Penicillin Treatment Accelerates Middle Ear Inflammation in Experimental Pneumococcal Otitis Media

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Received 2 December 1991/Accepted 18 February 1992

Most Streptococcus pneumoniae strains are killed by very low concentrations of penicillin and other β-lactam antibiotics, yet middle ear inflammation and effusion persist for days to weeks after treatment in most cases of pneumococcal otitis media. To study the effect of β-lactam antibiotic treatment on pneumococci and the middle ear inflammatory response during pneumococcal otitis media, we measured concentrations of pneumococci, inflammatory cells, and lysozyme in middle ear fluid (MEF) by using the chinchilla model. Procaine penicillin G given intramuscularly 12 and 36 h after inoculation of pneumococci into the middle ear caused a significant acceleration in the MEF inflammatory cell concentration compared with that in untreated controls, with a significant peak in the inflammatory cell concentration 24 h after pneumococcal inoculation. The lysozyme concentration in MEF also increased more rapidly in treated than in control animals. Viable pneumococci were not detected in MEF after the second dose of penicillin, but the total pneumococcal cell concentration remained unchanged for at least 45 days. Therefore, penicillin treatment accelerated middle ear inflammation while killing pneumococci, but treatment did not accelerate clearance of the nonviable pneumococcal cells from MEF. Further studies will need to define the contribution of these responses to acute and chronic tissue injury.

Studies of otitis media pathogenesis have explored inflammatory responses to pneumococci because Streptococcus pneumoniae is frequently the cause of acute otitis media, which leads to chronic tissue injury in some persons. Kinetic studies of middle ear inflammation in an animal model have demonstrated increased middle ear vascular permeability and accumulation of lysozyme and arachidonic acid metabolites in middle ear fluid (MEF) as soon as 2 h after inoculation of nonviable pneumococci into the middle ear (14, 17). Oxidative metabolic products were released from phagocytic cells into MEF as soon as 24 h after inoculation of nonviable as well as viable pneumococci (9).

These inflammatory responses to pneumococci were initiated by pneumococcal envelope components, especially the wall cell, which lies beneath the capsule (3, 18, 23, 24). Treatment with β-lactam antibiotics significantly increased inflammation in experimental pneumococcal meningitis because inflammatory cell wall debris was released (22). We sought, therefore, to determine whether penicillin treatment affects the middle ear inflammatory response during pneumococcal otitis media, since products of inflammation, especially oxidative and nonoxidative bactericidal products from neutrophils, might contribute to acute and chronic otitis media pathogenesis.

MATERIALS AND METHODS

This study was performed in accordance with the Public Health Service Policy on Human Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (Public Law 89-144, as amended); the animal use protocol was approved by the Institutional Animal Care and Use Committee.

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

Encapsulated type 7F pneumococci were grown to the mid-log phase and diluted to approximately 40 CFU/ml in sterile 0.01 M phosphate-buffered saline (pH 7.4) as described previously (9). A volume of 1 ml of the pneumococcal inoculum was placed directly into both middle ear hypotympanic bullae by passing a catheter through the cephalad bullae as described previously (14). Intramuscular procaine penicillin G (50,000 U/kg; Eli Lilly & Co., Indianapolis, Ind.) was given to 14 animals 12 and 36 h after pneumococcal inoculation (treated group). Another 10 chinchillas were not treated (untreated group).

MEF (50 μl) and 1 ml of peripheral blood were sampled at 0, 6, 12, 18, 24, 36, and 48 h after pneumococcal inoculation from 10 treated chinchillas and 10 untreated chinchillas. MEF and peripheral blood were also sampled from these treated chinchillas at 4, 10, 23, and 45 days after inoculation. MEF (20 μl) was aspirated from the remaining four treated chinchillas at 6 h and every 2 h between 18 and 32 h after inoculation.

Quantitative culturing of MEF and peripheral blood was performed by inoculating 10-μl aliquots of serial MEF dilutions on 5% sheep blood agar. Inflammatory cells in MEF were counted by use of a hemacytometer. The number of pneumococcal cells in MEF was enumerated microscopically in a Petroff-Hauser chamber.

MEF samples obtained from treated chinchillas 45 days after pneumococcal inoculation were stained immunohistochemically to identify pneumococci in MEF. Approximately 15 μl of MEF was placed on a cleaned glass slide, air dried, fixed with 100% ethanol for 1 min, and stained with a specific monoclonal antibody (D3114/63; immunoglobulin M isotype; kindly provided by Larry McDaniel, Birmingham, Ala.) against pneumococcal teichoic acid (11) and fluorescein...
isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Tago Immunologica1s, Burlingame, Calif.).

MEF samples were centrifuged at 500 × g, and supernatants were collected for lysozyme quantitation by a modified 18-h enzymatic assay (20). Samples were frozen at −70°C, and all samples were assayed simultaneously. The reaction mixture contained a *Micrococcus lysodeikticus* suspension, 0.1% sodium azide (Sigma Chemical Co.), and 1.0 mg of bovine albumin (Sigma) per ml in phosphate-buffered saline, to which was added the sample; the mixture was then incubated at 37°C for 18 h. The *M. lysodeikticus* suspension was prepared in 0.05 M phosphate buffer to an optical density at 450 nm of approximately 1.0. The optical density was measured at 450 nm, and the net optical density was calculated by subtracting the optical density of an identical mixture lacking the MEF sample. The lysozyme concentration was expressed as units per milliliter of sample relative to a standard (200 U/ml; from human milk; Sigma). In this assay, 1 U of lysozyme activity per ml corresponded to 6 ng of lysozyme activity per ml measured by the Quantiplate method (Kallestad, Austin, Tex.).

Data were analyzed for statistical significance by use of Student’s *t* test (two-tailed) for paired and nonpaired data.

**RESULTS**

Inflammatory cells gradually accumulated in MEF from untreated chinchillas beginning 18 h after pneumococcal inoculation, with a continued linear increase through 48 h (Fig. 1). All 10 untreated animals died between days 3 and 7 with pneumococccemia. More inflammatory cells accumulated in MEF from penicillin-treated animals than in MEF from untreated animals as soon as 18 h after inoculation (6 h after the first penicillin dose) (Fig. 1). At 24 h after inoculation (12 h after the first penicillin dose), there were significantly more inflammatory cells in MEF from penicillin-treated animals than in MEF from untreated animals (*P* < 0.05); however, the response was quite variable among animals whose MEF was sampled frequently between 18 and 32 h after inoculation. The peak inflammatory cell response occurred, on the average, 28 h after pneumococcal inoculation (16 h after the first penicillin dose) (Fig. 2). The second dose of penicillin, 36 h after inoculation, did not cause a similar acceleration in the inflammatory cell response.

In untreated animals, the lysozyme concentration in MEF increased logarithmically between 12 and 24 h after inoculation and reached a plateau between 24 and 48 h (Fig. 3). The MEF lysozyme concentration in treated animals was significantly higher 18 h after pneumococcal inoculation (6 h after penicillin treatment) than that in untreated animals (*P* < 0.001) (Fig. 3). This lysozyme peak occurred about 6 h before the peak inflammatory cell response to penicillin.

The number of viable pneumococci in MEF from untreated animals increased logarithmically during the first 18 h after inoculation and thereafter reached a plateau of about 10⁵ CFU/ml. Penicillin treatment caused an abrupt reduction in the number of viable pneumococci, and all ears yielded sterile MEF 12 h after the second penicillin dose (Fig. 4).

The total pneumococcal cell count in MEF from untreated animals increased during the first 24 h after inoculation, parallel to the increase in the number of viable pneumococci. Penicillin treatment at 12 h arrested pneumococcal growth but did not reduce the number of nonviable pneumococcal cells, which were detected in MEF as late as 45 days after inoculation (Fig. 5). Immunofluorescence staining of MEF sampled at 45 days clearly demonstrated intact pneumococcal cells.

**DISCUSSION**

Pneumococci remain the most common bacteria causing acute otitis media (4). They are the most frequently cultured bacteria from MEF in cases of acute otitis media, and pneumococcal capsular polysaccharide has been detected in as many as 50% of acute otitis media MEF samples, including 36% of culture-negative samples (10). Evidence for the
Persistence of pneumococcal cells in chronic otitis media with effusion is found in reports demonstrating gram-positive diplococci in culture-negative chronic effusions (6). These pathogen-positive effusions had more neutrophils than pathogen-negative effusions (6).

Nonviable pneumococci and the pneumococcal cell wall induce inflammation in rabbit cerebrospinal fluid (23, 24) and in the chinchilla middle ear (3, 14, 16, 18), with increased concentrations of inflammatory cells, especially neutrophils, and lysozyme within 48 h after pneumococcal inoculation.

β-Lactam antibiotic treatment of experimental pneumococcal meningitis caused a significant increase in the concentration of cerebrospinal fluid inflammatory cells (22), and ceftriaxone treatment of experimental *Haemophilus influenzae* type b meningitis caused greatly increased concentrations of endotoxin and tumor necrosis factor alpha in cerebrospinal fluid; in both experimental models, inflammatory reactions were suppressed by dexamethasone (12).

Using the chinchilla pneumococcal otitis media model, we observed that penicillin treatment caused a significant accel-
ACCELERATION OF MIDDLE EAR INFLAMMATION BY PENICILLIN

FIG. 4. Geometric mean (± standard error of the mean) number of viable pneumococci in ears after inoculation of pneumococci. Solid line, ears treated with penicillin at 12 and 36 h after pneumococcal inoculation; broken line, untreated ears.

eration in the middle ear inflammatory reaction, as evidenced by high lysozyme and inflammatory cell concentrations in MEF 6 to 12 h after the first dose of penicillin. The accelerated inflammatory response suggests that some pneumococci in the middle ear space were lysed by penicillin. The persistence of pneumococci in MEF indicates that not all pneumococci were lysed. Lysis failure may have been (i) due to an inability of chinchilla phagocytic cells to clear killed pneumococci, an observation previously made for rabbit neutrophils (21), (ii) due to an inadequate penicillin concentration that did not exceed the bactericidal concentration for the organism for a sufficient period, or (iii) because the middle ear environment affected pneumococcal autolysin activity.

Middle ear inflammatory responses cause neutrophils to release their oxidative products into MEF, as previously described (9), and these products may further aggravate middle ear tissue injury. Lysozyme accumulation in MEF preceding significant inflammatory cell influx has been observed before in this model (15). Since epithelial cells are a

FIG. 5. Geometric mean (± standard error of the mean) total pneumococcal cell count in ears after inoculation of pneumococci. Solid line, ears treated with penicillin at 12 and 36 h after pneumococcal inoculation; broken line, untreated ears.
source of lysozyme (7), it seems reasonable that the early release of lysozyme represents middle ear epithelial cell injury by pneumococci.

If antibiotic-induced pneumococcal lysis and cell wall debris accumulation occur in humans as in chinchillas, down-modulation of the inflammatory response should be further investigated for otitis media as it has for meningitis. A small study with chinchillas revealed less middle ear mucoperiosteal histopathology in animals with pneumococcal otitis media and treated with penicillin plus corticosteroid versus penicillin alone (8). Clinical trials with systemic corticosteroid treatment for chronic otitis media with effusion, a condition that often follows antibiotic treatment of acute otitis media, have revealed a short-term beneficial effect of treatment (1, 5, 13, 17, 19). Future studies will need to better define the inflammatory pathways leading to antibiotic-induced inflammation and the relative inflammatory activities of different antibiotic classes and to study the use of concurrent antibiotic and anti-inflammatory drug treatment of acute otitis media as well as of nonsteroidal anti-inflammatory drugs.

ACKNOWLEDGMENT

This study was supported by grant P50-DC00133 from the National Institute on Deafness and Other Communication Disorders.

REFERENCES