Induction of Meningeal Inflammation by Diverse Bacterial Cell Walls

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Bacterial cell walls have been shown to be potent inflammatory agents. Cell wall components from various bacterial species can i) immunomodulate the activity of macrophages, T cells and B cells (1, 2), ii) trigger the alternative pathway of complement activation (3–5), iii) interact with polymorphonuclear leukocytes (6, 7), and iv) bind with humoral immune factors such as C-reactive protein (8). Such activities presumably contribute to inflammation provoked by cell walls in models of post-infectious and immune diseases (9, 10). Recently, however, it has become increasingly clear that host reactions to cell walls can also contribute very significantly to the course of *acute* bacterial infections such as bacteremia (11–13) and meningitis (14–16).

Given the enormous diversity in chemical structures of bacterial cell walls it is not clear to what extent biological activities are restricted to chemical structures unique to particular bacterial species. In fact, despite structural similarities between cell walls of different bacterial species, antigenic diversity appears to produce variation in the site and nature of wall-induced inflammation in vivo (17). Pneumococcal cell walls are powerful inducers of inflammation in the rabbit model of experimental meningitis (15). However, this finding can not be assumed to apply to other cell walls since pneumococcal cell walls have a most unusual chemical structure. The wall teichoic acids contain phosphorylcholine, galactosamine and 2,4,6-tri-deoxydiaminohexose as well as ribitol phosphate (18). The purpose of the present study was to determine to what extent the inflammatory activity of pneumococcal walls and wall components was unique to the peculiar chemistry of these walls. Using the rabbit model of meningitis, we investigated whether or not cell walls from other bacterial species which have well known, chemically characterized differences to pneumococcal walls could also induce an inflammatory response characteristic of meningitis.

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Bacterial cell walls were prepared from four species: unencapsulated *Streptococcus pneumoniae* strain R6, *Micrococcus lysodeikticus* strain ATCC 4698, *Bacillus subtilis* strain 168, and *Escherichia coli* strain W7. These species were chosen for their specific differences in cell wall structure compared to the pneumococcus. *Micrococcus lysodeikticus* contains teichuronic acid instead of teichoic acid (19); *Bacillus subtilis* has a polyglycerophosphate instead of a ribitol-containing teichoic acid (20), and *Escherichia coli* contains no teichoic acid. Insoluble cell wall was extracted as described (14, 15) by treatment of cells with boiling sodium dodecyl sulfate, washing and lyophilization. *Escherichia coli* peptidoglycan containing lipoprotein was fractionated further to remove lipoprotein by treatment with alpha amylase and pronase (21). This preparation was documented to be free of lipoprotein by amino acid analysis (Durram D-500 amino acid analyser) and to contain less than 1 pg/ml endotoxin by reverse phase high performance liquid chromatography (21) and the limulus lysate assay (E-toxate kit, Sigma Chemical, USA).

Groups of four rabbits were anaesthetized and placed in stereotaxic frames as described (14). Cell walls were suspended in pyrogen free saline at concentrations between 0.4 and 1000 μg/0.2 ml, and after removal of 0.3 ml cerebrospinal fluid preparations were inoculated into the cisterna magna. Control animals received saline alone. Cytochemical determinations were made serially in each animal over a 24 h period to document changes in density of leukocytes and concentrations of glucose, protein and lactic acid (14). Cell walls did not interfere with these assays. Polymorphonuclear leukocytes were differentiated from lymphocytes by morphology in Wright stained preparations of cerebrospinal fluid.

When injected into the subarachnoid space, inflammation ensues if the concentration of pneumococcal cell walls exceeds a threshold of 10^6 cell equivalents (i.e. 0.1 μg cell wall) (14). When tested at ten times this amount or more, all of the structurally diverse cell walls rapidly produced inflammation in the cerebrospinal fluid (Table 1). As seen by comparison of leukocyte densities in Table 1 (1 mg cell wall/dose) and Figure 1 (20 μg cell wall/dose), a dose response was apparent for each wall preparation tested. For example, in the case of *Escherichia coli* cell wall free of lipoprotein, leukocyte density decreased (21,000, 7060, and 4230 cells/μl) as the dose of cell walls decreased (1 mg, 4 μg, and 0.4 μg respectively). In all cases, leukocytosis was accompanied by striking increases in protein and lactic acid concentrations although no strict correlation between these parameters was observed. Interestingly, however, only in the case of *Micrococcus lysodeikticus* and *Bacillus subtilis* walls was a decrease in CSF glucose observed. The degree and time course of inflammation differed strikingly depending on the structure of the cell walls

Table 1: Comparison of cerebrospinal fluid cytochemistry profile five hours after intracisternal inoculation of 1 mg of various bacterial cell walls (standard error of mean in parenthesis).

	Leukocytes (cells/μl CSF)	Protein (mg/100 ml CSF)	Glucose (mg/100 ml CSF)	Lactic acid (mg/100 ml CSF)
Control (saline)	30 (± 10)	34 (± 12)	86 (± 4)	15 (± 5)
Micrococcus lysodeikticus	3730 (± 50)	220 (± 5)	62 (± 2)	79 (± 1)
Bacillus subtilis	9026 (± 1500)	380 (± 50)	48 (± 5)	125 (± 5)
Streptococcus pneumoniae	3133 (± 300)	430 (± 84)	81 (± 9)	64 (± 9)
Escherichia coli with lipoprotein	16800 (± 200)	526 (± 175)	72 (± 15)	108 (± 20)
Escherichia coli without lipoprotein	21000 (± 800)	321 (± 150)	93 (± 20)	78 (± 25)

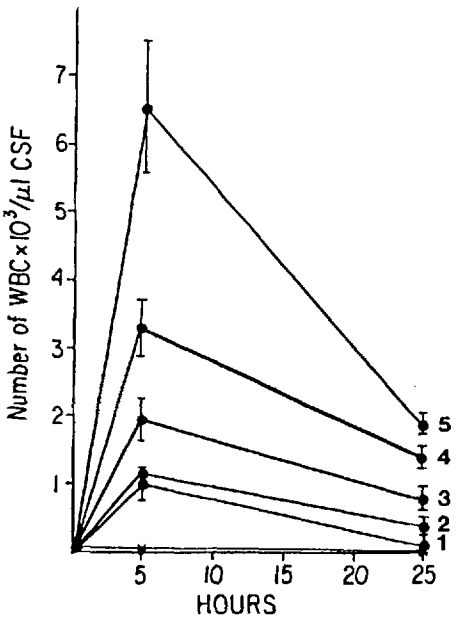

Figure 1: Cerebrospinal fluid leukocytosis induced by bacterial cell walls. *Micrococcus lysodeikticus* (1), *Bacillus subtilis* (2), and *Streptococcus pneumoniae* (3) cell walls were injected intracisternally at a concentration of 20 μg/0.2 ml at time 0. *Escherichia coli* with (4) and without (5) lipoprotein were injected at a concentration of 4 μg/0.2 ml. Saline control (X).

(Figure 1; all values for leukocyte number are statistically significantly different, $p < 0.01$). Walls with polyribitol-containing teichoic acid (*Streptococcus pneumoniae*) or teichuronic acid (*Micrococcus lysodeikticus*) showed the lowest inflammatory specific activity. The more common polyglycerophosphate containing teichoic acid-peptidoglycan complex represented by *Bacillus subtilis* cell wall had the highest specific activity of the gram-positive cell walls tested. Surprisingly, the gram-negative peptidoglycan was the most potent inducer of leukocytosis particularly if preparations were free of lipoprotein. Under the conditions tested, the maximum leukocytosis occurred on the first day for all cell wall types; inflammation resolved by 48 h. In all cases leukocytosis was initially predominantly polymorphonuclear (> 80 %) and evolved over 24 h to a predominantly lymphocytic (> 80 %) response. This sequence mimics that of natural meningitis when bacterial multiplication is arrested by antibiotics. Taken together these results suggest that while all the wall preparations tested exhibited a common ability to induce cerebrospinal fluid cytochemical abnormalities characteristic of acute bacterial meningitis, considerable differences in the kinetics of the inflammatory response and the specific activity of bacterial cell walls exist depending on chemical structure. Further experiments will be necessary to extend the basis of these observations.

The peptidoglycan-teichoic acid network of gram-positive bacteria is accessible to the environment despite the presence of an overlying capsule, and inflammation can be incited equally well by living encapsulated or unencapsulated cells (14). In contrast, the peptidoglycan of gram-negative bacteria is hidden under the differentially permeable outer membrane. Bacterial lysis would thus be required to release gram-negative peptidoglycan into the subarachnoid space. Beta-lactam antibiotics, currently the most useful antibiotics for the treatment of bacterial meningitis, induce bacterial lysis in both gram-positive and gram-negative bacteria. Thus, release of cell walls occurs during therapy of bacterial meningitis and in this context, inflammation may actually be transiently increased as bacterial killing is ongoing. This common response to bacterial cell walls in the meningeal compartment may contribute to the poor prognosis of meningitis which persists despite development of antibiotics highly bacteriolytic and bactericidal to all bacteria causing this disease.

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