude inner ear tissue extracts together with complete Freund's adjuvant [2, 14], which, by itself, could lead to an autoimmune response [15]. These studies have led to the characterization of a 68-kD protein as possible causative antigen which was shown to be identical to the ubiquitous heat shock protein 70 [16, 17]. Since this experimental design is not organ specific but patients with this proposed autoimmune process only exhibit inner ear involvement [18], the possibility of an epiphenomenon, in this design not related to the inner ear, makes necessary a change in experimental design.

In this study, autoimmune disease was induced via transfer of sensitized T lymphocytes with the advantage of a more precise control of causative factors. In addition, this approach enables in vitro selection of the possible causative cochlear protein by using the lymphocyte transformation test and should lead to the identification of the autoantigen in the near future. This knowledge will be a useful tool for the diagnosis of human autoimmune disease of the inner ear.

Material and Methods

Animals

Inbred Lewis rats aged 6–8 weeks and weighing 250–280 g (Charles River Wiga GmbH, Sulzfeld, Germany) were used for all experiments. The animals were checked otoscopically and excluded if they showed any sign of middle ear infection. The animals were anesthetized with ether for all procedures.

Antigen

Fresh cochleas from swine were used to prepare the antigen. After removal from the skull, the temporal bone was immediately frozen in liquid nitrogen. The cochleas were then microscopically dissected, and tissue from the membranous labyrinth and modiolus was taken separately in sterile phosphate-buffered saline (PBS; pH = 7.4). A quantitative protein estimation using the Lowry method was performed.

Immunization History

Donor animals were divided into three groups (5 animals in each group) and immunized in the hind footpad with 100 µg of the respective antigen dissolved in 50 µl PBS and emulsified in an equal volume of complete Freund's adjuvant containing 4 mg/ml *Mycobacterium tuberculosis* H37a (Difco Laboratories Inc., Detroit, Mich., USA). Group A was immunized with modiolus antigen, group B was immunized with membranous labyrinth antigen and a control group C was immunized with tuberculin purified protein derivative (PPD) in complete Freund's adjuvant.

Primary Cell Culture

Ten days following injection the draining lymph nodes of the animals were removed and stored in sterile Eagle's Hepes medium (EH). The tissue was homogenized with a glass homogenizer (Belco, Newark, N.Y., USA) in a volume of 40 ml EH. The cells were centrifuged (1,200 rpm at 4°C for 10 min), washed twice with EH and their viability was assessed by trypan blue exclusion. These cells were cultured for 3 days in bulk with a concentration of 10^7 cells/ml restimulation medium together with 20 µg/ml of the respective antigen. The cells were incubated at 37°C in a humidified incubator (5% CO_2/95% air). After 3 days, the cells were centrifuged, washed with EH and cultured at a concentration of 2.5×10^8 cells in 20 ml T-cell growth factor medium.

Restimulation

Peptide-specific T cell lines were derived from bulk cultures of the lymph node cells as previously described [19]. Antigen-specific T cell lines were selected by repeated cycles of propagation in T cell growth factor (50 U IL-2/100 µl) containing medium followed by antigen- specific restimulation using irradiated (4,000 rad), syngeneic thymocytes as antigen-presenting cells. This procedure was repeated 3 times at 8-day intervals.

Lymphocyte Transformation Test

Successful antigen-specific stimulation of cells could be demonstrated by the lymphocyte transformation test. [^{3}H]Thymidine (1 µCi/well) was added to the culture for the last 16 h of a 72-hour culture. [^{3}H]Thymidine incorporation was determined on filter mats using a Packard Matrix 96 Direct Beta counter. For this test a single-cell suspension was prepared from cell culture, and the cells were cultured at a concentration of 10^6/ml in 200 µl DMEM supplemented with glutamine, penicillin/streptomycin, sodium pyruvate, essential amino acids and 1% rat serum in flat-bottom 96-well tissue culture plates. Additional positive and negative tests were performed with 20 µl concanavalin A and 20 µl PPD. The lymphocytes were incubated at 37°C in a humidified environment with 5% CO_2 for 72 h. This test was repeated each time in parallel with the restimulation process.

Cytofluorographic Analysis

Before passive transfer of the proliferating blasts, cell typing of the lymphocyte subtypes was performed with a fluorescein-activated cell sorter (FACS) by indirect immunofluorescence staining. The following cell surface markers were tested with their specific primary antibodies: W3/13 (CD3), W3/25 (CD4), OX8 (CD8), OX22 (CD45RO), R73 [constant region of T cell receptor (TCR) α/β] and OX39 (IL-2 receptor; Serva, Heidelberg, Germany). The T cell blasts were used after the third restimulation and diluted in FACS buffer at a concentration of 2×10^6 cells/ml buffer. The absorption was measured at 495 nm.

Passive Cell Transfer

Following the third restimulation with their specific antigens the cells were aspirated by gently pipetting and then washed twice. Viability was determined by trypan blue exclusion. The cells were resuspended in EH at 10^7–10^8 viable cells/ml and then injected into the tail vein of the animals. The recipient animals were naive Lewis rats without immunization (10 animals each group). Ten to 14 days following the cell transfer the animals were anesthetized with ether and perfused intracardially with 4% paraformaldehyde before the cochlea and control tissues such as the kidney, liver and brain were removed.

Histology

The cochleas were decalcified in 10% EDTA at room temperature for 2 weeks. The control organs were fixed and stored in 4% paraformaldehyde. The cochleas were paraffin embedded and sectioned with a microtome. One set of the slides was used for hematoxylin-eosin staining and another set for immunohistochemistry using the peroxidase technique. Specific antibodies against cell surface antigens were employed to identify and classify lymphocytes in the cochlea and control tissues. The following antibodies were used (Serva): W 3/25 (1:10) = CD4 (T helper cells); ED1 (1:20) = monocytes and macrophages; W3/13 (1:20) = pan T cells and granulocytes.

After deparaffinization, endogenous peroxidase was blocked with 0.6% H_2O_2/methanol for 20 min. Each section was incubated with 50 µl of the diluted primary antibody at 4°C in a humid box overnight. The following day the slides were washed twice with PBS and incubated with peroxidase-coupled secondary antibody (goat anti-mouse IgG, 1:50) at 37°C for 30 min. After washing with PBS twice, the sections were stained with 1 µl H_2O_2/1 ml amino-ethylcarbazole (1:75) and lightly counterstained with Meyer's hematoxylin.

Western Blot Analysis

Serum from the animal group with histologically evident labyrinthitis or neuritis was tested by Western blot analysis against various fractions of cochlear tissue. Proteins from the membranous labyrinth or modiolus were first separated with SDS-PAGE and then blotted onto a Mini-Protean II Dual Slab membrane (Bio-Rad, Munich, Germany). The neural protein P_0 from the myelin sheath was used as negative control in the same analysis. Serum of the above-mentioned rats was used as the primary antibody, spun at 4°C for 1 h at 3,000 rpm and the supernatant diluted at 1:100 and 1:500. It was then placed on a nitrocellulose membrane and incubated at 4°C overnight. Goat anti-rat IgG peroxidase-coupled secondary antibody (Serva; 1:50) was added for 1 h at room temperature. For development 3,3′-diaminobenzidine (DAB) was chosen. Additionally protein weight markers were run as size standards.

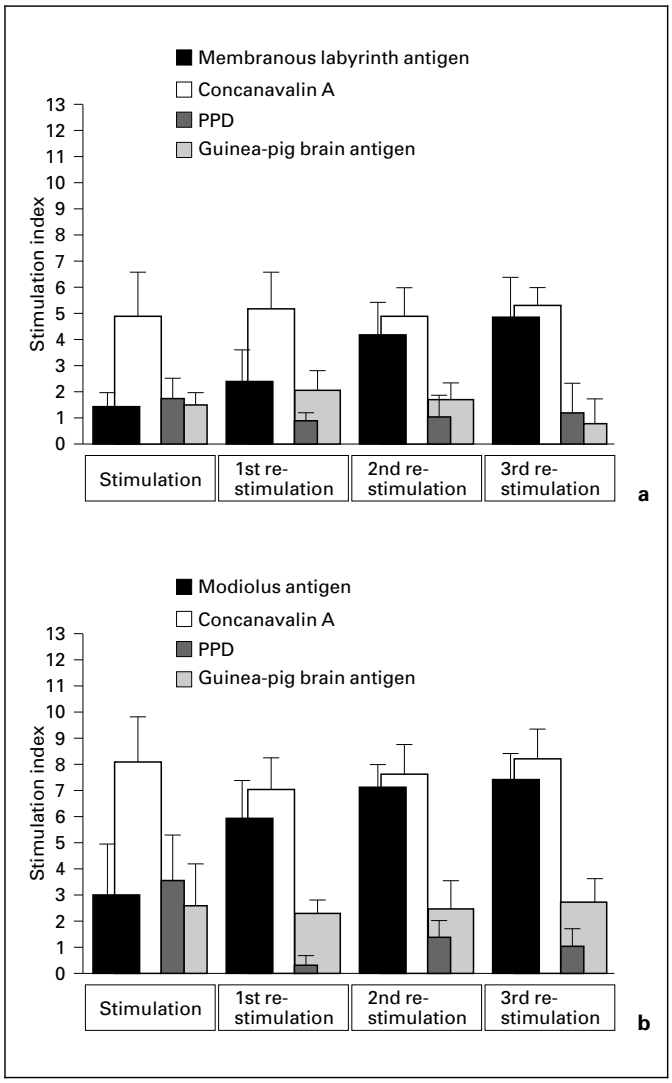

Fig. 1. Results of the lymphocyte transformation test for the membranous labyrinth cell line (**a**) and the modiolus cell line (**b**) after repeated stimulation.

Results

Lymphocyte Transformation Test

Our first results demonstrate the successful selection and stimulation of lymphocytes with the respective inner ear antigens as shown in the lymphocyte transformation test (fig. 1). After three restimulation cycles, the T cell lines were highly specific for inner ear antigen which was evident by the increasing stimulation index and low residual response to PPD. We observed this for both the membranous labyrinth cell line and the modiolus cell line (fig. 1). To exclude shared epitopes of both cell lines, T cells se-

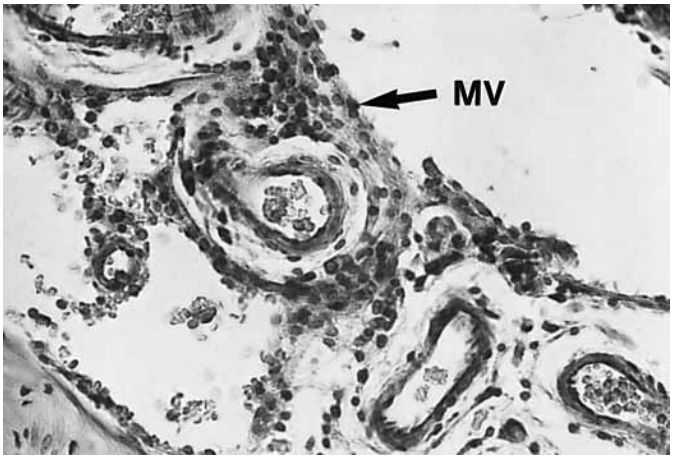

Fig. 2. Perivasculitis of modiolar vessels (MV), depicted by an arrow, in the cochlea of a rat which received T cells raised against modiolus antigen 10 days after cell transfer.

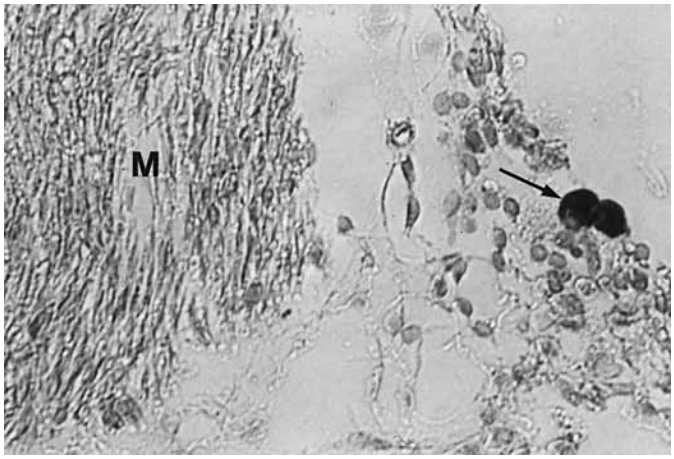

Fig. 4. Immunohistochemical identification of T helper cells (arrow), stained with monoclonal antibody W3/25, close to the modiolus (M) in the modiolus group 4 days after transfer.

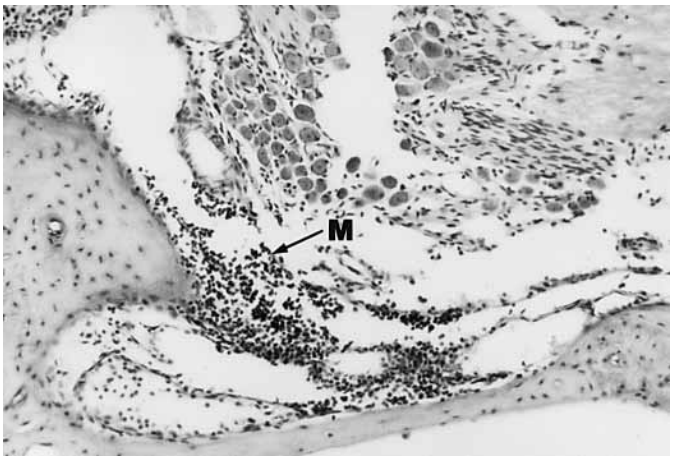

Fig. 3. Cellular infiltration of the modiolus (M), depicted by an arrow, in the area of the internal auditory canal of a rat 10 days after adoptive transfer of modiolus T cells.

Table 1. Expression of lymphocyte differentiation antigen (%) on T cell lines by FACS

T cell line	Monoclonal antibody						Second antibody control
	W3/13	W3/25	OX8	OX22	R73	OX39	
HL	97	95	2	3	69	96	3
Mod.	96	93	3	4	72	95	4

(HL = Membranous labyrinth cell line, mod. = modiolus cell line).

lected with membranous labyrinth were tested with modiolus antigen and vice versa; they exhibited very low reactivity in both cases (data not shown). Additionally, low cross-reactivity of the modiolus and labyrinth cell population with guinea pig brain antigen was seen, demonstrating that inner ear antigen epitopes are different from those of the brain.

FACS Analysis of the Transferred Cells

FACS analysis results were similar for both cell lines indicating that approximately 95% of transferred cells were T cells (CD3+) and belong to the T helper cell population (CD4+; table 1). Only a very small fraction consisted of T suppressor or B cells (CD8+, CD45+). Almost all of the cells expressed the IL-2 receptor (OX39+) and about 70% the TCR-α/β (R73+; table 1).

Histology/Immunohistochemistry

In the animal group receiving lymphocytes raised against modiolus antigen, the modiolar vessels showed a perivascular lymphocytic infiltration not seen in the control group (figure 2). The modiolus itself and particulary the area of the internal auditory canal showed a severe lymphocyte infiltration with evident perineural hemorrhage and edema (fig. 3). Predominantly T helper cells could be detected immunohistochemically at early stages (4 days after transfer; fig. 4). Seven of 10 animals exhibited these inflammatory changes.

In the group with membranous labyrinth as antigen, cellular infiltration of lymphocytes into the perilymph

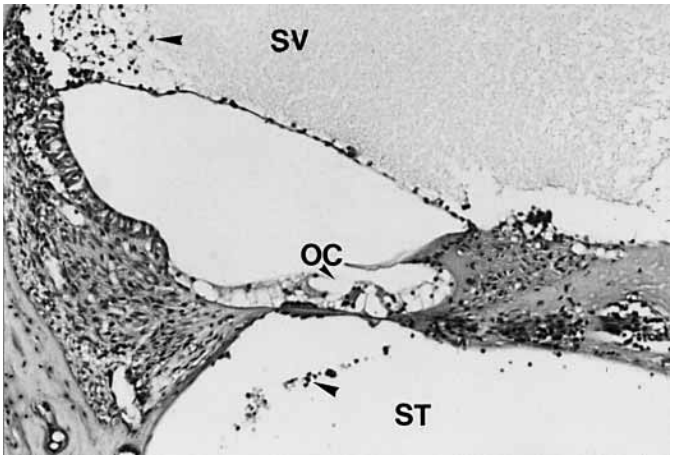

Fig. 5. Cellular infiltration of perilymph compartments (arrowheads), 10 days after transfer, in the cochlea of a rat which received T cells raised against membranous labyrinth. SV = Scala vestibuli; OC = organ of Corti, ST = scala tympani.

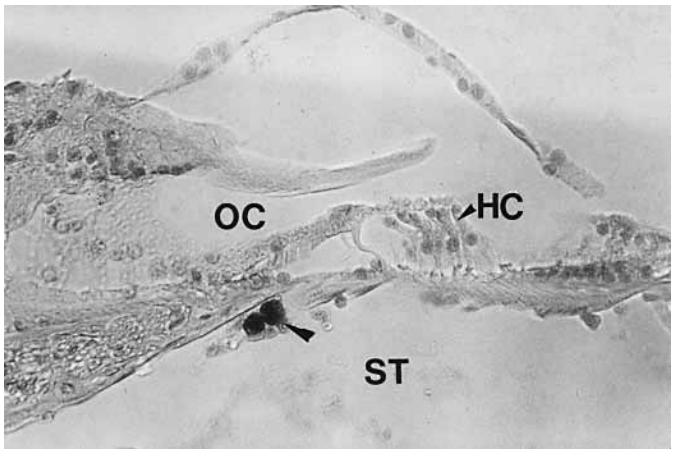

Fig. 6. Immunohistochemical identification of T helper cells (arrowhead), stained with monoclonal antibody W3/25, in the cochlea of the membranous labyrinth group 4 days after transfer. ST = Scala tympani, OC = organ of Corti; HC = hair cells.

compartments was observed especially in the scala vestibuli (fig. 5). In early stages of inflammation (4 days after transfer), the main lymphocyte subtype in this group consisted also of T helper cells (fig. 6). A total of 8 of 10 rats developed labyrinthitis bilaterally. Control tissues from the brain, liver and kidney exhibited no cellular infiltration or pathological change. Controls injected with the same dose of PPD-specific T cell blasts developed no inflammation of the inner ear or other organs.

Autoantibodies Identified by Western Blot

Western blot analysis of serum from animals with histologically confirmed labyrinthitis showed strong bands against cochlear tissues at 30 kD in the modiolus group and 30 and 42 kD in the membranous labyrinth group (fig. 7). In both animal groups 30-kD bands were detected against the myelin sheath protein P_0. Control serum from animals with transfer of PPD cells or nonimmunized rats showed no reactivity.

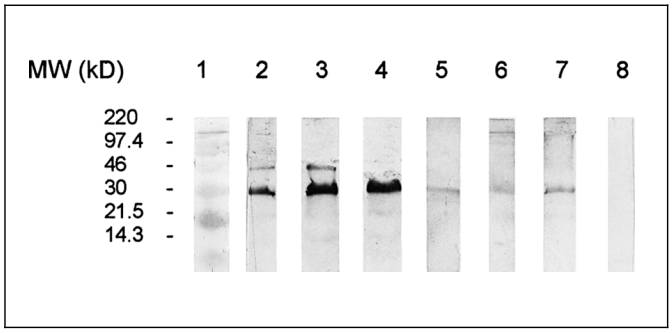

Fig. 7. Western blot analysis to detect autoantibodies in serum of rats with histologically evident labyrinthitis/neuritis: bands at 30 and 42 kD in the labyrinthitis group and at 30 kD in the neuritis group; cross-reactivity against myelin sheath protein P_0 in both groups. Lane 1 = Molecular-weight marker; lanes 2–4 = serum of rats suffering from labyrinthitis run against membranous labyrinth antigen (2/3) or P_0 protein (4); lanes 5–7 = serum of rats suffering from neuritis run against modiolus antigen (5/6) or P_0 protein (7), lane 8 = control.

Discussion

The aim of this study was to establish an animal model for autoimmune disease of the inner ear. In order to classify a disease as autoimmunologically mediated, it is mandatory to prove that the host immune system reacts against an endogenous protein [20]. Most of the proteins of the inner ear are not presented to the thymus during ontogeny, since they are protected by a blood- labyrinth barrier and, under normal conditions, are not in contact with the systemic circulation. However, there is clinical and experimental evidence that during trauma, surgery or viral infection the inner ear proteins may be exposed to the immune system and are then recognized as foreign [3, 21]. This leads to the induction of inner-ear-specific lymphocytes which are capable of recirculation as memory cells to the contralateral cochlea, thus starting an immune response resulting in a sympathetic cochleolabyrinthitis.

Previous results have given evidence that this process is cell mediated [5, 6] and that the ES functions as an immunodefensive organ for the inner ear. Recurrent inflammation of the ES leads to dysfunction of the inner ear's fluid homeostasis and to conditions resembling Ménière's disease [11–13].

It is generally accepted that autoreactive B lymphocytes cause systemic non-organ-specific autoimmune diseases while autoreactive T cells are capable of inducing an organ-specific process [22]. Clinical observation of patients with hypothetically autoimmunologically mediated inner ear dysfunction indicates that other organs are not involved [16, 23]. This points to a T-lymphocyte-mediated process.

In this study two inner-ear specific T cell lines were raised using heterologous immunization of Lewis rats. For practical purposes the inner-ear-specific T cell line was selected for reactivity against swine inner ear proteins. Since these proteins are phylogenetically conserved and exhibit the same bands in SDS-PAGE [17], this approach seems reasonable. These autoaggressive cells express CD4+/CD8– and use the TCR-α/β. We were also able to show that the cells were truly self-reactive as seen in their proliferative response to either modiolus or membranous labyrinth proteins. Adoptive transfer experiments established that this inner-ear-specific T cell line is highly pathogenetic and can induce two different histopathological patterns: a perivasculitis and neuritis in the modiolus group and a labyrinthitis in the group with membranous labyrinth as antigen. The first cells which invaded the inner ear were T helper cells, as demonstrated immunohistologically, followed by macrophages and neutrophils. Since previous studies were able to show a close correlation between the amount of infiltration by lymphocytes and impaired function, especially hearing loss [24], it is likely that rats in this study exhibited also hearing loss.

The invading T cells are probably carrying the autoantigenic determinants as shown in other models of autoimmune disease such as experimental autoimmune encephalitis or autoimmune uveitis [25, 26]. Once the autoimmune cascade is started, plasma cells were stimulated to secrete immunoglobulins which could be detected as autoantibodies at 30 and 42 kD in the membranous labyrinth group and 30 kD in the modiolus group. Just recently Suzuki et al. [27] have been able to demonstrate that the 30-kD band observed in patients with inner ear disorders is identical to the P_0-protein of the myelin sheath, a finding which supports our study. In addition Matsuoka et al. [19] induced hearing loss in mice sensitized with P_0.

The experimental design of our study serves as an animal model for cell-mediated autoimmune disease of the inner ear and has direct relevance for our understanding of the early immunopathogenesis of putative autoimmune-mediated diseases of the inner ear, e.g. initial Ménière's disease. With the establishment of a T cell line, an in vitro control of possible causative cochlear proteins seems possible by use of the lymphocyte transformation test. This approach has the advantage that it isolates the cochlear proteins and can therefore identify the protein with the strongest autoimmunological effect as in other animal models [28]. After biotechnical production of this protein, sufficient amounts for clinical tests should be available to allow diagnosis of autoimmune diseases of the inner ear in man. This animal model will also be very useful to test different therapeutic strategies for the prevention of damage to the cochlea with resulting hearing loss during autoimmune labyrinthitis.

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