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Breakdown of the Round Window Membrane Permeability Barrier Evoked by Streptolysin O: Possible Etiologic Role in Development of Sensorineural Hearing Loss in Acute Otitis Media

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Sensorineural hearing loss is a common sequela of acute and chronic otitis media, and the round window membrane (RWM) is currently being considered as a major route for noxious agents to pass from the middle ear cavity to the cochlea. *Streptococcus pneumoniae*, a major causative agent of otitis media, and *Streptococcus pyogenes* A produce molecularly related toxins, pneumolysin and streptolysin O (SLO), that form large pores in target membranes. In this study, we analyzed the effects of SLO on the permeability of the RWM. Resected RWMs from a total of 104 guinea pigs were embedded between two chambers of an in vitro system. One chamber was designated as the tympanal (*cis*) compartment, and the other was designated as the inner ear (*trans*) compartment. The permeability of normal and SLO-damaged RWMs towards Na^+, [^{14}C]mannitol, and proteins was investigated. SLO evoked permeability defects dose dependently in the RWM with fluxes of both Na^+ and [^{14}C]mannitol being demonstrable over a time span of up to 8 h. Serum proteins and radioiodinated SLO were also shown to pass through the damaged RWM. Scanning electron microscopy revealed the morphological correlates to these results. We propose that damage to the RWM by potent pore-forming cytolysins leads to leakage of ions from the perilymph. Ionic disequilibrium and passage of noxious macromolecules to the cochlea could contribute to disturbances of the inner ear function.

Otitis media (OM) in both the acute and chronic forms is one of the most common ear diseases in children and adults (6, 34). The disease occurs in 2 to 3% of all children under 16 years of age (21) and is caused most frequently by *Streptococcus pneumoniae* and *Haemophilus influenzae* (17, 34). OM induces a variety of pathological changes in the middle ear, including hyperemia and edema, granulocyte and monocyte infiltration, and irreversible mucosal changes such as generation of granulation tissue, cholesteatoma formation, tympanosclerosis, and ossicular destruction (20, 25, 34). A serious sequela of OM is damage to the inner ear leading to sensorineural hearing loss (16, 32, 33, 36, 38, 39, 41), which is limited to the cochlear basal turn (35). Especially in children, hearing loss leads to severe damage of development of speech, language, and cognitive functions (27). The etiology of sensorineural hearing loss has remained elusive, but previous investigations indicate that permeability of the three-layered round window membrane (RWM) is of pivotal importance. The RWM is the major permeability barrier between the middle ear cavity and the cochlea (19, 36, 37). It is permeable by water (2) but not by ions and macromolecules (13, 18, 22). This guarantees maintenance of the characteristic ionic milieu in the perilymph and hinders the passage of noxious agents from the middle ear to the inner ear. A few previous investigations indicate that the RWM becomes permeable by macromolecules (22) in OM, but the mechanisms underlying this phenomenon remain largely unknown.

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Some studies have directly addressed the effects of certain bacterial products on the permeability of the RWM. The RWM has been reported to be permeable by *Staphylococcus aureus* pyrogenic exotoxin (19, 42) and *Salmonella typhimurium* endotoxin (26) but, somewhat oddly, not by *H. influenzae* endotoxin (30). Application of *Escherichia coli* endotoxin or *Staphylococcus aureus* exotoxin into the round window niche (23) or induction of pneumococcal OM (24) increases the passage of tetraethylammonium across the RWM. *Pseudomonas aeruginosa* exotoxin A is able to permeate the RWM provided that very high doses (1 mg/ml) are applied (29).

In 1987, Lundman et al. (31) described a novel experimental system for investigating the permeability characteristics of the RWM. The RWM surrounded by an intact bony frame is excised from experimental animals and embedded in a Plexiglas sheet that separates two buffer chambers. Transmembrane flux of ions and macromolecules can then be measured directly. The authors confirmed that the RWM was impermeable to high- and low-density lipoproteins over a period of 3 h ex vivo.

In the present study, we utilized the experimental system of Lundman et al. to determine whether streptolysin O (SLO), the major cytotoxin elaborated by *Streptococcus pyogenes* A, might directly alter the permeability characteristics of the RWM. SLO was employed because this toxin is related molecularly to pneumolysin, the major cytotoxin of *Streptococcus pneumoniae* (1, 5, 8, 9, 11, 12, 43) and it is available in a highly purified form in our laboratory (7, 10, 44). We report that low concentrations of this pore-forming cytotoxin evoke a rapid breakdown of the RWM permeability barrier. Such damage to the RWM, if occurring during infections with toxin-inducing

bacterial strains, could directly cause sensorineural hearing loss by evoking imbalances in the ionic composition of the perilymph. Furthermore, our data indicate that toxic macromolecules such as SLO might diffuse directly from the middle ear to the inner ear.

MATERIALS AND METHODS

Preparation of the RWM. One hundred twenty-five ears of healthy guinea pigs (250 to 400 g body weight) were used. Ear infection was excluded by examination of the external auditory canal and tympanic membrane. The bulla was removed and opened carefully (3). The RWM was then resected, leaving a small surrounding bony ring of cochlear material. For the resection, a Messmer universal drill and Horico diamond separation disks were used. The RWM was embedded between the two chambers of an in vitro system with two separate chambers by use of Espe Duralon dental cement as described by Lundman et al. (31). One chamber was designated as the tympanal (*cis*) compartment, and the other was designated as the cochlear (*trans*) compartment. The chambers were made of transparent plastic material and had a volume of 200 μl each. The chambers had two metal tubes for instillation of fluids and air evacuation. These were closed after filling and sampling. The entire preparation took no longer than 15 min.

Preparation of SLO. Two toxin preparations were used with the same results. SLO was either purified from streptococcal culture supernatants as described (7) or obtained in recombinant form from *E. coli* (44). Each toxin preparation yielded a single major polypeptide of M_r 55,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Solutions containing 20 μg of native SLO per ml contained no detectable lipopolysaccharide (LPS) as determined by a quantitative *Limulus* assay (Kabi Vitrum Diagnostica, Stockholm, Sweden). The proteins were stored lyophilized and reconstituted with buffer plus 1% bovine serum albumin and 2 mM dithiothreitol. The toxin was radioiodinated to a specific activity of 0.4 μCi/mg, with functional activity retained as described in detail elsewhere.

Determination of Na^+ flux through the RWM. A sodium gradient of 92 mM was established between the *cis* and *trans* compartments by use of a standard nutrient mixture in the *cis* chamber and a 0.15 M K_2H- or KH_2-phosphate buffer (pH 7.4) in the *trans* chamber. The Na^+ concentration of samples drawn from the *trans* chamber was always less than 6 mM. After the addition of 0.032, 0.16, 0.8, and 4.0 μg of SLO per ml to the *cis* compartment, the chambers were incubated at 38°C. Controls received no cytolysin. Five-microliter samples were removed from the *trans* chamber after 10 min and 1, 4, and 8 h. Na^+ concentrations were determined by flame photometry. pH and osmolarity were also determined.

Measurements of [^{14}C]mannitol flux through the RWM. The *cis* and *trans* compartments were filled with Hanks balanced salt solution, and 0.8 μg of SLO per ml plus 1 μCi of [^{14}C]mannitol was applied to the *cis* chamber. Controls received 1 μCi of [^{14}C]mannitol without the cytolysin. Five-microliter samples were collected after 10 min and 1 and 2 h from the *trans* compartment, and radioactivity was measured with a beta counter (Packard Tri-Carb 2500TR).

Permeability of RWM by macromolecules. (i) Protocol 1. The *trans* chamber was filled with Hanks balanced salt solution. The membrane was treated with SLO by application of 0.16, 0.8, and 4.0 μg/ml in the *cis* chamber. After 30 min, 50 μl of human serum from healthy individuals was added to the same compartment. After 10 min and 1 and 2 h, 5-μl samples were taken from the *trans* chamber. The presence of serum proteins in the latter samples was assessed by quantitative immunoelectrophoresis (4). Positive controls, with 2% Triton X-100 in the *cis* chamber instead of SLO, and negative controls, with neither SLO nor Triton X-100, were performed. In a third group of control experiments, SLO was inactivated by preincubation with human serum before application to the *cis* chamber.

(ii) Protocol 2. SLO was added to the *cis* chamber at 0.8 μg/ml for 60 min. Thereafter, both chambers were emptied and filled with fresh Hanks balanced salt solution. Fifty microliters of human serum was applied additionally to the *cis* chamber. After 10 min and 1 and 2 h, 5-μl samples were collected from the *trans* chamber. Quantitative rocket immunoelectrophoresis was performed with polyclonal rabbit antibodies against human serum proteins (Dakopatts code 100SF), anti-human albumin (Dakopatts code A001), and anti-human α2-macroglobulin. For calibration, samples from the *cis* chamber were diluted 1:10 and electrophoresed similarly; this was done so that identical rocket heights in the *cis* and *trans* samples reflected the flux of 10% of the respective protein through the RWM.

(iii) Protocol 3. One microcurie of radioiodinated SLO plus 0.8 μg of unlabelled SLO per ml was applied to the *cis* chamber. After 10 min and 1 and 2 h, 5-μl samples were collected from the *trans* compartment, and radioactivity was determined with a gamma counter (Packard Cobra II).

Scanning electron microscopy. The RWMs were decalcified, desiccated, and dried with a critical-point drying procedure. They were mounted onto a scanning electron microscope carrier with conductive carbon cement and gold-sputtered.

RESULTS

Na^+ flux through the RWM. As shown in control experiments, spontaneous flux of Na^+ through the intact RWM was minimal. Application of SLO at concentrations above 0.16 μg/ml evoked an initially rapid flow of Na^+ from the *cis* to the *trans* chamber (Fig. 1). After 4 h, the flow of Na^+ appeared to plateau. The ion gradient was stable over a period of 8 h in the controls. No changes in pH or osmolarity were observed (data not shown).

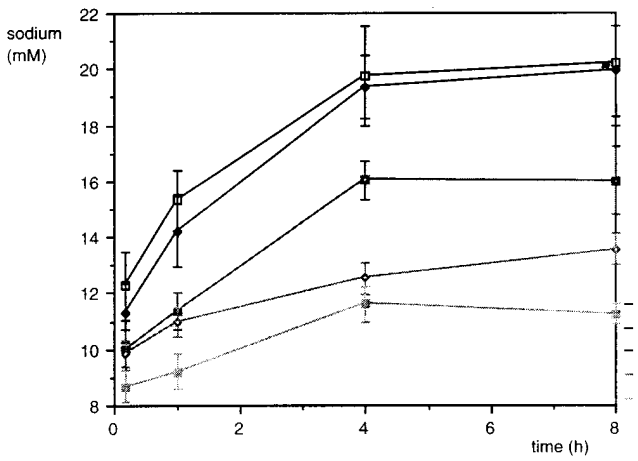

FIG. 1. Na^+ flux through the RWM evoked by SLO. The chamber corresponding to the tympanal side was designated *cis*, and the chamber corresponding to the cochlear compartment was designated *trans*. A sodium gradient of 92 mM was established between *cis* and *trans*; the Na^+ concentration of samples drawn from the *trans* chamber before toxin application was always less than 6 mM. After the addition of SLO at 4.0 (⊡), 0.8 (◆), 0.16 (■), 0.032 (◆), and 0 (▭) μg/ml to the *cis* chamber, 5-μl samples were removed from the *trans* chamber at the given times and Na^+ concentrations were determined. The mean values ± standard deviations of 15 experiments in each group are shown.

Flux of [^{14}C]mannitol through the RWM. [^{14}C]mannitol together with 0.8 μg of SLO per ml was applied to the *cis* chamber, and samples were removed from the *trans* chamber at 10 min and at 1 and 2 h. Controls without cytolysin application indicated that the RWM is not normally permeable by [^{14}C]mannitol. One of five RWMs remained impermeable by [^{14}C]mannitol after application of 0.8 μg of SLO per ml, whereas flux of the marker was discerned in the other four experiments, where an average of 4.3% of the [^{14}C]mannitol applied to the *cis* chamber entered the *trans* chamber (Fig. 2).

Permeability of RWM by macromolecules. Rocket immunoelectrophoresis with polyspecific antibodies against human serum proteins showed that at concentrations of SLO above 0.8 μg/ml, a rapid flow of macromolecules occurred through the RWM. Multiple rocket precipitates representing human serum proteins were detected in samples drawn from the *trans* chamber (Fig. 3). No proteins passed through the RWM when only 0.16 μg of SLO per ml was employed. Application of 2% Triton X-100 to the *cis* chamber also led to a breakdown of the RWM permeability barrier, so that serum proteins became detectable in the *trans* chamber. In one set of experiments, SLO was preincubated with human serum. It is known that human serum contains antibodies and lipoproteins that inactivate the cytolysin. In this case, no flux of serum proteins across the RWM was observed. Furthermore, no flux of macromolecules was detected when 1 μg of LPS from *E. coli* O55:B5 (Sigma, Deisenhofen, Germany) per ml was applied instead of SLO.

In another set of experiments, the RWMs were first incubated with 0.8 μg of SLO per ml for 1 h. Thereafter, the chambers were emptied and filled with fresh Hanks balanced salt solution, and 50 μl of human serum was added to the *cis*

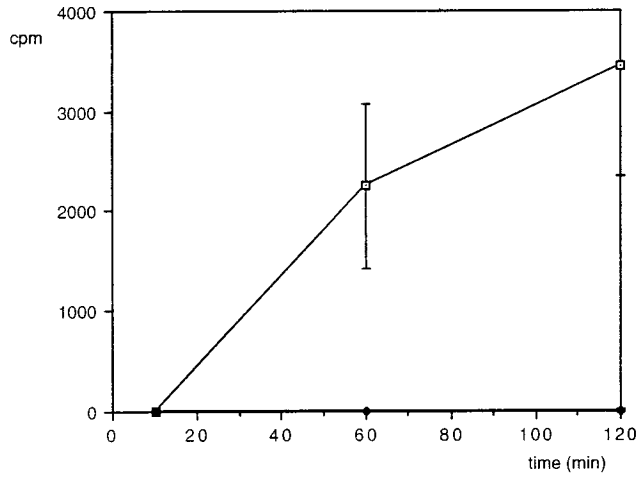

FIG. 2. Flux of [^{14}C]mannitol through the RWM. [^{14}C]mannitol together with 0.8 μg of SLO per ml was applied to the *cis* chamber, and 5-μl samples were removed for determination of radioactivity at the given times. Values are means ± standard deviations (n = 5 in each group). Symbols: ⊡, 0.8 μg/ml; ◆, 0 μg/ml.

chamber. At the given times, samples were removed from the *trans* compartment and analyzed by rocket immunoelectrophoresis with specific antibodies against albumin. In these experiments, passage of albumin through the RWM was detected in 75% of the RWMs (Fig. 4). An average of 4% of total albumin applied to the *cis* chamber was detected in the *trans* chamber, as inferred from the heights of the rocket immunoprecipitates.

In addition to the flux of serum albumin identified with specific antibodies, a similar flux of α_2-macroglobulin through RWMs treated with 0.8 and 4.0 μg of SLO per ml (data not shown) was observed. Membranes that had received 0.16 μg of SLO per ml were impermeable by this macromolecule. When 4 μg of SLO per ml was applied to the inner ear side of the RWM, leakage of macromolecules to the tympanal chamber was also observed (data not shown).

Permeability of RWM by SLO. The flux of a radioactive, functionally intact SLO tracer was also studied. Flux of the radiolabelled cytotoxin through the RWM was observed in 75% of the experiments in which 0.8 μl of SLO per ml was

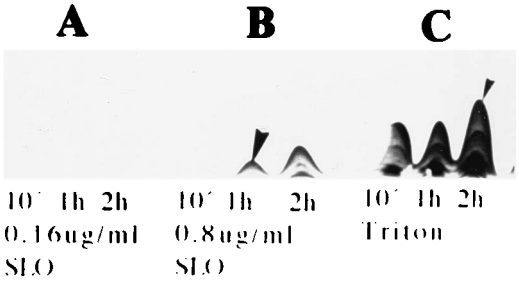

FIG. 3. Flux of human serum proteins through the RWM. SLO was applied to the *cis* chamber for 30 min, after which whole human serum was added to the same compartment. At the given times (i.e., 10 min and 1 and 2 h), 5-μl samples were taken from the *trans* chamber and applied to rocket immunoelectrophoretic analysis with polyspecific antibody to serum proteins. Results of three experiments in which the RWM was treated with 0.16 μg of SLO per ml (A), 0.8 μg of SLO per ml (B), or 2% Triton X-100 (C) (all final concentrations) are shown. Note the immediate breakdown of the membrane permeability barrier evoked by Triton X-100 and the slow breakdown of the permeability barrier evoked by 0.8 μg of SLO per ml. The major precipitate (arrows) represents albumin.

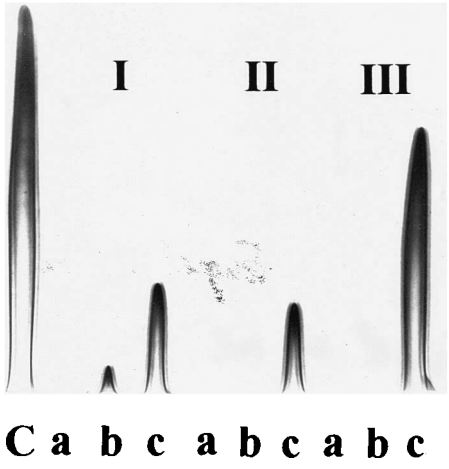

FIG. 4. Quantitation of albumin flux through the RWM evoked by 0.8 μg of SLO per ml. The results of three different experiments (I to III) in which the RWMs were treated with 0.8 μg of SLO per ml for 60 min and then whole human serum was added to the *cis* chamber are shown. Five-microliter samples were retrieved from the *trans* chamber after 10 (a), 60 (b), or 120 (c) min. C, control rocket immunoprecipitate obtained by electrophoresing 5 μl of a 10-fold-diluted sample from a *cis* chamber. A quantitative estimate of the albumin concentration in the *trans* chamber could be obtained by comparing the heights of the rocket-shaped lines with that of the control.

employed. The collective results are shown in Fig. 5, and an average of 6.82% of the radiolabelled toxin applied to the *cis* chamber appeared in the *trans* chamber after 2 h.

Scanning electron microscopy. Scanning electron microscopy was performed on a small number of RWMs treated with 4 μg of SLO per ml. Figure 6A shows an oblique view of the undamaged RWM after 8 h in a control experiment. The intact epithelial cells lining the tympanal side of the RWM and the undamaged microvilli are visible. After treatment with 4 μg of SLO per ml, marked changes were observed. After a 20-min treatment, the epithelial cell nuclei were shrunk, and large defects in the cell membrane were observed. Figure 6B is a micrograph taken en face; the virtual absence of microvilli and the gross damage to the epithelial cells are visible. After 8 h,

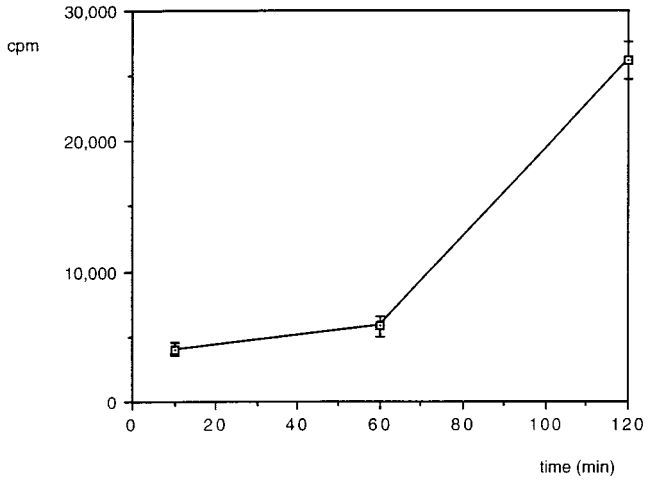

FIG. 5. Flux of radioiodinated, functionally intact SLO through the RWM. RWM preparations were treated with 0.8 μg of SLO per ml (⊡) spiked with 1 μCi of radioiodinated tracer in the *cis* chamber. At the depicted times, 5-μl samples were removed from the *trans* chamber and radioactivity was determined. Values are means ± standard deviations from eight experiments.

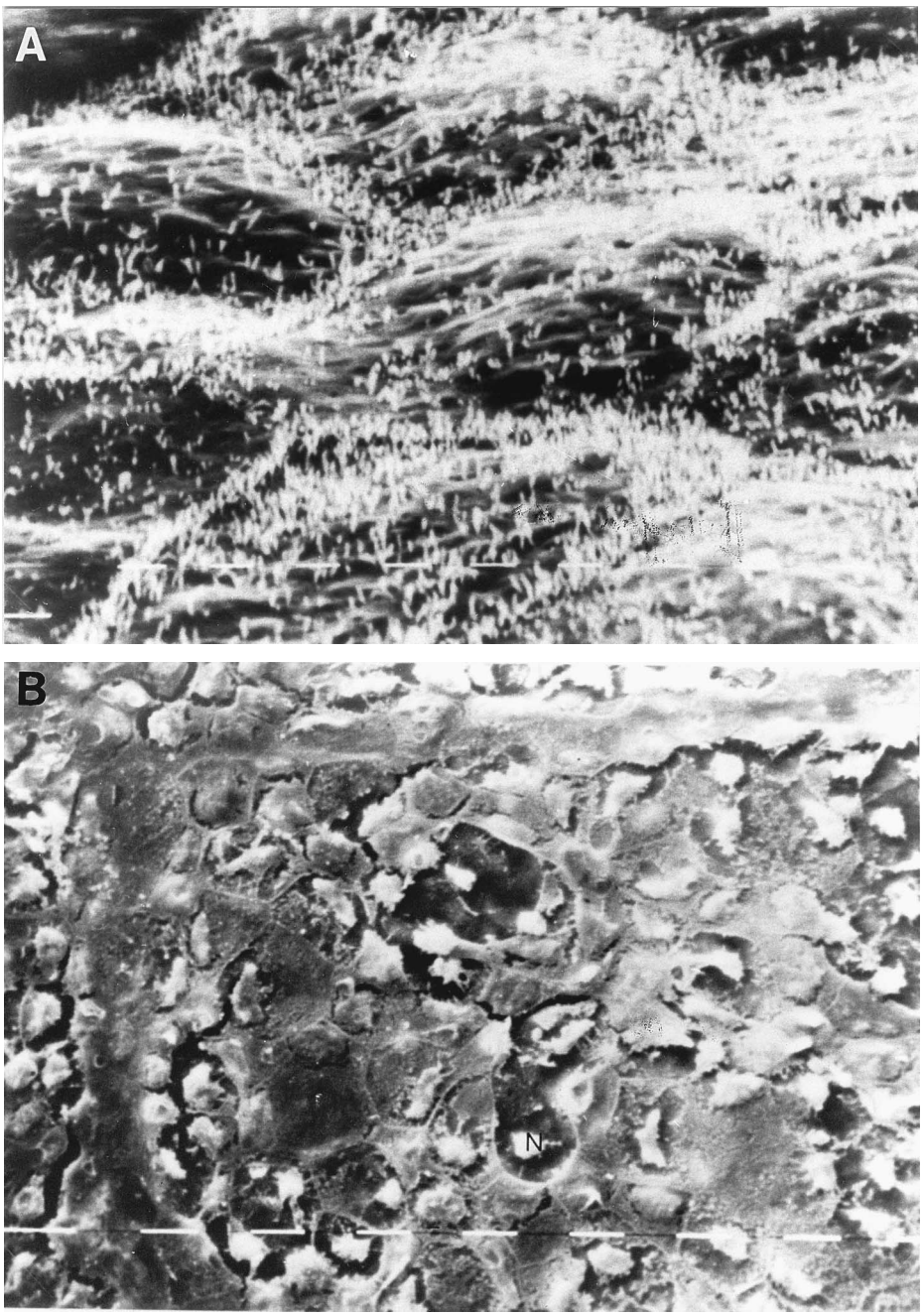

FIG. 6. (A) Scanning electron micrograph of control RWM seen obliquely after 8 h. Intact epithelial cells (presenting as the large mounds) and undamaged microvilli are visible. (Magnification, ×3,970). (B) Scanning electron micrograph of RWM viewed en face following a 20-min treatment with 4 μg of SLO per ml. The epithelial cells are shrunk, large defects in the cell membrane can be observed, and cell nuclei (N) are exposed. The microvilli have virtually disappeared. (Magnification, ×3,970).

the epithelial cells became detached, and the basal membrane was denuded (data not shown).

DISCUSSION

SLO is the prototype of a large family of bacterial cytolysins that bind to cholesterol and form large pores with 30-nm diameters in the lipid bilayer of erythrocytes (1, 8, 9, 11, 14). We presume that similar pores are created in the membranes of epithelial cells lining the tympanal side of the three-layered RWM, thus leading to cytolysis and disruption of the permeability barrier. As middle ear effusion constantly bathes the RWM (28), disturbances of perilymphatic and endolymphatic ion concentrations due to uncontrolled ion fluxes through the RWM may be expected. Furthermore, a flux of macromolecules including noxious bacterial products could occur. Since *Streptococcus pneumoniae* bacteria elaborate a homologous and functionally similar cytolysin (1, 8, 9, 11, 12, 43), we believe that analogous processes may be operative in OM caused by this agent.

Our conclusions are based on a large number of experiments in which the permeability of guinea pig RWM was studied in an in vitro model. The advantage of the model is that no contamination of samples via other pathways (40) from the middle to the inner ear occurs. We found that SLO evoked permeability defects dose dependently in the RWM, with fluxes of both Na^+ and [^{14}C]mannitol being demonstrable over a time span of several hours. Control experiments without SLO showed that the undamaged RWM was not permeable for [^{14}C]mannitol or macromolecules, and only a low flux of Na^+ that was far below the levels found after toxin application was observed. That the observed effects were due to SLO rather than to contaminating substances such as LPS was inferred from the findings that preincubation of toxin preparations with serum neutralized toxicity. It is known that SLO is inactivated by serum components, including lipoproteins. Furthermore, application of LPS at the relatively high concentration of 1 μg/ml caused no impairment of the RWM permeation barrier towards macromolecules. Quantitative *Limulus* assays excluded the possibility that preparations of native SLO contained detectable amounts of LPS.

As our investigations with human serum proteins showed, even macromolecules were able to pass through the RWM following treatment with ≥0.8 μg of SLO per ml. No leakage occurred in control preparations that received no toxin or in those receiving SLO after preincubation with human serum. In positive control experiments, 2% Triton X-100 was applied to the *cis* chamber, and this caused immediate breakdown of the RWM permeability barrier. The rationale underlying the protein flux experiments was straightforward, and quantitative immunoelectrophoresis permitted the amounts of protein diffusing to the *trans* chamber to be approximated with ease. The method represented an alternative to measurements of total protein or SDS-PAGE analyses and permitted direct identification of different proteins in the mixture (e.g., α_2-macroglobulin as a very large molecular marker). With this method, we estimated that 3 to 5% of the albumin applied to the *cis* chamber diffused to the *trans* chamber when an RWM was damaged by SLO. Experiments using ^{125}I-radiolabelled SLO demonstrated that even the cytolysin itself was able to permeate the damaged RWM, and the degree of flux was comparable to that found for albumin. This finding was somewhat unexpected, since we originally thought that the active toxin might become quantitatively trapped in the RWM as a result of avid binding to cholesterol.

These results could be demonstrated for the majority (75%) of RWMs treated with 0.8 μg of SLO per ml, but it must be noted that toxin-dependent permeability increases were absent in a few experiments. The causes for the relative intrinsic resistance of certain RWM preparations towards toxin action are unknown.

Overall, our present findings indicate that SLO and, by inference, other pore-forming bacterial exotoxins may directly damage the RWM, causing leakage of ions and even macromolecules from and to the perilymph. Ionic disequilibrium ensuing in the latter could lead to severe disturbances of inner ear function. Passage of macromolecules such as bacterial proteases or even the cytolysin itself could be responsible for toxic damage to the organ of Corti. In this regard, it is of particular interest to note that pneumolysin has recently been shown to be potently toxic towards hair cells of guinea pig cochlea (15). If, as we expect, pneumolysin can eventually be shown to produce similar effects on the RWM as those produced by SLO, this would provide a relatively simple explanation for the well-known clinical syndrome of sensorineural hearing loss that frequently occurs during pneumococcal otitis media. It is noteworthy that pneumolysin is probably present mainly in an intracellular or cell-bound form (12), so that lysis of bacteria by antibiotics may even enhance the local levels of this damaging product. Bacterial cell lysis is not a requisite for release of SLO from group A streptococci, which are also occasionally causative agents of otitis media.

The present study became feasible through the development by Lundman et al. (31) of a reliable, albeit technically demanding, method for studying the permeability characteristics of the RWM in vitro. It is, to our knowledge, the first demonstration that a bacterial cytolysin can disrupt the permeability barrier of a complex, multilayered membrane. The results raise the possibility that bacterial cytolysins such as SLO may be significant in the pathogenesis of inner ear diseases of tympanal origin.

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REFERENCES

1. **Alouf, E., and C. Geoffroy.** 1991. The family of the antigenically-related, cholesterol-binding ("sulphydryl-activated") cytolytic toxins, p. 147–186. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, Inc., New York.
2. **Arslan, M.** 1969. Modification of the osmotic pressure of perilymph and endolymph. Acta Otolaryngol. **67:**360–377.
3. **Asarch, R., M. Abramson, and W. B. Litton.** 1975. Surgical anatomy of the guinea pig ear. Ann. Otol. **84:**250–255.
4. **Axelsen, N. H.** 1983. Electroimmunoassay (rocket immunoelectrophoresis). Scand. J. Immunol. **17:**103–106.
5. **Bernheimer, A. W.** 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta **344:**27–50.
6. **Bernstein, J. M.** 1985. Recent advances in otitis media with effusion. Ann. Allergy **55:**544–551.
7. **Bhakdi, S., M. Roth, A. Sziegoleit, and J. Tranum-Jensen.** 1984. Isolation and identification of two hemolytic forms of streptolysin-O. Infect. Immun. **46:**394–400.
8. **Bhakdi, S., and J. Tranum-Jensen.** 1987. Damage to mammalian cells by proteins that form transmembrane pores. Rev. Physiol. Biochem. Pharmacol. **107:**147–223.
9. **Bhakdi, S., and J. Tranum-Jensen.** 1988. Damage to cell membranes by pore-forming bacterial cytolysins. Prog. Allergy **40:**1–43.
10. **Bhakdi, S., J. Tranum-Jensen, and A. Sziegoleit.** 1985. Mechanism of membrane damage by SLO. Infect. Immun. **47:**52–60.
11. **Bhakdi, S., U. Weller, I. Walev, E. Martin, D. Jonas, and M. Palmer.** 1993. A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. Med. Microbiol. Immunol. **182:**167–175.
12. **Boulnois, G. J., T. J. Mitchell, F. K. Saunders, F. J. Mendez, and P. W. Andrew.** 1990. Structure and function of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*. Zentralbl. Bakteriol. Suppl. **19:**43–51.
13. **Brady, D. R., J. P. Pearce, and S. K. Juhn.** 1976. Permeability of round window membrane to ^{22}Na or RISA. Arch. Otolaryngol. **214:**183–184.
14. **Buckingham, L., and J. L. Duncan.** 1983. Approximate dimensions of membrane lesions produced by streptolysin-S and streptolysin-O. Biochim. Biophys. Acta **729:**115–122.
15. **Comis, S.-D., M. P. Osborne, J. Stephen, M. Tarlow, T. L. Hayward, T. J. Mitchell, P. W. Andrew, and G. J. Boulnois.** 1993. Cytotoxic effects on hair cells of guinea pig cochlea produced by pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*. Acta Otolaryngol. **113:**152–159.
16. **English, G. M., J. L. Northern, T. J. Fria, and M. S. Denver.** 1973. Chronic otitis media as a cause of senorineural hearing loss. Arch. Otolaryngol. **97:**18–22.
17. **Giebink, G. S., and P. G. Quie.** 1977. Comparison of otitis media due to types 3 and 23 *Streptococcus pneumoniae* in the chinchilla model. J. Infect. Dis. **136**(Suppl. 8)**:**191–195.
18. **Goldberg, B., M. V. Goycoolea, P. M. Schlievert, D. Shea, P. Schachern, M. M. Paparella, and A. M. Carpenter.** 1981. Passage of albumin from the middle ear to the inner ear in otitis media in the chinchilla. Am. J. Otolaryngol. **2:**210–214.
19. **Goycoolea, M. V., M. M. Paparella, B. Goldberg, P. Schlievert, and A.-M. Carpenter.** 1980. Permeability of the middle ear to *Staphylococcus pyrogenic*

exotoxin in otitis media. Int. J. Pediatr. Otorhinolaryngol. **1:**301–308.
20. **Goycoolea, M. V., M. M. Paparella, S. K. Juhn, and A.-M. Carpenter.** 1980. Oval and round window changes in otitis media. Laryngoscope **90:**1387–1391.
21. **Gregg, J. B., K. Meyer-Roberts, and M. J. Colleran.** 1983. Ear disease and hearing loss, Pierre, South Dakota, 1962–1982. S. D. J. Med. **10:**9–17.
22. **Hamaguchi, Y., T. Morizono, and S. K. Juhn.** 1988. Round window membrane permeability to human serum albumin in antigen-induced otitis media. Am. J. Otolaryngol. **9:**34–40.
23. **Ikeda, K., and T. Morizono.** 1988. Changes of the permeability of round window membrane in otitis media. Arch. Otolaryngol. Head Neck Surg. **114:**895–897.
24. **Ikeda, K., M. Sakagami, T. Morizono, and S. K. Juhn.** 1990. Permeability of the round window membrane to middle-sized molecules in purulent otitis media. Arch. Otolaryngol. Head Neck Surg. **116:**57–60.
25. **Juhn, S. K., M. M. Paparella, M. V. Goycoolea, C. S. Kim, and G. S. Giebink.** 1977. Pathogenesis of otitis media. Ann. Otol. **86:**481–492.
26. **Kawauchi, H., T. F. DeMaria, and D. J. Lim.** 1988. Endotoxin permeability through the round window. Acta. Otolaryngol. Suppl. **457:**100–115.
27. **Klein, J. O.** 1984. Otitis media and the development of speech and language. Pediatr. Infect. Dis. **3:**389–391.
28. **Lee, S. H., H. W. Woo, T. T. K. Jung, C. Lee, S. K. Miller, Y. M. Park, and S. K. Hwang.** 1992. Permeability of arachidonic acid metabolites through the round window membrane in chinchillas. Acta Otolaryngol. Suppl. **493:**165–169.
29. **Lundman, L., D. Bagger-Sjöbäck, S. K. Juhn, and T. Morizono.** 1992. *Pseudomonas aeruginosa* exotoxin A and *Haemophilus influenzae* type B endotoxin. Effect on the inner ear and passage through the round window membrane of the chinchilla. Acta Otolaryngol. Suppl. **493:**69–76.
30. **Lundman, L., S. K. Juhn, D. Bagger-Sjöbäck, and C. Svanborg.** 1992. Permeability of the normal round window membrane to *Haemophilus influenzae* type b endotoxin. Acta Otolaryngol. **112:**524–529.
31. **Lundman, L. A., L. Holmquist, and D. Bagger-Sjöbäck.** 1987. Round window permeability—an *in vitro* model. Acta Otolaryngol. **104:**472–480.
32. **Moore, D. C., and G. F. Best.** 1980. A sensorineural component in chronic otitis media. Laryngoscope **90:**1360–1366.
33. **Morizono, T., G. S. Giebink, M. M. Paparella, M. A. Sikora, and D. Shea.** 1985. Sensorineural hearing loss in experimental purulent otitis media due to *Streptococcus pneumoniae*. Arch. Otolaryngol. **12:**794–798.
34. **Paparella, M. M.** 1983. Current concepts in otitis media. Henry Ford Hosp. Med. J. **31:**623–629.
35. **Paparella, M. M.** 1984. Review of sensorineural hearing loss. Am. J. Otolaryngol. **5:**311–314.
36. **Paparella, M. M., D. R. Brady, and R. Hoel.** 1970. Sensorineural hearing loss in chronic otitis media and mastoiditis. Trans Am. Acad. Ophthalmol. Otolaryngol. **74:**108–115.
37. **Paparella, M. M., M. Goycoolea, P. A. Schachern, and J. Sajjadi.** 1987. Current clinical and pathological features of round window diseases. Laryngoscope **97:**1151–1160.
38. **Paparella, M. M., F. Hiraide, M. Oda, and D. Brady.** 1972. Pathology of sensorineural hearing loss in otitis media. Ann. Otol. **81:**636–647.
39. **Paparella, M. M., T. Morizono, C. T. Le, F. Mancini, P. Sipilä, Y. B. Choo, G. Lidén, and C. S. Kim.** 1984. Sensorineural hearing loss in otitis media. Ann. Otol. Rhinol. Laryngol. **93:**623–629.
40. **Sando, I., R. P. Wood, Y. Masuda, and W. G. Hemenway.** 1971. Perilymphatic communication routes in guinea pig cochlea. Ann. Otol. **80:**826–834.
41. **Schachern, P. A., M. M. Paparella, and M. Goycoolea.** 1987. The permeability of the round window membrane during otitis media. Arch. Otolaryngol. Head Neck Surg. **113:**625–629.
42. **Schachern, P. A., M. M. Paparella, M. Goycoolea, B. Goldberg, and P. Schlievert.** 1981. The round window membrane following application of staphylococcal exotoxin: an electron microscopic study. Laryngoscope **91:**2007–2017.
43. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. Boulnois.** 1987. Molecular cloning, characterization and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55:**1184–1189.
44. **Weller, U., et al.** Unpublished data.