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Production of Interleukin-1 but Not Tumor Necrosis Factor by Human Monocytes Stimulated with Pneumococcal Cell Surface Components

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While there is considerable evidence that both interleukin-1 (IL-1) and tumor necrosis factor (TNF) are central mediators of inflammation caused by gram-negative bacteria and endotoxin, the roles of these two mediators in gram-positive infection are unknown. Pneumococcal infections are characterized by an intense inflammatory reaction in infected tissues. Current evidence suggests that the component of the pneumococcus which causes this inflammation in many body sites is the cell wall. We determined the ability of native pneumococcal cell wall, lipoteichoic acid, and cell wall subcomponents to stimulate secretion of IL-1 and TNF from human monocytes. Each pneumococcal cell surface component was found to have a different specific activity for induction of IL-1. Teichoication was an important determinant of this activity: teichoicated species were at least 10,000-fold more potent than endotoxin and 100-fold more potent than teichoic acid-free peptidoglycan. IL-1-inducing activity was greatly reduced by chemical alteration of the teichoic acid. In contrast to endotoxin, cell wall did not induce production of TNF. This dissociation of the production of IL-1 and TNF during the response of the human monocyte to pneumococcal surface components suggests that, in at least some circumstances, the mechanisms for generation of an inflammatory response to infection may be fundamentally different between gram-positive and gram-negative disease.

Pneumococcal infections are characterized by an intense inflammatory reaction in infected tissues. The deleterious effects of this phenomenon are illustrated by the fact that, in pneumococcal meningitis, the inflammatory mass in the subarachnoid space correlates directly with the morbidity and mortality of disease (12). The evidence is strong that the pneumococcal components responsible for the generation of this inflammatory response lie underneath the capsule. Instillation of the pneumococcal cell wall or lipoteichoic acid (Forssman antigen) into the subarachnoid space of rabbits recreates the entire symptom complex of meningitis, while the capsule is relatively inactive (23, 25). Similar evidence has been gathered for pneumonia (24), otitis media (17), and vasculitis (purpura) associated with sepsis (5).

The mechanisms by which pneumococcal cell surface components interact with host defenses are only beginning to emerge. The gram-positive cell wall is composed of a peptidoglycan backbone and a covalently linked teichoic acid. Both the pneumococcal peptidoglycan and teichoic acid and the lipid-linked teichoic acid congener, Forssman antigen, fix complement via the alaternative pathway in vitro (26, 27), and complement plays a role in inflammation due to these components in the lung and the central nervous system (22, 24). Recent studies in animal models suggest that the pneumococcal surface interacts with the arachidonic acid cascade since inhibitors of the cyclo-oxygenase pathway inhibit the generation of inflammation in the subarachnoid space, while lipoxygenase inhibitors are effective in the lung and ear (17, 21, 24). However, we have not been able to demonstrate direct stimulation of the arachidonate pathway in macrophages by pneumococcal surface components in vitro (20).

Cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor (TNF), have been shown to be central mediators of inflammation stimulated by gram-negative endotoxin in vitro and in vivo (2, 6). IL-1 and TNF have been detected in the course of pneumococcal meningitis in humans (13). We have demonstrated that these cytokines can induce meningeal pathology when tested in a rabbit model of meningitis and that antibodies to these two cytokines can significantly down-modulate inflammation in pneumococcal meningitis (K. Saukkonen, S. Wolpe, B. Sherry, A. Cerami, E. Tuomanen, manuscript in preparation). This suggests that at least two cytokines play a role in inflammation during invasive gram-positive infection. In vitro studies exploring induction of cytokine production by gram-positive bacterial components have been limited. One published report has suggested that a heterogeneous mixture of teichoic acid-free peptidoglycan precursors which is secreted from penicillin-treated *Streptococcus faecalis* can induce IL-1 production in human monocytes (8). Although such fragments are produced only by the small subset of gram-positive bacteria which are tolerant to penicillin (4, 9) (i.e., most gram-positive bacteria respond to penicillin by cell wall degradation and lysis rather than by tolerance and secretion of murein precursors), this observation suggests that at least some gram-positive cell wall components can incite secretion of cytokines from the monocyte. Muramyl dipeptide, a structural component of pneumococcal peptidoglycan, also induces IL-1 production (1). As a first step to understanding how cytokines are generated during gram-positive infections, we investigated the ability of the human monocyte to secrete IL-1 and TNF in response to a series of well-characterized, inflammatory, pneumococcal surface components which are relevant to in vivo infection. Major advances in the purification and structural identification of the pneumococcal cell wall have occurred recently (7). Using these techniques, we prepared and tested the abilities of

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native pneumococcal cell wall, lipoteichoic acid, and purified cell wall subcomponents to stimulate production of IL-1 and TNF from human monocytes. We report that pneumococcal cell surface components had different specific activities for IL-1 induction. In this regard, teichoicated species appeared to be more potent than endotoxin. However, in contrast to endotoxin, cell wall did not directly induce production of TNF.

MATERIALS AND METHODS

Bacterial components. Unencapsulated *S. pneumoniae* R6 was used for all studies (23). Heat-killed bacteria, crude cell wall, and purified Forssman antigen were prepared from cultures growing logarithmically in choline-containing chemically defined medium as described previously (23, 25). Crude cell wall was also prepared from cells in which the choline of the teichoic acid was biosynthetically replaced by ethanolamine (23). Choline-containing cell wall was further fractionated into teichoic acid-linked and teichoic acid-free peptidoglycan by affinity chromatography (7). The teichoicated fragments were separated by size, and the fragment of the largest size representative of 70% of the teichoic acid of native cell wall was chosen for study. Teichoic acid-free peptidoglycan was composed of a mixture of dissacharide peptide monomers, dimers, and trimers containing <2% teichoicated material (7).

Whole bacteria were washed and suspended in pyrogen-free saline for further testing. Cell wall fractions were lyophilized and suspended in pyrogen-free saline by sonication in a water bath before testing. All cell wall preparations were documented to contain <10 ng of endotoxin in solutions of 10 μg of wall per ml by the *Limulus* lysate test (Sigma Chemical Co., St. Louis, Mo.). Endotoxin from *Escherichia coli* (strain O111:B4; Ribi ImmunoChem, Hamilton, Mont.) was suspended in pyrogen-free saline by sonication in a water bath. Lipoteichoic acid from *S. sanguis* was prepared as described before (23), lyophilized, and suspended in pyrogen-free saline. Monoclonal antibody TEPC-15 against choline was generously provided by M. Potter, National Institutes of Health, Bethesda, Md.

Isolation and culture of human monocytes. Blood was drawn from normal healthy volunteers with informed consent. Mononuclear cells were isolated by Ficoll-Hypaque separation (LymphoPak; Nycamed, Oslo, Norway) and cultured in petri dishes for 1 h at 5×10^6 cells per ml of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) with 20% fetal calf serum (M. A. Bioproducts, Walkersville, Md.) and the supplements minimum essential medium-penicillin/streptomycin, minimum essential medium-nonessential amino acids, and minimum essential medium-sodium pyruvate (GIBCO) (13). After a wash with at least 10 culture volumes of RPMI 1640, adherent cells were cultured in serum-free RPMI, supplemented as above. Bacteria or cell wall fragments were added to the cultures in a volume of 10 μl. Tests always included a standard challenge with 1 μg of endotoxin. To reduce interference with IL-1 production by inhibitors such as prostaglandins generated by the monocytes during the stimulation, assays were performed in the presence of 5×10^6 M indomethacin (Sigma) (I. Sahdev, R. O'Reilly, and M. Hoffmann, Blood, in press). After 24 h, the culture supernatants were collected and stored at −20°C until they were assayed for IL-1 or TNF activities. Each titration of stimulatory activity of a given pneumococcal component was tested in at least four independent monocyte challenges.

Assay for IL-1. IL-1 was quantitated in monocyte culture supernatants by the lymphocyte activating factor (LAF) assay in the absence of exogenous stimulus (13; Sahdev et al., in press). Each culture supernatant was tested at multiple dilutions in 96-well cell culture plates (Becton Dickinson Labware, Oxnard, Calif.) and each dilution was tested in triplicate in at least two independent tests. The uptake of tritiated thymidine (Dupont, NEN Research Products, Boston, Mass.) in 24 h by the mouse thymocytes was determined by harvesting cells with an LKB cell harvester and counting in an LKB Betaplate counter. Control wells containing recombinant IL-1α (rIL-1α) (0.1 to 10^5 U; courtesy of P. Lomedico, Hoffmann-La Roche, Inc., Nutley, N.J.) and no stimulus (background) were included in each test series. Specificity of the test for IL-1 was assured by the fact that >99% of the uptake of tritiated thymidine stimulated by rIL-1 was inhibited by the addition of monoclonal anti-IL-1α and -β antibodies (courtesy of R. Chizzonite, Hoffmann-La Roche). The assay was not responsive to rIL-6.

Assay for TNF. Quantitation of TNF in monocyte supernatant fluids was performed by using the TNF kill-sensitive and kill-resistant L929 cell lines as described previously (3). Samples were added to cells in 96-well culture plates and incubated for 18 h in the presence of actinomycin D (1 μg/ml) (18). Uptake of a mitochondrial dye (MTT) was assessed by using a standard enzyme-linked immunosorbent assay reader (14).

RESULTS

Pneumococci and pneumococcal cell surface components induce IL-1 release by human monocytes. Intact pneumococci and pneumococcal cell wall or lipoteichoic acid induced large amounts of IL-1 production by human peripheral monocytes. Detection of induction of IL-1 secretion in response to intact pneumococci required stimulation of the monocytes by a threshold concentration of 10^5 intact cells

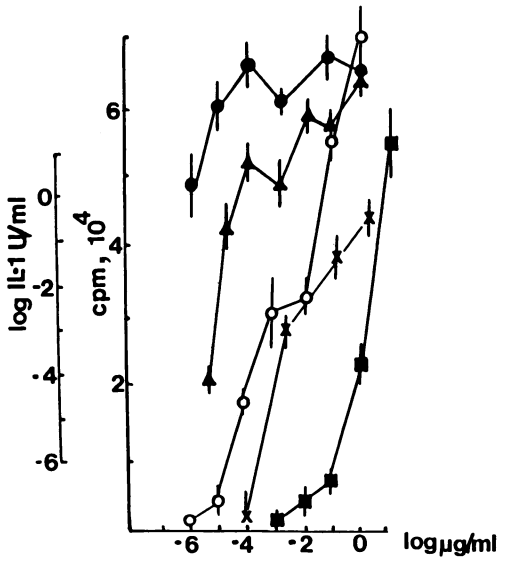

FIG. 1. Stimulation of IL-1 production from human monocytes by bacterial surface components: mean ± standard deviation of titration of IL-1 activity in monocyte supernatant fluids diluted 1:8. Symbols: X, intact pneumococci, 10^7 cells = 1 μg of wall; 0, lipopolysaccharde; ●, teichoicated peptidoglycan; ▲, teichoic acid-free peptidoglycan; ■, ethanolamine-containing teichoicated peptidoglycan. Counts per minute were converted to IL-1 units by comparison with standard curve of rIL-1 run with each experiment.

TABLE 1. Relative ability to induce IL-1 in vitro

Component	Endpoint of activity[a]	
	pg/ml	Cell equivalents[b] per ml
Lipopolysaccharide	1	10^3
Intact pneumococcus		10^4
Cell wall		
Teichoicated fragments	0.0001	1
Peptidoglycan monomers	0.01	10^2
Ethanolamine-teichoicated fragments	1,000	10^4
Lipoteichoic acid		
Pneumococcus	1	
S. sanguis	>1,000	

[a] Concentration of bacterial component at which ability to induce IL-1 was lost.
[b] Number of whole bacteria equivalent to the concentration shown in preceding column.

per ml (10^{-2} μg of cell wall per ml) (Fig. 1 and Table 1). The specific activities of purified pneumococcal cell wall components differed greatly depending on the chemical species tested (Fig. 1 and Table 1). Purified teichoicated cell wall had the highest specific activity, greatly exceeding those of endotoxin and intact pneumococci. This activity was attributable to the cell wall fragment and not to a bioactive contaminant for three reasons. (i) Biosynthetic substitution of ethanolamine for choline in the teichoic acid of identical preparations dramatically reduced bioactivity (Fig. 1). (ii) Monoclonal antibody directed against the choline moiety inhibited (>80%) IL-1 production stimulated by either wall or lipoteichoic acid-containing choline; the activity for ethanolamine wall or endotoxin was unchanged. (iii) Soluble peptidoglycan components >98% free of teichoic acid demonstrated bioactivity 100-fold less than teichoicated cell wall, a value still 100-fold greater than for endotoxin. The residual activity of this preparation may be due to the 1 to 2% contamination by teichoic acid-containing species.

The pneumococcal lipid-linked teichoic acid congener Forssman antigen caused increases in IL-1 production by monocytes similar to those seen with endotoxin (1 μg/ml caused maximal incorporation; loss of activity was seen at 1 pg/ml) (Table 1). In contrast, the structurally distinct lipoteichoic acid of *S. sanguis* (polyglycerophosphate rather than ribitol-containing backbone) was relatively inactive (no IL-1 release detectable at 1 ng [Table 1] or 1-μg/ml challenge; maximal IL-1 release at 10 μg/ml).

The ability of supernatant fluids to stimulate tritiated thymidine uptake by thymocytes was >97% inhibited by monoclonal antibody to rIL-1β (Table 2). Only a modest reduction was achieved by addition of antibody to rIL-1α. Complete inhibition of activity of the supernatant fluid was achieved by addition of both antibodies.

TABLE 3. Relative ability to induce TNF in vitro

Component	Endpoint of activity[a]	
	pg/ml	Cell equivalents per ml
Lipopolysaccharide		
Cell wall		
Teichoicated fragment	$>10^7$	$>10^8$
Peptidoglycan monomers	$>10^7$	$>10^8$
Ethanolamine-teichoicated fragment	$>10^7$	$>10^8$
Intact pneumococcus		10^6

[a] See Table 1, footnotes *a* and *b*.

Absence of TNF production by monocytes stimulated with pneumococcal cell surface components. When monocytes were challenged with whole bacteria, TNF production was detected, but only at cell densities above 10^6 cells per ml (1.0 μg of wall per ml) (Table 3). Even at concentrations as high as 10 μg of purified teichoicated or teichoic acid-free cell wall per ml, no TNF production was detected in monocyte supernatants at 24 h (lowest dilution, 1:2) (Table 3). The same supernatant fractions contained IL-1 activity. Furthermore, recombinant TNF was readily detected in controls at concentrations ranging from 1 μg to 0.1 ng/ml, and TNF activity was detected in supernatants of monocytes stimulated with lipopolysaccharide as a control.

DISCUSSION

In contrast to gram-negative sepsis, the role of cytokines such as IL-1 and TNF in gram-positive infections is not well characterized (2, 15). The results we present document that intact pneumococci and several pneumococcal cell surface components are powerful inducers of IL-1 production by human peripheral monocytes. The teichoic acid-peptidoglycan complex present on the pneumococcal cell surface is several orders of magnitude more powerful than endotoxin in inducing IL-1 production in vitro. However, in contrast to cytokine production induced by endotoxin in which production of both IL-1 and TNF occurs together, gram-positive bacterial surface components did not directly stimulate production of TNF when IL-1 was detected. Thus, it appears that, at least under the assay conditions we tested, the inflammatory cascades engaged by gram-positive bacteria may differ substantially from those activated directly by gram-negative bacterial products. This may have implications for understanding clinical differences in the courses of the two types of sepsis. Our findings support the proposal

TABLE 2. Effect of antibodies to IL-1 on activity of monocyte supernatants in LAF assay

Monocyte stimulus	Antiserum (1:500; 10^3 cpm ± SD)[a]				
	Control	Normal goat	Anti-IL-1α	Anti-IL-1β	Anti-IL-1α + anti-IL-1β
Cell wall (10 μg)	17.3 ± 2.3	16.9 ± 3.4	9.7 ± 0.3	0.2 ± 0.07	0.06 ± 0.01
Lipopolysaccharide (1 μg)	31.3 ± 4.3	35.4 ± 3.5	25.1 ± 1.6	3.2 ± 1.5	0.07 ± 0.01
IL-1 (1 U)	24.8 ± 5.0	30.0 ± 4.8	0.4 ± 0.08	15.8 ± 0.9	ND

[a] Supernatants from human monocytes were tested at a final dilution of 1:8. ND, Not determined.

that IL-1 occupies a central role in inflammation caused by the pneumococcus (20).

Both IL-1 and TNF have been detected in at least some cases of severe pneumococcal infection (meningitis) in humans (11), yet TNF was not detected in our in vitro assay. Several explanations can be suggested to reconcile these observations. The threshold density of pneumococci required to initiate inflammation in vivo during pneumococcal meningitis (25) is 10^5 cell equivalents, a value of the same order of magnitude as that required for induction of IL-1 in vitro, but 100-fold less than that for TNF. This supports the view that IL-1 production during pneumococcal disease in vivo may be caused by direct interactions between pneumococcal components and mononuclear phagocytes as measured in vitro. On the other hand, TNF production during pneumococcal infection may occur later in disease when bacterial densities are higher. Alternatively, if TNF plays a role early in the inflammatory response, its production may be secondary to other mediators or it may require interactions between pneumococci and cell types other than that studied here.

The teichoic acid, particularly the phosphorylcholine moiety, on the pneumococcal cell surface appears to be a major determinant of the ability of cell walls to induce IL-1 secretion from monocytes. Chemical alteration of or antibody against this structure dramatically reduced IL-1 production. Antibody to phosphorylcholine is common in humans, and C-reactive protein also binds to this structure (16, 19). It is possible that masking of the teichoic acid by such ubiquitous natural components in vivo might serve to downmodulate the inflammatory potential of this highly reactive moiety both on intact bacteria and on cell wall fragments released during pneumococcal lysis induced by antibiotics (21).

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LITERATURE CITED

1. **Abehsira-Amar, O., C. Damais, M. Parant, and L. Chedid.** 1985. Strain dependence of muramyl dipeptide-induced LAF (IL 1) release by murine-adherent peritoneal cells. J. Immunol. **134:** 365–368.
2. **Beutler, B., and A. Cerami.** 1987. Cachectin: more than a tumor necrosis factor. N. Engl. J. Med. **316:**379–385.
3. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72:** 3666–3670.
4. **Chaterjee, A. N., W. Wong, F. E. Young, and R. W. Gilpin.** 1976. Isolation and characterization of a mutant of *Staphylococcus aureus* deficient in autolytic activity. J. Bacteriol. **125:** 961–979.
5. **Chetty, C., and A. Kreger.** 1985. Generation of purpura-producing principle from pneumococcal cell walls. J. Bacteriol. **163:**389–391.
6. **Dinarello, C. A.** 1984. Interleukin-1. Rev. Infect. Dis. **6:**55–63.
7. **Garcia-Bustos, J. F., and A. Tomasz.** 1987. Teichoic acid-containing muropeptides from *Streptococcus pneumoniae* as substrates for the pneumococcal autolysin. J. Bacteriol. **169:** 447–453.
8. **Gold, M. R., C. L. Miller, and R. I. Mishell.** 1985. Soluble non-cross-linked peptidoglycan polymers stimulate monocyte-macrophage inflammatory functions. Infect. Immun. **49:**731–741.
9. **Handwerger, S., and A. Tomasz.** 1985. Antibiotic tolerance among clinical isolates of bacteria. Rev. Infect. Dis. **7:**368–386.
10. **Holtje, J. V., and A. Tomasz.** 1975. Biological effects of lipoteichoic acids. J. Bacteriol. **124:**1023–1027.
11. **Leist, T. P., K. Frei, S. Kam-Hansen, R. M. Zinkernagel, and A. Fontana.** 1988. Tumor necrosis factor alpha in cerebrospinal fluid during bacterial, but not viral, meningitis. J. Exp. Med. **167:**1743–1748.
12. **McAllister, C. K., J. M. O'Donoghue, and H. N. Beaty.** 1975. Experimental pneumococcal meningitis. II. Characterization and quantitation of the inflammatory process. J. Infect. Dis. **132:**355–360.
13. **Mizel, S. B., J. J. Oppenheim, and D. L. Rosenstreich.** 1978. Characterization of lymphocyte-activating factor (LAF) produced by the macrophage cell line, P388d1. I. Enhancement of LAF production by activated T lymphocytes. J. Immunol. **120:**1497–1502.
14. **Mossmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J. Immunol. Methods **65:**55–63.
15. **Okusawa, S., J. A. Gelfand, T. Ikejima, R. J. Connolly, and C. A. Dinarello.** 1988. Interleukin 1 induces a shock-like state in rabbits. J. Clin. Invest. **81:**1162–1172.
16. **Oliveira, E. B., E. C. Gotschlich, and T.-Y. Liu.** 1980. Comparative studies on the binding properties of human and rabbit C-reative proteins. J. Immunol. **124:**1396–1402.
17. **Ripley-Petzoldt, M. L., G. S. Giebink, S. K. Juhn, D. Aeppli, A. Tomasz, and E. Tuomanen.** 1988. The contribution of pneumococcal cell wall to the pathogenesis of experimental otitis media. J. Infect. Dis. **157:**245–255.
18. **Ruff, M. I., and G. E. Gifford.** 1980. Purification and physicochemical characterization of rabbit TNF. J. Immunol. **125:** 1671–1677.
19. **Szu, S. C., S. Clarke, and J. B. Robbins.** 1983. Protection against pneumococcal infection in mice conferred by phosphocholine-binding antibodies: specificity of the phosphocholine binding and relation to several types. Infect. Immun. **39:**993–999.
20. **Toumanen, E.** 1987. Molecular mechanisms of inflammation in experimental pneumococcal meningitis. Pediatr. Infect. Dis. **6:**1146–1149.
21. **Tuomanen, E., B. Hengstler, R. Rich, M. A. Bray, O. Zak, and A. Tomasz.** 1987. Nonsteroidal anti-inflammatory agents in the therapy for experimental pneumococcal meningitis. J. Infect. Dis. **155:**985–990.
22. **Tuomanen, E., B. Hengstler, and A. Tomasz.** 1986. The role of complement in inflammation during experimental pneumococcal meningitis. Microb. Pathogen. **1:**15–32.
23. **Tuomanen, E., H. Liu, B. Hengstler, O. Zak, and A. Tomasz.** 1985. The induction of meningeal inflammation by components of the pneumococcal cell wall. J. Infect. Dis. **151:**859–868.
24. **Tuomanen, E., R. Rich, and O. Zak.** 1987. Induction of pulmonary inflammation by components of the pneumococcal cell surface. Am. Rev. Respir. Dis. **135:**869–874.
25. **Tuomanen, E., A. Tomasz, B. Hengstler, and O. Zak.** 1985. The relative role of bacterial cell wall and capsule in the induction of inflammation in pneumococcal meningitis. J. Infect. Dis. **151:** 535–540.
26. **Winkelstein, J. A., and A. Tomasz.** 1977. Activation of the alternative pathway by pneumococcal cell wall teichoic acids. J. Immunol. **118:**451–454.
27. **Winkelstein, J. A., and A. Tomasz.** 1978. Activation of the alternative pathway by pneumococcal cell wall teichoic acids. J. Immunol. **120:**174–178.