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Type II Collagen–Induced Autoimmune Endolymphatic Hydrops in Guinea Pig

Abstract. *Endolymphatic hydrops was induced in guinea pigs by immunizing them with native bovine type II collagen. Histopathologic changes consisted of moderate extension of the Reissner's membrane, spiral ganglion degeneration, atrophied organ of Corti, and mild atrophy of the surface epithelium in the endolymphatic duct. These findings suggest that an immune response directed against type II collagen—a type of collagen found in the membranous labyrinth, subepithelial layer of the endolymphatic duct, spiral ligament, and enchondral layer of the otic capsule—may induce endolymphatic hydrops.*

In 1861, a Paris physician, Prosper Ménière, described an illness characterized by vertigo, deafness, and tinnitus (*1*). This disease, the cause of which is still unknown, has been linked to a failure of the mechanism regulating the production and disposal of endolymph. The occurrence of endolymphatic hydrops in the ears of animals with bacterial labyrinthitis was recognized as early as 1926 by Wittamaac (*2*). Most of the occurrences were spontaneous and reflected the inflammatory reaction of the epithelial lining of the endolymphatic space (*2, 3*). Various methods have been used to induce endolymphatic hydrops in animals, including intravenous injection of horse serum (*4*), tubercle bacilli (*4*), egg albumin (*5*), or horseradish peroxidase (*6*); injection of human serum through the stylomastoid foramen has also been used (*7*). Other methods include surgical obstruction of endolymphatic duct (*8, 9*), injection of ethacrynic acid (*10*), and infrasound acoustic trauma (*11*). The immunologic methods, although better than the surgical methods, produced only limited success.

We described autoimmune hearing loss, vestibular dysfunction, and otospongiotic lesions in the rat (*12, 13*). In addition, we studied guinea pigs for the occurrence of the inner ear lesions (*14*). All of the abnormalities appeared to be associated with autoimmunity to type II collagen. Antibody titers to type II collagen were higher in the patients with Ménière's disease than in controls, an indication that autoimmunity to type II collagen might have a role in the etiology of Ménière's disease (*15*).

It was, therefore, of interest to develop a well-defined animal model of autoimmune endolymphatic hydrops by immunization with type II collagen. We have now developed such a model in guinea pigs. Bovine type II collagen was isolated and purified as described (*16*). The purity of the collagen preparations was determined by amino acid analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and uronic acid analysis (*16*). In each case, there was no detectable contamination by noncollagenous proteins, by proteoglycans, or by other types of collagen.

Collagen for immunization was stirred overnight in 0.1*N* acetic acid at 4°C. An emulsion was made by adding collagen to an equal volume of incomplete Freund's adjuvant and homogenizing the mixture (VirTis homogenizer). Hartley guinea pigs received intradermal injections (into the footpad) of 400 μg of native bovine type II collagen emulsified with incomplete Freund's adjuvant. All of the guinea pigs were given boosters of an identically prepared emulsion injected into the foot at 7-day intervals.

Serums were collected by cardiac puncture and antibody titers were assayed by an enzyme-linked immunosorbent assay (ELISA) method (*17*). Disposable polystyrene 96-well microtiter plates were coated with collagen dissolved in 0.4–ionic strength phosphate buffer (*p*H 7.6). Collagen-coated plates were incubated with tris-NaCl containing 1 percent bovine serum albumin (BSA) to block nonspecific binding. Serum, diluted 1:100 and 1:1000 in tris-NaCl containing 1 percent BSA and 0.05 percent Tween 20, was added to the plates, which were then incubated at room temperature for 2 hours. The plates were washed, and peroxidase-conjugated goat antiserum to guinea pig immunoglobulin G (IgG) (Cappel Laboratory, Inc., Cochranville, Pennsylvania) was added at a predetermined dilution. After a 2-hour incubation at room temperature, the plates were washed three times, and 100 μl of orthophenylene diamine (OPD) substrate (25 mg of OPD in 25 μl of 30 percent H_2O_2 in phosphate citrate buffer, *p*H 5). Sixty minutes later, the colorimetric reaction was read at 490 nm (Dynatec MR 580 micro-ELISA autoreader). Analyses were performed in duplicate, and the results were expressed as absorbance.

The inner ear fluid was collected as follows: the thoracic cavity of the anesthetized animal was opened and the blood was removed from the heart. In order to avoid blood contamination of the inner ear fluid and cerebrospinal fluid, we perfused the animals through the left ventricle of the heart with physiological saline. The cerebrospinal fluid was

Table 1. Histologic changes in the inner ear of 13 guinea pigs immunized with type II collagen. Changes were characterized as profound (+++), moderate (++), or slight (+). A dash indicates a normal condition.

Changes	Finding in animals			
	Killed 2 weeks after last immunization		Killed 4 weeks after last immunization	
	N = 1	*N* = 3	*N* = 2	*N* = 7
Hydrops	++	+	+	–
Degeneration of organ of Corti	++	++	++	+
Precipitation in cochlear duct	++	++	+	+
Degeneration of spiral ganglion	++	+	–	–
Dilation of endolymphatic duct and sac	+	+	+	–

collected, and temporal bones were then dissected as a unit.

Under a surgical microscope, the tympanic bulla of the temporal bone was opened to permit visualization of the oval window and round window. A pointed micropipette was inserted into the round window to collect the perilymph from the scala tympani (*18*).

After removal of the stapes, another pointed micropipette was inserted into the oval window to collect the perilymph from the vestibule. A small piece of filter paper was put into the oval window to soak up the remaining perilymph. The shining saccular membrane was seen at the bottom of the oval window and another micropipette was inserted into the saccular membrane to collect the endolymph. This fluid contained levels of potassium and sodium identical to that of endolymph, indicating that there was no contamination from the perilymph.

The temporal bones were immediately fixed in 10 percent buffered Formalin, decalcified with 5 percent tricholoracetic acid, and embedded in paraffin and celloidin. Sections were cut at 10 μm horizontally and stained with hematoxylin and eosin. For the immunofluorescence studies, we slightly modified a paraffin-embedding technique described by Sainte-Marie (*19*). Temporal bones were removed, fixed in cold 95 percent ethanol, decalcified with 5 percent EDTA solution (*p*H 7.4) for 2 to 3 weeks at 4°C, and then embedded in melted paraffin at 56°C. Sections were cut at 7 μm horizontally and dried overnight at room temperature. Nonspecific background staining was reduced by an incubation with normal rabbit serum diluted 1:5 with 1 percent bovine serum albumin. All prepared sections were incubated for 45 minutes with a 1:20 dilution of goat antiserum to guinea pig IgG (Cappel) and the third component of complement (C3) (Cappel), then for 30 minutes with a 1:20 dilution of fluorescein isothiocyanate–conjugated rabbit antiserum to goat IgG (Cappel). The sections were then washed in phosphate-buffered saline and mounted in glycerin. Control sections were treated as described but with normal goat serum or second antibody only.

Table 2. Activity of antibody to collagen tested with ELISA in eight guinea pigs immunized with bovine type II collagen. Control animals showed no antibody activity against type II collagen. S.D., standard deviation.

Substance	Absorbance (mean ± S.D.)
Serum	0.683 ± 0.393
Cerebrospinal fluid	0.036 ± 0.017
Endolymph	0.098 ± 0.045
Perilymph	
Scala tympani	0.143 ± 0.101
Scala vestibuli	0.114 ± 0.048

The guinea pigs immunized with bovine type II collagen showed spiral ganglion cell degeneration characterized histologically by the swelling of cell bodies, vacuolated cytoplasm, and pyknotic and atrophied nuclei. A loss of 30 to 40 percent of the ganglion cell population was observed and was more conspicuous in the apical turn than in the basal turn (Table 1). Some guinea pigs immunized with bovine type II collagen showed an atrophied organ of Corti, a cystic lesion of the stria vascularis, and moderate extension of Reissner's membrane (Fig. 1). Endolymphatic hydrops was observed in about half of the treated animals and was more profound in the lower turns of the cochlea than in the upper turns. There were no changes in the middle ear. Guinea pigs killed 2 weeks after booster injection had more distinct and larger fluid accumulations than those killed 4 weeks after booster was given (Table 1). Almost all of the immunized animals had a degeneration of the organ of Corti showing the edema of lysis of the sensory cells. This finding was more marked in the lower turns than in the upper turns. The main cochlear artery showed no histological changes in the sections stained with hematoxylin and eosin. The endolymphatic duct of the guinea pigs immunized with bovine type II collagen contained a large number of macrophages and precipitates and showed mild atrophy of the surface epithelium in the middle portion of the duct. The proximal portion of the duct and the sac appeared to be normal. The immunofluorescence study showed positive reactions to guinea pig IgG and C3 in the epithelial layer of the middle portion of the endolymphatic duct (where the subepithelium is composed of type II collagen), on the vessel wall of the main cochlear artery, and an internal surface of the spiral ligament in the scala vestibuli. Control animals did not show any fluorescence in this region. We do not know the reason for this; however, it might play some pathogenic role for poor resorption of fluid in this ductal area, possibly resulting in hydrops.

Precipitates were a very common finding in both endolymphatic and perilymphatic spaces. In the animals killed 2 weeks after the final immunization, there was much more precipitation in the scala media (cochlear duct) than in the scala tympani or scala vestibuli.

Likewise, a large quantity of precipitation and cells were seen in the endolymphatic sac. Precipitation in the endolymphatic space seemed to decrease with time. In the animals killed 4 weeks after the final immunization, there was less precipitation in the endolymphatic space than in the scala tympani or scala vestibuli.

No changes other than the precipitation were observed in saccule, utricle, and semicircular canals. The immunized guinea pigs had increased antibody activity against type II collagen in the serum and in the endolymphatic and perilymphatic fluid (Table 2). Inner ear structures, for example, the spiral ligament, the subepithelial layer of endolymphatic duct, and the labyrinthine membrane also contain type II collagen.

Thus, our animal model is characterized by the following pathological lesions: spiral ganglion degeneration, atrophied organ of Corti, cystic lesions of the stria vascularis, mild to moderate dilation of scala media, mild atrophy of surface epithelium of endolymphatic duct, large numbers of macrophages and precipitates in the endolymph, immunofluorescent deposits (IgG and complement) in the epithelial layer of the middle part of the endolymphatic duct, and increased antibody activity against type II collagen, a component of the inner ear tissues.

Whether or not autoimmunity to colla-

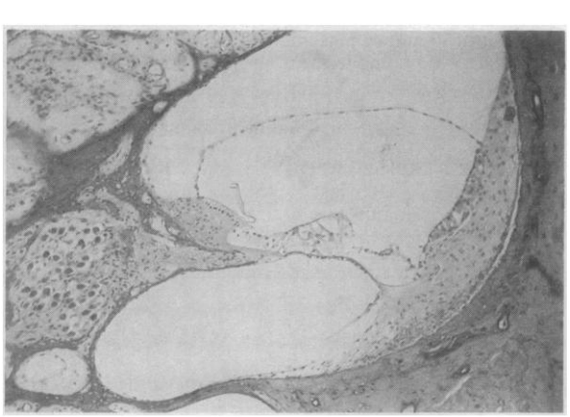

Fig. 1. Second turn of the cochlea in a guinea pig immunized with bovine type II collagen. Note the moderate extension of Reissner's membrane, the mild degeneration of the spiral ganglion cells and organ of Corti, and the dilated capillaries in the stria vascularis. Stain is hematoxylin and eosin. Original magnification, ×60.

gen is involved in human ear diseases is not known. Preliminary studies performed in our laboratory suggest that 50 percent of patients with Ménière's disease have collagen autoimmunity (*15*). On this basis, we propose that the hydrops may be initiated by autoimmunity to collagen. Our animal model may thus be useful in defining the pathogenesis of human Ménière's disease.

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Rapid Degradation of "New" Acetylcholine Receptors at Neuromuscular Junctions

Abstract. *Acetylcholine receptors at innervated neuromuscular junctions are very stable, with half-lives reported to be 6 to 13 days. Their turnover is described as a first-order process, implying a single population of receptors. In this study, two subpopulations of acetylcholine receptors at normally innervated junctions have been identified. One has a rapid turnover rate with a half-life of 18.7 hours, similar to that of extrajunctional receptors, and the other has a slow turnover rate with a half-life of 12.4 days. The rapidly turned over subpopulation represents approximately 20 percent of the total junctional receptors. This finding may account for the discrepancies in previous reports of turnover rates and may explain the rapid reversibility in vivo of agents that "irreversibly" block acetylcholine receptors. This finding also implies that the synthesis rate of junctional acetylcholine receptors may be higher than previous estimates. The rapidly turned-over subpopulation may represent receptors that were newly inserted into the neuromuscular junction and that were not yet stabilized by an influence of the motor nerve.*

Acetylcholine (ACh) receptors at normally innervated neuromuscular junctions are very stable, in contrast to the ACh receptors at extrajunctional regions of denervated muscle (*1, 2*). The turnover of extrajunctional ACh receptors has been described as a single-order process (*3*); the receptors are rapidly degraded, with a half-life of 15 to 30 hours (*4*). Junctional ACh receptors have a far slower rate of degradation, also described as a single-order process, with a half-life of 6 to 13 days (*2, 3, 5–7*). However, scrutiny of the data in several earlier studies suggested that a subpopulation of the junctional receptors may be degraded more rapidly (*3, 5, 8*). We examined the turnover of ACh receptors at intact neuromuscular junctions and now report that a sizable fraction of junctional receptors have a fast turnover rate. This population of ACh receptors may represent newly inserted ACh receptors that have not yet been stabilized at the postjunctional membrane.

We determined the rate of ACh receptor degradation by monitoring the loss of ^{125}I-labeled α-bungarotoxin (^{125}I-α-BuTx) that was specifically bound to junctional ACh receptors (*3, 9*). The loss of bound ^{125}I-α-BuTx was found to correspond to the loss of ACh receptor sites (*9*). The ^{125}I-α-BuTx is degraded along with the ACh receptor and is released from the muscle in the form of ^{125}I-tyrosine (*3, 6, 9*).

Binding of ^{125}I-labeled or unlabeled α-BuTx to ACh receptors of the mouse diaphragm was carried out as described (*6, 10*). Adult female (C57BL/6 × DBA/2)F_1 hybrid mice (18 to 20 g) were anesthetized with chloral hydrate (0.4 mg per gram of body weight) for all surgical procedures. Mice were given unlabeled α-BuTx (1 μg per 20 g of body weight) or ^{125}I-α-BuTx (1.4 μg per 20 g of body weight; specific activity, 3.91×10^4 to 7.96×10^4 Ci/mole) in 140 μl of Ringer solution; half the dose was injected into each thoracic cavity. The mice were then maintained in an upright position for 1 to 2 hours to allow the solution to gravitate to the diaphragm and block or label the ACh receptors.

At various times after the blocking and labeling procedures, diaphragms were removed from groups of three or four mice. Care was taken to account for any background radioactivity (due to radioactive material diffusely bound along the muscle membrane). First, the diaphragms, in groups of three or four, were washed repeatedly for 48 hours with large volumes of buffered Ringer solution until no further radioactivity was detected in the final wash. Second, correction was made for background radioactivity in the junction-containing strip. For this purpose, the diaphragm was cut into junction-containing and extrajunctional strips. The radioactivity measured in the extrajunctional strip was subtracted (on a per-weight basis) from the radioactivity in the junction-containing strip. At all time points, this background radioactivity was a small fraction of the total radioactivity in the junctional portion of the muscle; at its highest, 1 day after the labeling procedure, background radioactivity was less than 10 percent of the total counts. To evaluate the contribution of the perijunctional regions to the turnover of receptors, we also used quantitative light autoradiography to examine the distribution of ^{125}I-α-BuTx binding (*11*).

The radioactive material remaining bound to the neuromuscular junctions was determined for groups of mice at each time point. Because it takes several hours for binding of ^{125}I-α-BuTx and washout of unbound ^{125}I-α-BuTx to occur in vivo, measurements were begun 1 day after the labeling procedure. All subsequent counts were expressed as a percentage of the total counts present at day 1 after labeling, and the means were plotted on a logarithmic scale against time. Straight lines were fitted to the points by the method of least squares, and half-lives of ACh receptors were calculated from the slopes of the lines.