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Roles of Autolysin and Pneumolysin in Middle Ear Inflammation Caused by a Type 3 *Streptococcus pneumoniae* Strain in the Chinchilla Otitis Media Model

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Streptococcus pneumoniae **cell wall and pneumolysin are important contributors to pneumococcal pathogenicity in some animal models. To further explore these factors in middle ear inflammation caused by pneumococci, penicillin-induced inflammatory acceleration was studied by using three closely related pneumococcal strains: a wild-type 3 strain (WT3), its pneumolysin-negative derivative (P-1), and its autolysin-negative derivative (A-1). Both middle ears of chinchillas were inoculated with one of the three pneumococcal strains. During the first 12 h, all three strains grew in vivo at the same rate, and all three strains induced similar inflammatory cell responses in middle ear fluid (MEF). Procaine penicillin G was given at 12 h to one-half of the animals in each group, and all treated chinchillas had sterile MEF at 24 h. Penicillin significantly accelerated MEF inflammatory cell influx into WT3- and P-1-infected ears at 18 and 24 h in comparison with the rate for penicillin-treated A-1-infected ears. Inflammatory cell influx was slightly, but not significantly, greater after treatment of WT3 infection than after treatment of P-1 infection. Interleukin (IL)-1β and IL-6, but not IL-8, concentrations in MEF at 24 h reflected the penicillin effect on MEF inflammatory cells; however, differences between treatment groups were not significant. Results suggest that pneumococcal otitis media pathogenesis is triggered principally by the inflammatory effects of intact and lytic cell wall products in the middle ear, with at most a modest additional pneumolysin effect. Investigation strategies that limit the release of these products or neutralize them warrant further investigation.**

Streptococcus pneumoniae strains are principal contributors to acute otitis media (AOM) and chronic otitis media with effusion (OME). Pneumococci have been cultured from approximately 40% of middle ear fluid (MEF) samples from children with AOM (4, 10) and 7% of MEF samples from children with chronic OME (4). Moreover, pneumococci were observed by Gram stain in 17% of sterile MEF samples from chronic OME cases (12), and pneumococcal capsular polysaccharide antigens and pneumococcal DNA have been detected in sterile MEF from chronic OME and AOM cases (21, 40).

Pneumococcal pathogenicity is not fully understood, but cell wall (7, 31, 36–38), pneumolysin (2, 3, 8, 17, 22, 28, 30), and autolysin (2, 6, 22) are major candidates for virulence factors. Beta-lactam antibiotic treatment accelerated type 3 *S. pneumoniae*-induced inflammation in the rabbit meningitis model (36) presumably because treatment lysed pneumococci, releasing cell envelope fragments such as cell wall or cytoplasm products such as pneumolysin into the surrounding environment. We observed a similar acceleration of inflammation in the chinchilla otitis media (OM) model using type 3 and 7F *S. pneumoniae* (19, 34). Timing of penicillin treatment with regard to bacterial concentration in MEF was a critical determinant of the middle ear (ME) inflammatory response (34).

In the present study, two insertion-inactivation derivatives of a type 3 *S. pneumoniae* strain and the wild-type parent strain were used to further explore the pathogenicity of pneumococcal cell wall, pneumolysin, and autolysin in vivo and in vitro. One derivative strain lacked pneumolysin production, and the other lacked autolysin production. All three strains have been previously characterized and used in pathogenesis studies in a murine peritonitis model (2).

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MATERIALS AND METHODS

Three closely related type 3 *S. pneumoniae* strains were used in this study: a pneumolysin-negative insertion-inactivation derivative (P-1), an autolysin-negative insertion-inactivation derivative (A-1), and their wild-type parent (WT3). The strains were kindly provided by James C. Paton, Department of Microbiology, Women's and Children's Hospital, North Adelaide, Australia. Bacteria were stored at −70°C in 60% Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 30% bovine serum albumin and 10% glycerol. Pneumococci were prepared by inoculating aliquots of the frozen bacterial suspensions on 5% sheep blood agar plates and incubating the plates overnight at 37°C in 10% CO_2; 10 CFU was inoculated into 10 ml of Todd-Hewitt broth enriched with 0.5% yeast extract (Difco Laboratories) (THY) and incubated in a 37°C water bath. Optical density at 660 nm (OD_{660}) was measured every hour from 3 to 7 h after inoculation, and the concentration of viable pneumococci (CFU per milliliter) was measured by quantitative culturing on sheep blood agar.

The penicillin MICs for the three pneumococcal strains were measured by the microdilution method, and the bactericidal effect of penicillin was studied by using five concentrations of penicillin (0.5, 1, 2, 4, and 8 times the MIC), added after 4 h of growth in THY. To measure the in vitro effects of penicillin on growth and cell lysis, the OD_{660} and viable-pneumococcus CFU per milliliter were measured for 4 h after penicillin addition, and the ratios of OD and $\log_{10}$ CFU per milliliter to pretreatment values were calculated; analysis of variance (ANOVA) for repeated measures was used to compare differences in the penicillin effects on pneumococcal growth and lysis.

For in vivo experiments, 69 healthy adult chinchillas weighing 400 to 600 g with normal MEs, as ascertained by otoscopy and tympanometry, were used. Eustachian tube obstruction was performed 24 h before inoculation to prevent the inoculum from flowing out of the Eustachian tube (5). All procedures were performed on ketamine hydrochloride-anesthetized animals and were performed

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in accordance with the Public Health Service *Policy on Human Care and Use of Laboratory Animals*, the National Institutes of Health *Guide for 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 of the University of Minnesota. Animals were divided into the following six groups: three penicillin treatment groups inoculated with WT3 (n = 12), P-1 (n = 12), or A-1 (n = 12) and three control groups not treated with penicillin (WT3, n = 11; P-1, n = 11; A-1, n = 11). Pneumococcal suspensions were grown to mid-log phase in THY and diluted in 0.01 M phosphate-buffered saline (pH 7.4) to approximately 40 CFU/ml. The actual inoculum was confirmed by performing quantitative cultures of the inoculum. A volume of 1 ml of the pneumococcal inoculum was placed directly into both ME hypotympanic bullae (25). Procaine penicillin G (50,000 U/kg of body weight; Crysticillin 300 A. S.; E. R. Squibb & Sons Inc., Princeton, N.J.) was given intramuscularly 12 h after bacterial inoculation. Control groups were inoculated with pneumococci but not treated.

MEF (50 μl) was sampled every 6 h between 6 and 36 h. Quantitative MEF cultures were performed on sheep blood agar. Inflammatory cells in MEF samples were counted with a hemocytometer. Differential cell enumeration was not performed, since we previously demonstrated that polymorphonuclear leukocytes (PMNL) predominate in MEF during the first 72 h after pneumococcal inoculation (25). MEF samples were centrifuged at 500 × g and frozen for biochemical analysis. Lysozyme in MEF was assayed by a modified 18-h enzymatic assay (19). Concentrations of interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) in MEF were measured by using high-sensitivity human IL-1β, IL-6, and TNF-α enzyme-linked immunoabsorbant assay (ELISA) kits (Quantikine; R & D Systems, Minneapolis, Minn.). The IL-8 concentration in MEF was measured by using a standard sensitivity human IL-8 ELISA kit (Quantikine; R & D Systems). Detection thresholds in a sterile chinchilla MEF matrix blank were as follows: IL-1β, 0.0625 pg/ml; IL-6, 0.078 pg/ml; IL-8, 46.875 pg/ml; and TNF-α, 0.25 pg/ml.

Statistics. Bacterial (CFU per milliliter), inflammatory cell (cells per cubic millimeter), and lysozyme (units per milliliter) concentrations in MEF were log transformed, and differences among groups and over time were analyzed for statistical significance by using ANOVA for repeated measures. Geometric-mean (GM) data are reported. The inflammatory cell concentration change after penicillin treatment was analyzed by two-way ANOVA with the log ratios of 18-h/12-h and 24-h/12-h concentrations, followed by separate Student's t tests when interaction was significant. Concentrations of IL-1β, IL-6, IL-8, and TNF-α in MEF (picograms per milliliter) 24 h after pneumococcal inoculation were log transformed and analyzed by two-way ANOVA. MEF with undetected cytokine was assigned a value one-half of the detection threshold for that cytokine. Correlation coefficients were calculated to compare inflammatory cell and cytokine concentrations and to study relationships among cytokines after aggregating all 24-h MEF samples from the six experimental groups.

RESULTS

In vitro growth rates of the three pneumococcal strains in THY were compared through log phase (5 h) by comparing the areas under the 2- to 5-h OD_{660} curve (OD_{AUC}) and the areas under the 0- to 5-h $\log_{10}$ CFU-per-milliliter curve (CFU_{AUC}) from five replicate experiments. The A-1 strain grew significantly more slowly (mean OD_{AUC} = 0.660; mean CFU_{AUC} = 8.775) than did the WT3 strain (mean OD_{AUC} = 0.867, $P < 0.001$; mean CFU_{AUC} = 9.336, $P = 0.008$) or the P-1 strain (mean OD_{AUC} = 0.862, $P < 0.001$; mean CFU_{AUC} = 9.113, $P = 0.031$); the WT3 and P-1 strains had similar growth kinetics (OD_{AUC}, $P = 0.88$; CFU_{AUC}, $P = 0.09$). Because reduction of A-1 growth was parallel in the OD and CFU determinations, it is unlikely that the longer chain length of the A-1 strain explained the growth difference. Penicillin MICs for all three strains were 0.015 μg/ml. Penicillin concentrations between one and eight times the MIC had similar effects on growth and lysis of the WT3 and P-1 strains but a greatly reduced effect on both growth and lysis of the A-1 strain (Fig. 1).

After in vivo inoculation of pneumococci into chinchilla MEs, the concentration of pneumococci in chinchilla MEF increased from 31 to 52 CFU/ml at inoculation to 110 to 590 CFU/ml at 12 h in all six experimental groups. There was no significant difference in pneumococcal concentration at 12 h among the six groups ($F = 1.05$, $P = 0.392$). All treated chinchillas had sterile MEF within 24 h after the penicillin dose (Fig. 2). After penicillin injection, there was no significant difference in pneumococcal concentration among the three treated groups ($F = 0.90$, $P = 0.411$) or among the three untreated groups ($F = 0.26$, $P = 0.773$).

The concentration of inflammatory cells in MEF increased in all six groups between 6 and 24 h after bacterial inoculation (Fig. 3). Inflammatory cell concentrations prior to penicillin injection at 12 h were significantly different among the three pneumococcal strain groups ($F = 3.408$, $P = 0.036$) because of a trend for more cells in the WT3 group (GM = 195 cells per mm^3) than in the P-1 group (GM = 123 cells per mm^3) ($t = 1.851$, $P = 0.067$), but there was no significant difference between WT3 and A-1 (GM = 155 cells per mm^3) or between P-1 and A-1 groups.

Penicillin injection significantly accelerated inflammatory cell influx in WT3- and P-1-infected ears but not in A-1-infected ears (Fig. 3). Compared with results for no treatment, penicillin significantly increased the posttreatment-to-pretreatment inflammatory cell ratios in WT3-infected ears at 24 h ($t = 2.726$, $P = 0.009$) and had a marginal effect at 18 h ($t = 1.779$, $P = 0.082$). Compared with results for no treatment, the penicillin effect in P-1-infected ears at 18 h ($t = 1.222$, $P = 0.228$) and 24 h ($t = 1.384$, $P = 0.174$) did not reach significance. Penicillin treatment appeared to reduce the ME inflammatory cell concentration in A-1-infected ears, although differences between treated and untreated groups were not significant at 18 h ($t = 1.819$, $P = 0.076$) or at 24 h ($t = 1.369$, $P = 0.178$).

Analysis among penicillin-treated groups revealed similar inflammatory cell ratios in WT3- and P-1-infected ears at 18 h ($t = 0.048$, $P > 0.9$) and 24 h ($t = 0.646$, $P > 0.5$), but the WT3-infected penicillin-treated group had significantly higher inflammatory cell ratios than did the A-1 treated group at 18 h ($t = 2.680$, $P = 0.010$) and at 24 h ($t = 1.207$, $P = 0.032$). Similarly, the P-1 treated group had significantly higher inflammatory cell ratios than did the A-1 treated group at 18 h ($t = 2.497$, $P = 0.016$) but not at 24 h ($t = 1.562$, $P = 0.125$). Among the three untreated groups, inflammatory cell increases between 6 and 24 h were not significantly different ($F = 0.02$, $P > 0.9$).

The lysozyme concentration in MEF increased in all six groups between 6 and 24 h from an average of 521 to 1,949 U/ml, but penicillin treatment did not accelerate the accumulation of lysozyme in any of the three treated groups relative to levels in the respective untreated control groups (data not shown).

IL-1β, IL-6, IL-8, and TNF-α were detected in 40.3, 43.1, 98.4, and 3.9%, respectively, of MEF samples obtained 24 h after ME inoculation (12 h after treatment). The percentages of MEF samples with detectable IL-1β, IL-6, and IL-8 were not significantly different among the six groups. Aggregate GMs from all three groups of untreated chinchillas were as follows: IL-1β, 0.33 pg/ml; IL-6, 0.10 pg/ml; and IL-8, 141.71 pg/ml. Mean IL-1β and IL-6 concentrations were higher in penicillin-treated than control ears of WT3- and P-1-infected but not A-1-infected animals, although the differences were not significant (Fig. 4). Differences between bacterial inoculum groups for each cytokine were not significant among treated or untreated chinchillas. The inflammatory cell concentration in MEF was significantly correlated with concentrations of IL-6 ($r = 0.660$, $P = 0.001$) and IL-8 ($r = 0.322$, $P = 0.010$) but not IL-1β ($r = 0.169$, $P = 0.221$). The MEF IL-6 and IL-8 concentrations were significantly interrelated ($r = 0.487$, $P = 0.030$), but concentrations of IL-1β and IL-6 ($r = 0.315$, $P = 0.218$) and IL-1β and IL-8 ($r = 0.132$, $P = 0.613$) were not significantly correlated.

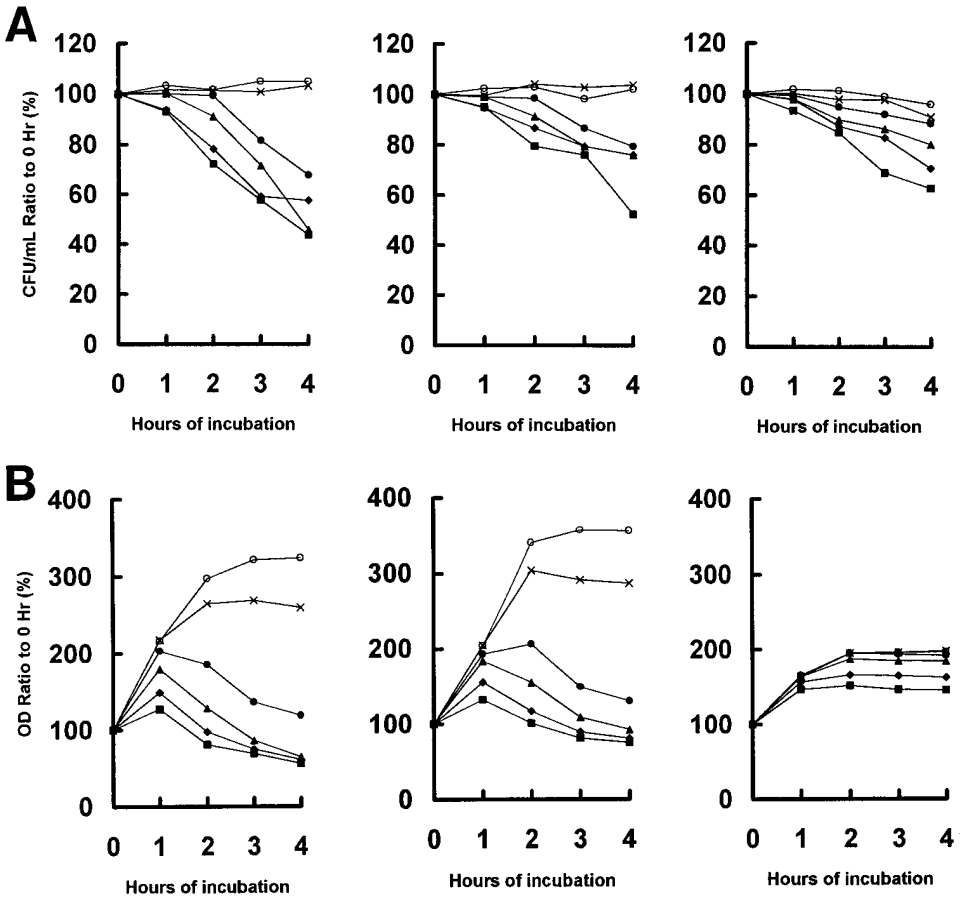

FIG. 1. (A) Ratio of mean $\log_{10}$ CFU per milliliter of viable type 3 pneumococci after penicillin administration in vitro; (B) ratio of mean OD_{660} after penicillin administration in vitro. (Left) WT3; (center) P-1; (right) A-1. Open circles, control; crosses, 0.5 times the MIC; closed circles, 1 times the MIC; closed triangles, 2 times the MIC; closed diamonds, 4 times the MIC; closed squares, 8 times the MIC.

DISCUSSION

S. pneumoniae strains have two bactericidal targets for penicillin, one that releases autolysin and another, autolysin-independent mechanism (24). The experiments described here show that in vitro, penicillin had a significantly smaller effect on growth and lysis of an autolysin-negative pneumococcal isogenic derivative strain (A-1) than it did on its autolysin-sufficient parent strain (WT3) and its isogenic pneumolysin-negative derivative strain (P-1). However, penicillin also affected growth and lysis of the A-1 derivative, probably by the autolysin-independent mechanism. Others have shown similar penicillin effects with an autolysin-negative derivative of the R6 *S. pneumoniae* strain (24, 33).

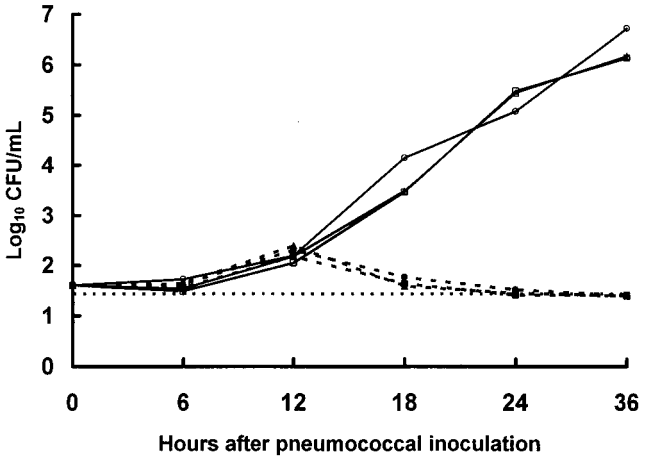

FIG. 2. Log_{10} mean CFU per milliliter of viable type 3 pneumococci in MEF after chinchilla ME inoculation. Solid lines, chinchillas treated with penicillin 12 h after pneumococcal inoculation; broken lines, untreated controls (circles, WT3; triangles, P-1; squares, A-1). The dotted line represents the assay detection threshold of 25 CFU/ml.

The relative contributions of pneumococcal cell envelope and cytoplasmic components to inflammation have been studied in vivo. Our present experiments with the chinchilla ME suggest that pneumococcal cell wall contributes more significantly to ME inflammation than does pneumolysin. During the first 12 h after bacterial inoculation, all three strains grew in MEF at equal rates. Although ANOVA suggested a trend for more inflammatory cells in MEF of WT3- than P-1-infected ears, the difference was very small. Thus, neither pneumolysin nor autolysin contributed to the inflammatory cell response during the first 12 h of ME infection. Friedland et al. (9) also observed than an insertion-inactivation pneumolysin-negative derivative type 2 pneumococcal strain (PLN-A) caused meningeal inflammation indistinguishable from that caused by its parent type 2 strain (D39), although recombinant pneumolysin and two pneumolysin toxoids injected intracisternally into the rabbit meningeal space produced meningeal inflammation. We previously showed that both heat-inactivated intact pneumococcal cells and isolated native cell wall, specifically, teichoic

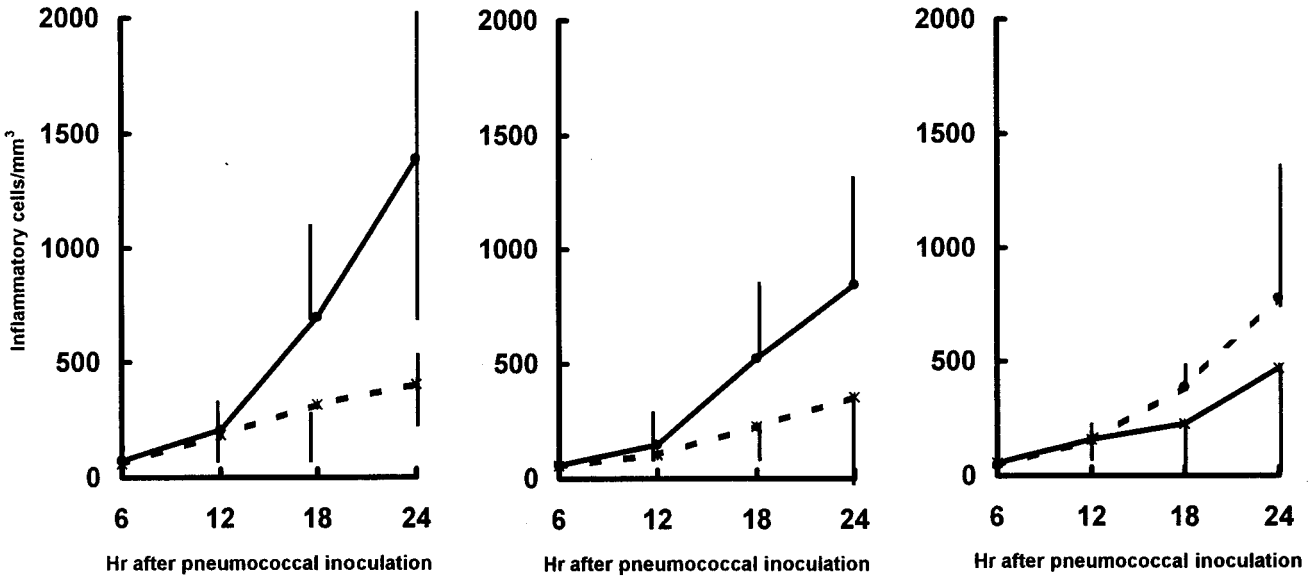

FIG. 3. GM numbers of inflammatory cells in chinchilla MEF after inoculation of type 3 pneumococci (± standard errors [error bars]). Solid lines, chinchillas treated with penicillin at 12 h after pneumococcal inoculation; broken lines, untreated controls. (Left) WT3; (center) P-1; (right) A-1.

acid-containing fragments, induced ME inflammation in the chinchilla model (7, 25, 31). In view of this, ME inflammation induced by the A-1 strain in our present experiment was probably due to the strain's intact wall. Similar experiments with the rabbit meningitis model suggested that pneumococcal wall is an important inflammatory stimulus in the meningeal space, as well (37, 38).

In vivo lysis of the WT3 and P-1 strains by penicillin in our present experiment induced similar increases in levels of MEF inflammatory cells, further evidence that pneumolysin does not have an appreciable effect on ME inflammation. Autolysin, however, had a significant effect, since penicillin treatment increased MEF inflammatory cells in ears infected with strains containing autolysin (WT3 and P-1) but not in ears infected with the autolysin-negative derivative, A-1.

In contrast to both the OM and meningitis models, a pneumolysin effect was demonstrated in rat and murine lungs with the wild-type 2 pneumococcus but not its PLN-A derivative (6, 32). These differences among models may be pneumococcal strain or serotype or animal species specific, since we have not been able to induce ME inflammation in chinchillas using either the D39 or the PLN-A strain (data on file but not reported). It will be important to study the type 3 parent and derivative strains in the rabbit meningitis and rat or murine lung models.

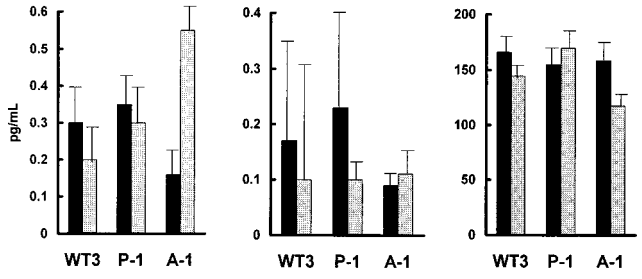

FIG. 4. GM concentrations of cytokines in chinchilla MEF sampled 24 h after inoculation of type 3 pneumococci (± standard errors [error bars]) (12 h after penicillin treatment). (Left) IL-1β; (center) IL-6; (right) IL-8. Dark bars represent penicillin-treated animals; light bars represent untreated animals.

The contribution of pneumolysin, a thiol-activated intracellular cytolysin, to pneumococcal disease pathogenesis has been suggested by the increased survival time of pneumolysin-immunized mice following pneumococcal intranasal challenge (28). Pneumolysin also had toxic effects in rat lungs (8) and rabbit corneas (17) in vivo, it lysed human PMNL and platelets (16), and it inhibited the PMNL respiratory burst in vitro (27). Mice injected intraperitoneally with the pneumolysin-negative type 3 *S. pneumoniae* derivatives survived longer than mice injected with the respective wild type (2); similar reduction of virulence was observed by survival time after intranasal and intraperitoneal challenge with type 2 *S. pneumoniae* and its pneumolysin-negative derivative (3). Damage to the human respiratory mucosa was greater with the pneumolysin-sufficient strain than with the pneumolysin-negative strain (30), suggesting that pneumolysin contributes to pneumococcal virulence.

Lysozyme activity in MEF was not accelerated by penicillin administration in our present experiment with type 3 strains as we previously demonstrated with a type 7F strain (19). Reduced lysozyme levels in MEF may be explained by a different interaction between ME epithelium and the type 3 versus type 7F strain, since ME epithelium is a known source of lysozyme (26). This theoretical difference in modes of epithelial interaction may also explain the lower virulence level of type 3 pneumococci (11).

Cytokine contribution to ME inflammation has been studied by using MEF obtained from children with chronic OME (13, 18, 23, 41, 42). MEF IL-1β (41) and IL-6 (42) concentrations were inversely correlated with age in children with chronic OME, and IL-1β levels were higher in purulent than serous or mucoid MEF (13, 18). IL-8 transcripts were detected in 75% of MEF samples from children and adults with chronic OME (35). MEF TNF-α concentrations were directly correlated with age of children with chronic OME (41), and both IL-1β and TNF-α concentrations in MEF were directly correlated with the concentration of IL-8 (23). Pneumolysin stimulated the

production in vitro of IL-1β and TNF-α from human monocytes (14).

MEF cytokine concentrations in chinchilla OM models have only recently been investigated. The presence of IL-1β in MEF after pneumococcal inoculation was related to its presence in MEF before inoculation (15). In the experiments described here, IL-1β and IL-6, but not IL-8, concentrations in MEF 24 h after bacterial inoculation directly paralleled the penicillin effect on MEF inflammatory cell influx. However, the penicillin effects on IL-1β and IL-6 were not as significant as was the effect on inflammatory cells, suggesting that MEF sample timing missed an earlier or later peak concentration of these proinflammatory cytokines or that other PMNL chemoattractant mediators were more active in the ME. Studying synthesis of these cytokines in ME resident and inflammatory cells, perhaps by using murine IL-1β, IL-6, and IL-8 reagents, will be useful in further characterizing mechanisms of PMNL influx into the ME and in understanding the relative contributions of pneumococcal virulence factors.

We observed that MEF inflammatory cell, IL-6, and IL-8 concentrations were significantly correlated. Others have reported that rates of IL-6 and IL-8 production are parallel (29, 39), and IL-8 is a known neutrophil chemoattractant (29). In recent experiments using the same chinchilla OM model, we observed 72 h after pneumococcal inoculation that the MEF inflammatory cell concentration correlated with concentrations of IL-1β in addition to IL-6 and IL-8; at this later stage of ME inflammation, there are significant relationships between IL-1β and IL-6 and between TNF-α and IL-8 (34a).

Because lytic cell wall products significantly accelerated chinchilla ME inflammation in the absence of pneumolysin, AOM treatment strategies should include both rapid elimination of bacteria from the ME cavity and attenuation of the ME inflammatory response. Reducing ME inflammation during antimicrobial treatment may be accomplished by strategies that limit the release of cell wall products, such as nonlytic antibiotics or autolysin immunization. Exogenous antiautolysin antibody blocked autolysin activity in vitro, and autolysin immunization prolonged survival in mice challenged intraperitoneally with a type 2 strain (D39) but not with the pneumolysin-negative derivative (PLN-A), suggesting that the protective effect of autolysin was due to the autolysin-induced release of pneumolysin (22). Immunization with a pneumolysin toxoid vaccine increased survival of mice infected with nine pneumococcal serotypes, suggesting that pneumolysin toxoid should be included in a future pneumococcal vaccine (1). A conjugated type 19F polysaccharide-pneumolysin toxoid vaccine was immunogenic and protective against septicemia in a murine model (20). Therefore, autolysin may be no more effective than pneumolysin as a protective antigen in a protein-conjugated pneumococcal vaccine.

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