

Endotoxin in Middle-Ear Effusions from Patients with Chronic Otitis Media with Effusion

THOMAS F. DEMARIA,^{1*} RICHARD B. PRIOR,² BRUCE R. BRIGGS,¹ DAVID J. LIM,¹ AND HERBERT G. BIRCK^{1,3}

Otological Research Laboratories, Department of Otolaryngology,¹ Division of Infectious Diseases, Department of Medicine,² The Ohio State University College of Medicine, and the Department of Otolaryngology, Children's Hospital,³ Columbus, Ohio 43210

Received 9 January 1984/Accepted 16 March 1984

Endotoxin concentrations were determined in middle-ear effusions (MEEs) from 89 children with chronic otitis media by using the *Limulus* amoebocyte lysate assay. Mean concentrations of endotoxin in *Haemophilus influenzae*-positive and *Streptococcus pneumoniae*-positive MEEs were 157 and 21.8 ng/ml, respectively, and were significantly different ($P < 0.01$). Endotoxin was also found in Gram stain-positive, culture-negative and Gram stain-negative, culture-negative MEEs, but the levels were not significantly different ($P > 0.05$). However, the endotoxin concentrations in both groups of culture-negative MEEs were significantly lower than those found in MEEs that grew either *H. influenzae* or *S. pneumoniae* ($P < 0.05$). These results show that endotoxin is present in a high percentage of human MEEs, including those that are culture negative, and may contribute to the pathogenesis of otitis media with effusion.

Haemophilus influenzae is considered the most common pathogenic organism associated with chronic otitis media with effusion (OME) (4). An earlier study of experimental OME in the chinchilla (2) indicated that killed *H. influenzae* organisms, when injected into the tympanum, induced clear, amber-colored middle-ear effusions (MEEs) and that endotoxin purified from these bacteria also induced MEE. Since endotoxins are lipopolysaccharide complexes present on the outer surface of most gram-negative bacteria (7), it is possible that endotoxin associated with *H. influenzae* may be responsible for the inflammation and fluid accumulation in the middle ear (2). The present study was undertaken to determine the possible role of endotoxin in chronic OME by determining endotoxin levels in human MEEs from children with chronic OME by using the *Limulus* amoebocyte lysate (LAL) assay.

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MATERIALS AND METHODS

Patient samples. The study group consisted of 89 patients, aged 6 months to 13 years, who underwent tympanostomy tube placement because of persistent MEE.

Bacterial culture from the MEEs. MEEs were collected, processed for Gram stain, and cultured for bacteria by protocols previously described (5). Briefly, samples were transported on ice to the laboratory, and 50- μ l aliquots from each effusion were plated on chocolate and blood agar plates and used to inoculate a thioglycolate broth. Presumptive *H. influenzae* isolates were identified by their typical colonial morphology on chocolate agar. Definitive identification was determined by their growth requirements for NAD and hemin by means of X-, V-, and XV-factor disks (Difco Laboratories, Detroit, Mich.). Definitive identification of *Streptococcus pneumoniae* isolates was made by sensitivity to optochin and sodium deoxycholate. All negative cultures were maintained for 48 h before being discarded.

LAL assay for endotoxin. Samples of MEEs from the 89

patients were stored at -80°C before LAL assay for endotoxin, and all MEEs were assayed within 6 months of collection. The endotoxin content in the MEEs was determined quantitatively by the microdilution LAL assay procedure (8). The *Limulus* lysate (Pyrogen) used for these studies was obtained from a commercial source (Mallinckrodt Inc., St. Louis, Mo.), and a single lot, 2AV, was used throughout. The lysate had a minimum sensitivity of 0.05 ng/ml (reference standard endotoxin lot EC-2). The microdilution LAL assay procedure was performed as previously described (8).

Because of the limited volume of the effusions, samples were thawed and initially diluted 1:5 (vol/vol) with pyrogen-free saline so that each titration could be performed in duplicate. All materials used throughout the test procedure were pyrogen-free. Preliminary control experiments were conducted to rule out exogenous endotoxin contamination by assaying for endotoxin in sterile, pyrogen-free water that was aspirated through the surgical collection devices. In separate experiments to rule out false-positive LAL results, selected LAL-positive MEEs were boiled for 10 min at 100°C and assayed to ensure that the substrate being measured was heat-stable endotoxin and not a proteolytic substance that might be present in the MEEs. MEEs were also assayed with the addition of a polyionic dispersing agent (Pyrosperser; Mallinckrodt Inc.) to test for the presence of endotoxin-masking components. Similarly, specific amounts of *Escherichia coli* endotoxin were added to LAL-negative MEEs. These "spiked" MEEs were then assayed to determine whether there were substances in the MEEs that would interfere with the sensitivity of the LAL assay.

All LAL assays were performed without previous knowledge of the microbiological findings.

Statistical analysis. The two-tailed Student *t* test was used to detect significant differences in endotoxin concentrations.

RESULTS

Of the 89 MEEs, 71 (80%) exhibited endotoxin activity, with concentrations ranging from 0.5 to more than 512 ng/ml. With one exception, significant levels of endotoxin were present in MEEs from which *H. influenzae* were cultured.

* Corresponding author.

TABLE 1. Endotoxin concentration in culture-positive human MEEs

Bacteria isolated	No. of specimens	Endotoxin concn (ng/ml) ^a	
		Mean ± SD	Range
<i>H. influenzae</i>	20	157 ± 190	0–512
<i>S. pneumoniae</i>	20	21.8 ± 29.5	0–128

^a Relevant to endotoxin reference standard EC-2.

The mean concentration of endotoxin in *H. influenzae*-positive MEEs was 157 ng/ml (Table 1). MEEs that contained culturable *S. pneumoniae* contained a mean of 21.8 ng of endotoxin per ml, approximately sevenfold less than that measured in MEEs from which *H. influenzae* were cultured ($P < 0.01$).

Endotoxin was also present in 67% of the culture-negative MEEs (Table 2). In effusions that did not contain viable bacteria but demonstrated bacteria by the Gram stain, the mean endotoxin concentration was 3.3 ng/ml. MEEs that did not contain bacteria either by culture or Gram stain had a mean concentration of 12.9 ng/ml. The fourfold difference in the endotoxin concentrations between these two groups was not statistically significant ($P > 0.05$). However, the concentrations of endotoxin in both groups of culture-negative MEEs were significantly lower than the levels of endotoxin in the MEEs that grew either *H. influenzae* or *S. pneumoniae* ($P < 0.05$).

The control experiments showed that endotoxin titers did not change after boiling, indicating that the substrate being measured was endotoxin. The addition of the dispersing agent to the samples did not increase endotoxin concentrations, which suggests the lack of endotoxin-masking components in the MEEs. The addition of known concentrations of *E. coli* endotoxin revealed that there were no substances in the MEEs that interfered with the sensitivity of the LAL assay.

DISCUSSION

Bacterial endotoxin is a biologically active material and a potent inducer of inflammation and modulator of the immune response. In addition, endotoxin is capable of interaction with complement and components of the clotting system. Endotoxin has been shown to effect the release of various vasoactive amines and other mediators of inflammation by direct interaction with macrophages, polymorphonuclear leukocytes, and other cell types (7). Therefore, it can be postulated that endotoxin trapped in the middle ear can be responsible for the persistency of the effusions long after the gram-negative bacteria are no longer viable.

The presence of the highest concentration of endotoxin in effusions containing viable *H. influenzae* was expected since this organism is gram negative and contains endotoxin. However, it was surprising to find that the effusions which grew *S. pneumoniae*, a gram-positive bacterium that does not contain endotoxin, contained a substantial amount of endotoxin. It is documented that the bacterial species may change during the course of treatment of otitis media (3), and it is possible that gram-negative bacteria (e.g., *H. influenzae*, *Neisseria* sp., or *Pseudomonas* sp.) had originally been present in the MEEs but were no longer viable at the time of culture.

The levels of endotoxin in the culture-negative MEEs were similar, regardless of the Gram stain results. Although the reason for this discrepancy is speculative, unreported antibiotic therapy may provide an explanation. Endotoxin-

TABLE 2. Endotoxin concentration in culture-negative human MEEs

Gram stain results	No. of specimens	Endotoxin concn (ng/ml) ^a	
		Mean ± SD	Range
Positive	25	3.3 ± 5.2	0–16
Negative	24	12.9 ± 31	0–128

^a Relevant to endotoxin reference standard EC-2.

containing bacteria, once present in the middle ear, could become fragmented by antibiotic therapy, thereby releasing endotoxin. Additionally, proteolytic enzymes may be present that could degrade gram-negative bacteria, subsequently releasing endotoxin.

The significance of these findings is enhanced by the fact that as little as 1.0 ng of purified endotoxin per ml, well below the lower level of endotoxin found in human MEEs, was capable of inducing inflammation and MEE in the chinchilla model (2). Endotoxin remaining in the middle ear might play an important role in the pathogenesis of OME by sustaining the inflammatory response after the acute primary infection has cleared. An earlier study (6) showed that over 80% of the MEEs demonstrated bacteria by Gram stain, whereas only 47% yielded viable bacteria on culture. The frequency and concentration of endotoxin observed in the culture-negative human MEEs in this study support this hypothesis.

The present study indicates that 67% of the sterile MEEs contain endotoxin, which is in contrast to the findings of Bernstein et al. (1). In their study, only 7% (1 of 14) of the sterile MEEs were positive by the LAL assay. Although this discrepancy cannot be fully explained, three factors must be considered: (i) their study was qualitative and did not include titration of the endotoxin, (ii) two different patient populations were used in the studies, and (iii) different commercially available sources of *Limulus* lysate were used, which may have resulted in different sensitivities. The *Limulus* lysate used in our studies has been shown to be more sensitive to native endotoxin than lysate commercially prepared by using chloroform extraction (9).

In summary, our results indicate that endotoxin is present in a high percentage of human MEEs, including those that are culture negative, and may contribute to the pathogenesis of OME.

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LITERATURE CITED

- Bernstein, J. M., M. D. Praino, and E. Neter. 1980. Detection of endotoxin in ear specimens from patients with chronic otitis media by means of the limulus amoebocyte lysate test. *Can. J. Microbiol.* **26**:645–648.
- DeMaria, T. F., B. R. Briggs, N. Okazaki, and D. J. Lim. 1984. Experimental otitis media with effusion following middle ear inoculation of nonviable *H. influenzae*. *Ann. Otol. Rhinol. Laryngol.* **93**:52–56.
- Howie, V. M., and J. H. Ploussard. 1971. Bacterial etiology and antimicrobial treatment of exudative otitis media: relation of antibiotic therapy to relapse. *South. Med. J.* **64**:233–239.
- Lim, D. J., and T. F. DeMaria. 1982. Panel discussion: pathogenesis of otitis media. *Bacteriology and immunology. Laryngo-*

- scope 92:278-286.
5. Liu, Y. S., R. W. Lang, D. J. Lim, and H. G. Birck. 1976. Microorganisms in chronic otitis media with effusion. *Ann. Otol. Rhinol. Laryngol.* 25(Suppl.):245-249.
 6. Liu, Y. S., D. J. Lim, R. W. Lang, and H. G. Birck. 1975. Chronic middle ear effusions: immunochemical and bacteriological investigation. *Arch. Otolaryngol.* 101:278-286.
 7. Morrison, D. C., and R. J. Ulevitch. 1978. The effects of bacterial endotoxin on host mediation systems. *Am. J. Pathol.* 93:427-617.
 8. Prior, R. B., and V. A. Spagna. 1979. Adaptation of a microdilution procedure to the *Limulus* lysate assay for endotoxin. *J. Clin. Microbiol.* 10:394-395.
 9. Prior, R. B., and V. A. Spagna. 1981. Response of several *Limulus* amoebocyte lysates to native endotoxin present in gonococcal and nongonococcal urethral exudates from human males. *J. Clin. Microbiol.* 13:167-170.