CONCISE COMMUNICATION

Effects of Antibiotic Class on the Macrophage Inflammatory Response to *Streptococcus pneumoniae*

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Antibiotic choice can alter host inflammation during invasive bacterial infections. Previous studies of gram-negative organisms concluded that antibiotic-mediated release of bacterial cell wall components amplifies inflammation. Less has been reported about antibiotic effect on gram-positive organisms. This study explored the hypothesis that *Streptococcus pneumoniae* would induce greater macrophage inflammatory mediator production when killed with cell wall active antibiotics rather than protein synthesis inhibitors. Stimulation of RAW 264.7 murine macrophages with pneumococci and oxacillin led to significantly higher inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF) accumulation than did the same concentrations of pneumococci and clindamycin. Neither antibiotic alone or in combination with lipopolysaccharide acted directly on macrophages to modify the immune response. Endotoxin contamination did not confound the results, as preincubation with polymyxin B did not change iNOS or TNF protein levels. Thus, the antimicrobial mechanism of action affects macrophage inflammatory mediator production after stimulation with pneumococci.

Antibiotics differ considerably in their mechanisms of action, which in turn affect bacterial viability, toxin release, and post-antibiotic effect. The β-lactam agents—penicillins and cephalosporins—are rapidly bactericidal and enhance cell wall component release from both gram-negative [1] and gram-positive bacteria [2]. Antibiotics that act by bacterial protein synthesis inhibition, such as clindamycin and erythromycin, are generally bacteriostatic and do not induce as much cell wall breakdown as their β-lactam counterparts [3].

Liberated bacterial cell wall elements can induce a potent host inflammatory response. Previous work in this area has focused on gram-negative bacterial components, specifically endotoxin. Lipopolysaccharide (LPS) up-regulates the production of proinflammatory cytokines in mononuclear cells in vitro [4] and in animal models [5]. Endotoxin injection into human volunteers leads to significant elevations in tumor necrosis factor (TNF), interleukin (IL)–6, IL–8, and IL–1 receptor antagonist as well as clinical evidence of sepsis: temperature dysregulation, hypotension, and leukocytosis [6].

Less is known regarding the inflammatory response to gram-positive cell wall. In experimental models, sterile site instillation of gram-positive cell wall components induces signs of meningitis, shock, and multorgan system failure [7, 8]. Purified gram-positive bacterial peptidoglycan fragments augment TNF release in human monocytes [9]. Furthermore, exposure to lipoteichoic acid, which is found only in gram-positive bacterial cell walls, increases macrophage production of TNF as well as nitric oxide (NO) [10].

Previous studies have shown that antibiotic choice can affect the inflammatory response after bacterial exposure. In a rabbit model of *Escherichia coli* meningitis, cefotaxime therapy induced greater endotoxin release and brain edema than did the protein synthesis inhibitor chloramphenicol [11]. Peripheral blood monocytes exposed to *Staphylococcus epidermidis* treated with penicillin or oxacillin produced more TNF than they did in response to the same strain treated with vancomycin or clindamycin [2]. An experimental murine pneumococcal meningitis model determined that lipoteichoic acid release and early mortality were increased with ceftriaxone rather than with rifampin [12]. In addition, certain antimicrobials have a direct immunomodulatory effect on host cells [13].

The high-output NO pathway plays a key role in the pathogenesis of sepsis; the inducible NO synthase (iNOS) enzyme is its rate-limiting step. We previously showed that RAW 264.7 murine macrophages stimulated with oxacillin-killed *Streptococcus pneumoniae* or purified pneumococcal cell wall induced iNOS protein, NO, and TNF production [14]. In this study, we compared iNOS and TNF production by RAW 264.7 macrophages after stimulation with 2 isolates of pneumococci and...
antibiotics acting by cell wall lysis (e.g., oxacillin) or by protein synthesis inhibition (e.g., clindamycin).

Materials and Methods

Reagents. Dulbecco’s MEM (DMEM) was purchased from Mediatech (Herndon, VA). L-glutamine was purchased from Gibco (Grand Island, NY). Fetal bovine serum containing <0.06 EU/mL by limulus amebocyte assay was purchased from HyClone Laboratories (Logan, UT). Pneumococcal strain 6303 (serotype 3) was purchased from American Type Culture Collection (ATCC; Rockville, MD). Pneumococcal strain 124 (serotype 23), a clinical isolate from a child with sepsis, was provided by J. Shene (St. Jude Children’s Research Hospital, Memphis). Lipopolysaccharide purified from E. coli O111:B4 was purchased from Sigma (St. Louis). Mouse recombinant (r) interferon (IFN)-γ was purchased from Genzyme (Cambridge, MA). Polymyxin B (PMB) was purchased from Pfizer (New York). Oxacillin, clindamycin, and cefotaxime were purchased from Sigma. Chloramphenicol was obtained from Parke-Davis (Morris Plains, NJ).

Cells and cell culture. RAW 264.7 cells were purchased from ATCC and cultured in antibiotic-free DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Experiments were done in 6-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) with 4.00–4.79 × 10^4 cells/well. Cells were stimulated with the appropriate reagents for 18 h. Negative controls of media alone and positive controls of LPS (10 ng/mL) plus rIFN-γ (10 U/mL) were used. All wells exposed to pneumococci were also stimulated with rIFN-γ (10 U/mL) as we previously showed that macrophage iNOS induction by pneumococci requires the presence of IFN-γ [14]. Antibiotics were added at the same time as live pneumococcal isolates. Concentrations of antibiotics were chosen to approximate achievable human serum levels at conventional dosing and greatly exceeded the MICs of the isolates (tube dilution MICs of both isolates were ~2.0 µg/mL for clindamycin and ~0.1 µg/mL for oxacillin).

Immunoblotting. For Western blotting, cells were lysed in extraction buffer (20 mM Tris, 100 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_2VO_3, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). Lysates, 100 µg of protein each, were electrophoresed on 7.5% SDS polyacrylamide gels, then transferred to nitrocellulose membranes and blocked overnight in 5% milk. Nitrocellulose membranes were reacted with mouse monoclonal antibody to macrophage iNOS (Transduction Laboratories, Lexington, KY). Blots were then reacted with a sheep anti-mouse IgG peroxidase-linked conjugate (Amersham, Arlington Heights, IL). Proteins were detected by enhanced chemiluminescence (Amersham). Proteins were quantitated by densitometry using the BioRad Quantity One software package and model GS-700 Imaging Densitometer (BioRad, Hercules, CA) as specified by the manufacturer.

Measurement of TNF concentrations. Cell-free supernatants were plated on a solid-phase sandwich ELISA specific for murine TNF as specified by the manufacturer (Genzyme, Endogen [Woburn, MA] or R&D Systems [Minneapolis]). Comparisons between the amounts of TNF secretion after exposure to each cell wall lytic agent and after each protein synthesis inhibitor were always made on the same plate to account for manufacturer variation.

Statistical analysis. Paired t tests were used to compare the iNOS and TNF levels produced in response to cell wall lytic antibiotics and protein synthesis inhibitors. For the comparison between oxacillin and clindamycin, the data from 14 experiments were expressed as mean percent of oxacillin control ± SE. The effects of other antibiotics were analyzed similarly from 4 or 5 experiments. P < .05 was considered significant.

Results

Pneumococci exposed to oxacillin stimulated greater iNOS production than pneumococci exposed to clindamycin. Live pneumococci of each strain at concentrations of 10^5–10^6 cfu/mL and rIFN-γ (10 U/mL) were added to RAW 264.7 cells. Oxacillin (40 µg/mL) or clindamycin (10 µg/mL) was added at the same time as the bacteria. Neither antibiotic alone or in combination with rIFN-γ induced the RAW cells to produce iNOS. Very little iNOS was detected after stimulation with 10^4

Figure 1. Oxacillin (Oxa) or clindamycin (Cm) was added at the same time as live pneumococci (PN) and recombinant interferon (rIFN)-γ to RAW 264.7 cells, then incubated 18 h (n = 14). With 10^5 cfu/mL of PN and either antibiotic, inducible nitric oxide synthase (iNOS) protein production was undetectable. At 10^6 cfu/mL of PN, iNOS production was significantly greater with the addition of Oxa vs. the addition of Cm (P < .003). At 10^6 cfu/mL of PN, the addition of Cm yielded greater iNOS protein than did the addition of Oxa (not significant). Neither Oxa nor Cm alone induced detectable iNOS production. Positive control, lipopolysaccharide (LPS) plus rIFN-γ. A. Representative experiment using strain 124. B. Cumulative data for both isolates compares mean percentage difference ± SE, with Oxa as standard. *P < .05.
Oxacillin (Oxa) or clindamycin (Cm) was added at the same time as live pneumococci (PN) and recombinant interferon (IFN)-γ to RAW 264.7 cells, then incubated 18 h (n = 14). At each PN concentration (10^3–10^6 cfu/mL), the addition of Oxa induced significantly more tumor necrosis factor (TNF) secretion than did the addition of Cm (P < .02). A, Representative experiment using strain 6303. B, Cumulative data for both isolates comparing mean percentage difference ± SE, with Oxa as standard. LPS, lipopolysaccharide. *P < .05.

Consistently, at 10^4–10^5 cfu/mL of pneumococci, addition of oxacillin led to significantly greater macrophage iNOS production than addition of clindamycin (P < .003). At 10^6 cfu/mL of pneumococci, there was no statistical difference in the amount of iNOS protein induced by the presence of oxacillin or the presence of clindamycin (figure 1). Results with both isolates were comparable.

In parallel experiments, iNOS production was measured after stimulation by pneumococci with cefotaxime (25 μg/mL) or chloramphenicol (10 μg/mL). Results with cefotaxime were analogous to those with oxacillin: Organisms exposed to cephalosporin rather than clindamycin induced significantly greater iNOS production at 10^3–10^6 cfu/mL (P < .02) but not at 10^8 cfu/mL. Likewise, addition of chloramphenicol induced less detectable iNOS protein accumulation at the 2 lower pneumococcal concentrations than either β-lactam agent (data not shown).

Pneumococci exposed to oxacillin stimulated greater TNF production than pneumococci exposed to clindamycin. RAW macrophages accumulated TNF in a dose-dependent fashion in response to increasing concentrations of pneumococci (10^3–10^6 cfu/mL) in the presence of either antibiotic. However, addition of oxacillin led to significantly greater TNF production at all concentrations of pneumococci than did clindamycin (P < .02) (figure 2). Results with both isolates were similar.

TNF production was also measured in RAW cells after stimulation by pneumococci in the presence of cefotaxime or chloramphenicol with the same antibiotic concentrations as in the iNOS experiments. Addition of cefotaxime led to significantly higher amounts of TNF at 10^3–10^6 cfu/mL of pneumococci than did addition of clindamycin (P < .04). TNF production by macrophages stimulated with pneumococci in the presence of chloramphenicol was less than that observed in the presence of oxacillin or cefotaxime, but the difference was not significant (data not shown).

**Immunomodulatory effect of tested antimicrobials on macrophage iNOS or TNF production.** To ascertain if any of the antibiotics directly affected macrophage iNOS or TNF production, RAW cells were stimulated overnight with 10–100 ng/mL of LPS in the presence of rIFN-γ (10 U/mL) alone or in combination with each antibiotic at the same concentrations as in the pneumococcal experiments. All of these experimental conditions induced a marked macrophage iNOS and TNF response. There was no difference in the production of either inflammatory mediator between the antibiotic-free control and any of the antibiotics (not shown).

**Production of macrophage inflammatory mediators in response to pneumococci was not due to endotoxin contamination.** In concurrent experiments, macrophages were pretreated for 1 h with PMB, a potent endotoxin inhibitor. There was no decrement in the amount of iNOS or TNF accumulation after stimulation of the RAW cells with pneumococci in the presence of PMB, as we previously demonstrated [14].

**Discussion**

Here we show that murine macrophages exposed to pneumococci produce more iNOS protein and TNF in the presence of oxacillin, a cell wall active antibiotic, than with clindamycin, a protein synthesis inhibitor. Of interest, the difference in TNF production was observed at all concentrations of pneumococci tested (10^3–10^6 cfu/mL), while the difference in iNOS protein accumulation was observed only at lower bacterial concentra-
tions ($10^3$–$10^5$ cfu/mL). There was a trend toward greater iNOS induction in the clindamycin group at the highest concentration of pneumococci tested ($10^6$ cfu/mL) for both isolates, although this was not statistically significant. The reasons for this discrepancy are unknown but provide additional evidence that TNF and iNOS/NO production are differentially regulated in macrophages.

Results with other tested antibiotics were comparable. Both cell wall lytic agents tested, oxacillin and cefotaxime, induced marked release of inflammatory mediators. The accumulation of both iNOS and TNF in cells exposed to pneumococci in the presence of the protein synthesis inhibitor chloramphenicol was generally less than cells stimulated with pneumococci and the β-lactams, similar to the findings with clindamycin.

The differences in macrophage inflammatory mediator accumulation in response to pneumococci killed with different classes of antibiotics may be related to the amount of cell wall release. Tuomanen et al. [7] suggested that components of the pneumococcal cell wall play a critical role in the induction of host inflammation. Stuertz et al. [15] reported that ceftiraxone and meropenem induced greater pneumococcal cell wall product release than did antibiotics with alternative mechanisms, such as rifampin, trovafloxacin, and quinupristin/dalfopristin. Furthermore, in experimental pneumococcal meningitis, Trostdorff et al. [16] found that ceftiraxone augmented lipoteichoic acid release and TNF production, compared with quinupristin/ dalfopristin. Alternatively, the discrepancy in macrophage mediator production could be related to an Eagle effect as Stevens et al. [17] found in experimental models of Streptococcus pyogenes infection that compared penicillin and clindamycin.

Differences in host inflammatory response could also be related to a direct modulatory effect of the antibiotic on the macrophage. A large body of evidence indicates that antimicrobials may have nonspecific effects on inflammation (both proinflammatory and anti-inflammatory) [13]. Stevens et al. [18] reported that human mononuclear cells stimulated with LPS induced 60%–70% less TNF when clindamycin was added to cell culture, while penicillin had no effect. In our model, we found no evidence that any of the tested antibiotics directly affected macrophage production of either TNF or iNOS.

Conventional wisdom states that we must treat life-threatening bacterial infections with the antimicrobial that most rapidly clears the offending organism. However, therapy with many bactericidal agents also increases the magnitude of the host inflammatory response. In this study, we found that this is indeed the case for macrophage accumulation of iNOS and TNF after exposure to S. pneumoniae. While normal host defense against bacterial invasion depends on the regulated production of these mediators and others—the pathophysiologic cascade leading to clinical sepsis occurs when inflammatory pathways are unhindered. It is possible that alternative treatment regimens, such as combinations of antibiotics of different classes or antibiotics with adjuvant anti-inflammatory drugs, might curb the uncontrolled host response. At minimum, further studies of the effect of antibiotic mechanism on the inflammation triggered during severe pneumococcal infections are warranted.

Acknowledgments

We thank Steven Buckingham for help with statistical analysis, Elizabeth Meals for excellent technical support, and Geli Gao, Tom Penfound, and Jerry Shenep for MIC determinations.

References


