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Oxidative Metabolic Products Released from Polymorphonuclear Leukocytes in Middle Ear Fluid during Experimental Pneumococcal Otitis Media

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To determine whether oxidative metabolic products of phagocytic cells are present in the middle ear during experimental pneumococcal otitis media, we measured the concentration of myeloperoxidase (MPO) in middle ear fluid (MEF) and the capacity of neutrophils isolated from MEF and peripheral blood to produce MPO and superoxide anion (O_2^-) after in vitro stimulation. Free MPO in MEF was significantly increased 24 and 48 h after either viable or nonviable pneumococci were inoculated into the middle ear. In vitro-stimulated production of MPO and O_2^- from middle ear neutrophils was significantly less than that from peripheral blood neutrophils 24 h after nonviable pneumococci were inoculated but similar to it after 48 h. Twenty-four hours after viable pneumococci were inoculated, middle ear neutrophils stimulated in vitro produced less MPO but the same amount of O_2^- as did blood neutrophils. Oxidative metabolic products, therefore, are released from phagocytic cells into the MEF during pneumococcal otitis media, and future studies will need to define the contribution of these products to acute and chronic middle ear tissue injury.

Studies of otitis media pathogenesis have explored inflammatory responses to the pneumococcus because *Streptococcus pneumoniae* is frequently the cause of acute otitis media, which initiates chronic tissue injury in some persons. Our kinetic studies of middle ear inflammation in an animal model have demonstrated increased vascular permeability for small serum proteins as early as 2 h after nonviable pneumococci are inoculated into the middle ear, followed by neutrophil influx, increasing vascular permeability for larger proteins, and lysozyme and arachidonic acid metabolites in middle ear fluid (MEF) (22, 23). The migrating neutrophils are essential elements of the pneumococcal host defense (13) and are abundant in the MEF of humans with acute purulent otitis media and in the MEF of chinchillas with experimental pneumococcal otitis media (22). The phagocytosis of bacteria by neutrophils stimulates intracellular oxidative responses, and bacteria are killed within phagosomes.

Others have shown that the intracellular oxidative reactions may also have adverse effects on local tissues (4, 16, 32). We therefore sought to determine whether middle ear neutrophils release their oxidative metabolic products into the middle ear space during the initial phase of middle ear inflammation caused by pneumococci, since the presence of these oxidative products might contribute to the pathogenesis of acute and chronic otitis media.

MATERIALS AND METHODS

A total of 46 healthy 1- to 2-year-old chinchillas weighing 400 to 600 g with normal middle ears (ascertained by otoscopy and tympanometry) were used. Eustachian tube obstruction was performed 24 h before inoculation to prevent the inoculum from flowing out the eustachian tube (5). All procedures were done with ketamine hydrochloride (20 mg/kg of body weight) intramuscular anesthesia.

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This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, National Institutes of Health guidelines (21a), and the Animal Welfare Act (Public Law 89-144, as amended). The animal use protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Encapsulated type 7F *S. pneumoniae* cells were inoculated into the peritoneal cavity of mice to enhance virulence, frozen at −70°C, and used within 2 months of mouse inoculation in all experiments. A suspension of viable pneumococci was prepared from mid-log-phase pneumococci grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) with 3.5% bovine albumin (Sigma Chemical Co., St. Louis, Mo.) and diluted to approximately 40 CFU/ml in sterile 0.01 M phosphate-buffered saline, pH 7.4 (PBS). A suspension of nonviable pneumococci was prepared by placing the twice-washed viable suspension in a boiling water bath for 10 min and then adjusting the suspension to a concentration of 2×10^8 cells per ml in PBS as previously described (22).

The nonviable suspension (0.8 ml) was inoculated directly into both middle ear hypotympanic bullae of 11 chinchillas, as previously described (22). For measuring myeloperoxidase (MPO) and O_2^- production by neutrophils, all of the MEF (approximately 700 μl per ear) and 10 ml of peripheral blood were obtained from 6 of these 11 chinchillas 24 h after inoculation and from the remaining 5 chinchillas 48 h after inoculation. The viable suspension (1.0 ml) was inoculated into both hypotympanic bullae of 22 chinchillas. All of the MEF and 10 ml of peripheral blood were obtained from eight of these animals 24 h after inoculation for measuring MPO and O_2^- production by neutrophils and from six of these animals for measuring total MPO in neutrophils. To count the number of bacteria in the MEF, the ears of the remaining eight chinchillas were aspirated 6, 18, 24, 36, and 48 h after inoculation.

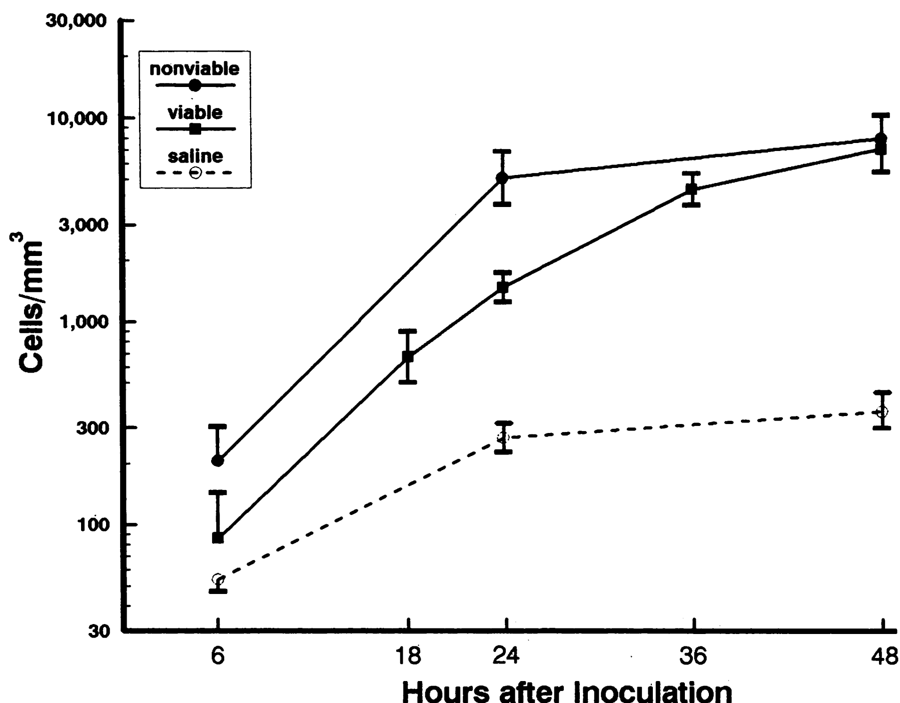

FIG. 1. Geometric mean (± standard error of the mean) number of inflammatory cells in MEF after inoculation of nonviable and viable pneumococci and saline.

Inflammatory cells in MEF were counted by using a hemocytometer. Peripheral blood was aspirated from 13 normal, uninoculated chinchillas for measuring in vitro production of MPO and O_2^- from normal neutrophils. Cultures of MEF and peripheral blood were performed by inoculating 10-μl serial dilutions of MEF or blood on 5% sheep blood agar.

MEF samples were centrifuged at 500 × *g* to isolate neutrophils. Supernatants were separated and assayed for free MPO. The cell pellet was resuspended in 0.87% ammonium chloride to hemolyze erythrocytes and then centrifuged, and the cells were washed twice in Hanks balanced salt solution containing 0.1% gelatin but without calcium and magnesium. Peripheral blood neutrophils were separated by a previously described method, which was modified by using 98% Histopaque 1077 (Sigma Chemical Co.) diluted in PBS (3, 27). The cell suspensions were diluted to 5×10^6 neutrophils per ml in Hanks balanced salt solution with 0.1% gelatin, Ca, and Mg. Cell viability was determined by trypan blue exclusion.

The ability of neutrophils obtained from MEF and peripheral blood to produce MPO in vitro was measured after neutrophils were stimulated for 15 min with 10^{-5} M (final concentration) *N*-formyl-methionyl-leucyl-phenylalanine. The neutrophil suspension was pretreated with cytochalasin B (final concentration, 5 mg/ml) for 10 min at 37°C. After incubation with *N*-formyl-methionyl-leucyl-phenylalanine for 15 min at 37°C, the supernatants were collected, and MPO activity was measured in triplicate samples by a colorimethoric method (12). Net MPO activity after the background (sodium azide pretreatment) was subtracted was expressed as units per 3.75×10^5 neutrophils relative to a lactoperoxidase standard (2.125 U/ml; Sigma) run on each plate. MPO in MEF supernatants was measured similarly.

The ability of neutrophils obtained from MEF and peripheral blood to produce O_2^- in vitro was measured by ferricytochrome *c* reduction (18, 28). The neutrophil suspension was added to ferricytochrome *c* (2.1 mg/ml, final concentration; Sigma) and stimulated with 0.2 mM (final concentration) phorbol myristate acetate (Sigma) for 15 min at 37°C. O_2^- activity was measured in triplicate samples as nanomoles of ferricytochrome *c* reduced per 2.5×10^5 neutrophils, and net O_2^- activity was reported after background activity (an identical reaction mixture with 3,000 U of superoxide dismutase [Sigma] per ml added before the neutrophil suspension) was subtracted.

To measure total MPO in neutrophils, suspensions of MEF and peripheral blood neutrophils, each containing 3.75 $\times 10^6$ cells/ml, were sonicated with a Sonifier Cell Disrupter (Branson Sonic Power Co.) at 40 W for 10 s. After centrifugation at 500 × *g*, supernatants were collected for MPO assay.

Data were analyzed for statistical significance by using the Student *t* tests for paired and nonpaired data.

RESULTS

The concentration of inflammatory cells in MEF was significantly increased in ears inoculated with nonviable and viable pneumococci compared with that in ears inoculated with saline ($P < 0.05$; Fig. 1). The modest accumulation of cells in saline-inoculated ears was caused by eustachian tube obstruction. Differences between ears inoculated with viable and nonviable bacteria were not significant. More than 95% of neutrophils from all MEF aspirations were viable.

The number of pneumococcal CFU in MEF from animals inoculated with viable pneumococci increased rapidly during the initial 18 h after inoculation (Fig. 2). Peripheral blood cultures from chinchillas inoculated with viable pneumococci were negative until 48 h after inoculation.

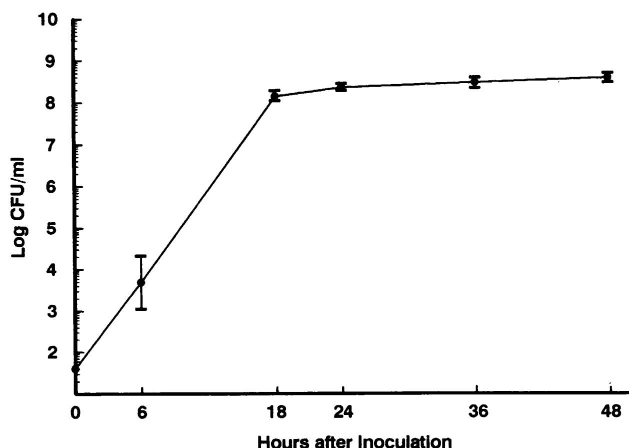

FIG. 2. Geometric mean (± standard error of the mean) concentration of viable pneumococci, measured as CFU, in MEF.

Neutrophils obtained from peripheral blood of chinchillas inoculated with viable pneumococci produced significantly more MPO, but not more O_2^-, than did blood neutrophils from healthy, uninoculated chinchillas after in vitro stimulation (Table 1). However, stimulated in vitro production of MPO and O_2^- by blood neutrophils from animals inoculated with nonviable pneumococci was not significantly different from production by neutrophils from uninoculated control animals.

Significantly higher titers of free MPO were measured in MEF 24 and 48 h after viable and nonviable pneumococci were inoculated than in MEF from saline-inoculated ears (Fig. 3). Neutrophils isolated from MEF produced both MPO and O_2^- after in vitro stimulation (Table 1). Twenty-four hours after nonviable pneumococci were inoculated, the stimulated in vitro production of both MPO and O_2^- by middle ear neutrophils was significantly lower than that by blood neutrophils from the same animals. Twenty-four hours after viable pneumococci were inoculated, the stimulated in vitro production of MPO by middle ear neutrophils was significantly lower than that by blood neutrophils, but in vitro production of O_2^- was not lower in the same animals. Forty-eight hours after nonviable pneumococci were inoculated, middle ear neutrophils produced as much MPO and O_2^- in vitro as did blood neutrophils from the same animals.

Sonicated middle ear neutrophils collected 24 h after viable-pneumococcus inoculation contained significantly less MPO (1.14 U) than did sonicated blood neutrophils (1.67 U; $P < 0.01$).

DISCUSSION

The cytotoxic effects of neutrophil metabolic products have been demonstrated in patients with rheumatoid, kidney, heart, vascular, lung, digestive tract, liver, and skin diseases (4, 10); superoxide anion may play a significant role in the ocular inflammatory response (7, 21). Experiments have also demonstrated that superoxide dismutase and oxygen free radical scavengers reduce inflammatory responses in experimental carrageenan foot edema (25), endotoxin-induced intravitreal inflammation (8), immune complex glomerulonephritis (29), and dextran- and UV-induced paw edema (2); these data, therefore, provide further evidence that neutrophil oxidative metabolites are cytotoxic. The determination of whether metabolic oxidants contribute to middle ear tissue injury in otitis media required an initial demonstration that these oxidants are present in the MEF.

Using a chinchilla model of pneumococcal otitis media, we observed that viable and nonviable pneumococci inoculated into the middle ear caused neutrophils to degranulate in the middle ear space, as evidenced by (i) high titers of free MPO in MEF, (ii) reduced MPO production by *N*-formyl-methionyl-leucyl-phenylalanine-stimulated middle ear neutrophils isolated from MEF 24 h after inoculation, and (iii) less MPO in sonicated middle ear neutrophils than in blood neutrophils obtained 24 h after inoculation. Dysfunction of exudate neutrophils, such as we observed, has been attributed to cell transformation during exudation and phagocytosis and to preferential mobilization of a neutrophil subpopulation (14). In our model, however, middle ear neutrophils recovered their ability to produce MPO in vitro 48 h after nonviable pneumococci were inoculated. Since the chemotactic stimulus persisted between 24 and 48 h, as evidenced by increasing numbers of neutrophils in the MEF during this interval, the mobilization of neutrophils did not appear to change in concert with improved MPO production. Thus, cell transformation during exudation and phagocytosis probably explained the acute decline in the MPO-producing capacity of middle ear neutrophils.

Superoxide anion production by middle ear neutrophils was also affected by pneumococcal inoculation, suggesting that O_2^- was also released into the middle ear space. However, viable and nonviable pneumococci seemed to have different effects on O_2^- production by neutrophils. Twenty-four hours after nonviable pneumococci were inoculated, the O_2^--producing capacity of middle ear neutrophils was reduced, whereas viable pneumococci had no apparent effect on O_2^- production by middle ear neutrophils sampled at this time. This discordant response to viable and nonviable pneumococci might have been due to greater neutrophil receptor availabilities for the heat-altered pneumococcal

TABLE 1. Geometric mean concentrations (95% confidence intervals) of MPO and O_2^- released from chinchilla polymorphonuclear leukocytes (PMNL) in peripheral blood and MEF after inoculation of encapsulated nonviable or viable pneumococci into the middle ear

Inoculum	Time (h) after inoculation	MPO release[a] (U/3.75 × 10^5 PMNL)		O_2^- release[b] (nM/2.5 × 10^5 PMNL)	
		Peripheral blood	MEF	Peripheral blood	MEF
Nonviable	24	0.65 (0.41, 0.90)	0.44[c] (0.11, 0.76)	6.27 (6.02, 6.52)	2.53[c] (2.25, 2.82)
Nonviable	48	0.46 (0.29, 0.63)	0.50 (0.35, 0.66)	5.36 (5.21, 5.51)	5.76 (5.71, 5.81)
Viable	24	1.02[d] (0.95, 1.08)	0.61[c] (0.43, 0.80)	6.37 (6.28, 6.45)	6.01 (5.86, 6.16)
None		0.49[d] (0.33, 0.66)		4.86 (4.75, 4.96)	

[a] In vitro MPO release after stimulation of polymorphonuclear leukocytes with *N*-formyl-methionyl-leucyl-phenylalanine.
[b] In vitro O_2^- release after stimulation of polymorphonuclear leukocytes with phorbol myristate acetate.
[c] $P < 0.05$.
[d] $P < 0.001$.

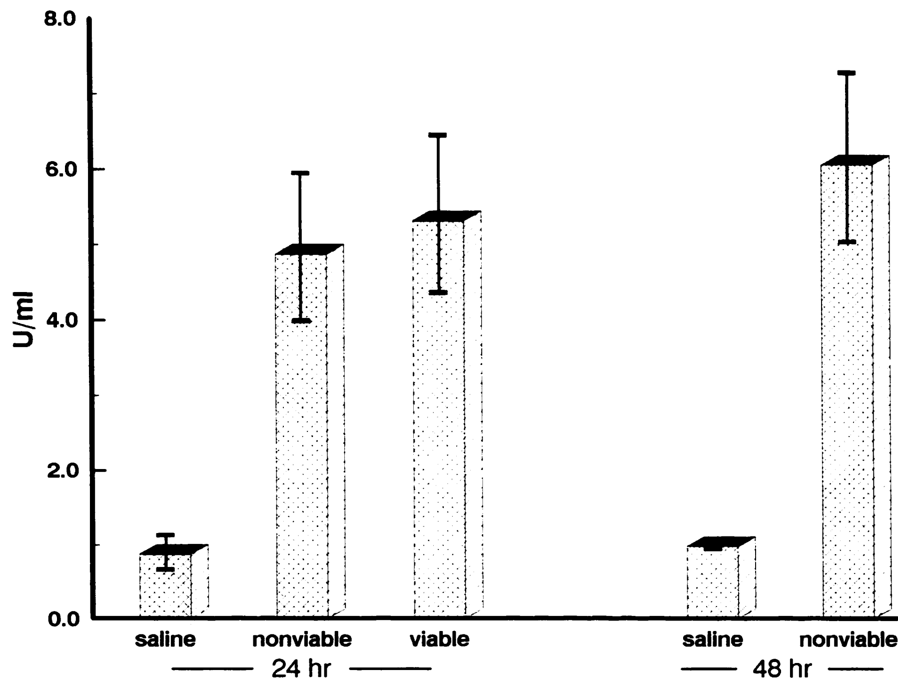

FIG. 3. Free MPO in MEF 24 and 48 h after inoculation of nonviable pneumococci and saline and 24 h after inoculation of viable pneumococci. Data are geometric means ± standard errors of the means.

surface than for the native surface. Capsular polysaccharide may have interfered with pneumococcal attachment to the surface or protein kinase C receptors on neutrophils, and heat may have altered the character permitting this attachment.

The discrepancy between levels of MPO and O_2^- production by middle ear neutrophils may be explained by characteristics of the stimuli used to elicit MPO and O_2^- responses. Others have shown that particulate and soluble stimuli elicit different responses from the proximal NADPH-oxidase metabolic cascade (17) and different O_2^- production capacities of guinea pig peritoneal exudate neutrophils (33). Alternatively, O_2^- release from middle ear neutrophils may have occurred in vivo before the 24-h sampling time.

This evidence of neutrophil degranulation in the middle ear during experimental pneumococcal otitis media expands our understanding of middle ear inflammation and is consistent with the early accumulation of neutrophil-derived lysozyme and arachidonic acid metabolites in MEF previously reported in this model (22–24). The reduced capacity of stimulated middle ear neutrophils to produce oxidative metabolites compared with that of blood neutrophils from the same animals is consistent with studies of exudate neutrophils in other species. Abscess-derived neutrophils from mice, for example, were less able than blood neutrophils to phagocytose and kill gram-negative bacilli in vitro (11), and a similar observation was made in rabbits (1). Likewise, zymosan-stimulated O_2^- production by casein-elicited peritoneal exudate guinea pig neutrophils was significantly decreased compared with that by blood neutrophils (33). In contrast, rabbit neutrophils harvested from 7-day-old sterile wounds had increased O_2^- and hydrogen peroxide production after in vitro stimulation with phorbol myristate acetate, *N*-formyl-methionyl-leucyl-phenylalanine, and zymosan compared with that of blood neutrophils (26), perhaps reflecting the different chemotactic stimuli. Further study of exudate neutrophils, especially in humans, is necessary to complement the extensive literature that has documented altered blood neutrophil function and oxidative product formation during acute bacterial infections (6, 9, 15, 19, 20, 30, 31).

In summary, neutrophil metabolic products, including lysozyme, arachidonic acid metabolites, and oxidants, are present in the middle ear space during the early phase of pneumococcal otitis media. Future experiments are needed to demonstrate the relative contributions of these products to acute and chronic middle ear tissue injury.

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