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The Capsular Polysaccharide Complex of *Bacteroides fragilis* Induces Cytokine Production from Human and Murine Phagocytic Cells

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To simulate early-phase immunologic events following *Bacteroides fragilis* infection in the peritoneal cavity, we examined the cytokine response of several cell types to purified capsular polysaccharide complex (CPC) and lipopolysaccharide (LPS) of this organism. Cytokines were produced from murine resident peritoneal (MRP) cells as well as human peripheral blood leukocytes. MRP cells cocultured with either *B. fragilis* CPC or LPS in vitro produced tumor necrosis factor alpha and interleukin-1α (IL-1α). In addition, MRP cells challenged with CPC produced IL-10. Human peripheral blood monocytes and polymorphonuclear leukocytes secreted IL-8 when cultured in the presence of CPC.

Bacteroides fragilis is frequently isolated from clinical cases of sepsis involving anaerobic gram-negative rods and accounts for the majority of infections that occur within the peritoneal cavity (13, 27). Soilage of the abdominal cavity with bowel contents often results in development of abscesses. The surface of *B. fragilis* is complex and includes a polysaccharide capsule, lipopolysaccharide (LPS), and surface-associated proteins. The capsular polysaccharide complex (CPC) is the major virulence factor of *B. fragilis* and has been demonstrated to be responsible for abscess formation in both the rat and mouse models of peritoneal sepsis (24, 31). Structurally, the CPC is a heterodimer composed of two component polysaccharides; each possesses an interesting charge motif which is directly responsible for the biologic activity of these structures (1, 33). When administered to animals via peritoneal challenge, the CPC in the presence of sterile cecal contents, as an adjuvant, can induce abscesses identical to those which develop following whole-organism challenge (24). In addition to the inductive property of this molecule, the CPC or component polysaccharides protect against abscess formation when administered to animals prior to CPC challenge (31, 35). As defined by Shapiro et al. (30), the immunologic response of mice to the CPC of *B. fragilis* depends on an as yet undefined subset of T cells on the basis of adoptive transfer experiments. In light of this later finding, we became interested in defining the immune response of host cells during initial contact with CPC.

Resident peritoneal cells, which consist primarily of macrophages, are the first line of immunologic defense for the host to bacterial challenge via this route. Following rupture of the intestine or perturbation of the peritoneal cavity, these cells perform several functions, including antigen presentation and secretion of cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) as a prelude to further immunologic response to infection (2, 3, 6, 10, 19). Cytokine production following microbial challenge has been well documented (4, 5, 12, 15, 21, 36). However, little information describing the cytokine response in animals or isolated host cells following pure polysaccharide challenge is available (25). Even less information dealing with bacterial capsular polysaccharides is available (18, 32). As proposed by Cross (7), there is interest in defining the immunologic events which follow *B. fragilis* infection. Work by Delahooke et al. (8) has demonstrated that phenol-water-extracted *B. fragilis* LPS can elicit induction of TNF-α from murine cells. Although these previous studies are important for developing an understanding of the immunologic impact of *B. fragilis*, we were interested in determining if CPC induced cytokines, as part of our efforts to understand the host immunologic response.

The present work is the first study that describes the effect of *B. fragilis* capsular polysaccharide on phagocytic cells, specifically describing a profile of early-phase cytokines produced in response to challenge with the *B. fragilis* capsular polysaccharide.

To obtain pure *B. fragilis* CPC and LPS, we cultured *B. fragilis* NCTC 9343 overnight in a pH-controlled, 20-liter batch culture of proteose-peptone yeast extract broth at 37°C as described previously (26). Briefly, cells were harvested after overnight growth and extracted by the hot-phenol-water method to liberate surface polysaccharides. The aqueous phase was collected, subjected to two ether extractions (crude CPC-LPS), dialyzed extensively, and stored frozen at −20°C. Phenol-water extracts were thawed when needed and enzymatically digested with DNase, RNase (Worthington Biochemical Corp., Freehold, N.J.), RNase T (Sigma, St. Louis, Mo.), and pronase to remove contaminating nucleic acids and proteins. Samples were then ethanol precipitated, resuspended in deoxycholate buffer (pH 9.8), and size fractionated by using an S-400 column (Pharmacia, Uppsala, Sweden) in deoxycholate buffer to separate LPS from CPC. Fractions containing CPC or LPS were pooled separately, dialyzed extensively, and lyophilized.

The purity of *B. fragilis* surface components was evaluated by using 1-mg/ml solutions of CPC to ensure that cross-contamination did not occur and that CPC fractions were free of endotoxin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on each lot of purified CPC or LPS. Samples of each lot were reduced in sample buffer containing SDS and 2-mercaptoethanol and were loaded onto a discontinuous polyacrylamide gel (16). Gels were then

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run at constant current at 20 mA, fixed, silver stained (Bio-Rad, Hercules, Calif.), developed, and visualized for product purity. Phenol-water-purified CPC visualized by SDS-PAGE exists as a high-molecular-weight product, while identically purified LPS is of low molecular weight. Each lot of CPC was tested by immunoelectrophoresis (pH 7.3), to screen for contaminating *B. fragilis* LPS. Following electrophoretic separation, the gels were incubated overnight with a rabbit antibody produced against whole *B. fragilis* NCTC 9343. Samples were scanned at A_{260} and A_{280} with a spectrophotometer for detection of nucleic acid and protein contamination. In addition, the *Limulus* amebocyte lysate assay (Endosafe, Charleston, S.C.) demonstrated that 1-mg/ml CPC samples were essentially free of endotoxin (≤0.05 endotoxin units). Only those samples of CPC that were confirmed to be pure and free of endotoxin by these four methods were used in cell challenge assays. A sample of CPC from each lot was analyzed by nuclear magnetic resonance spectroscopy to confirm structure and to maintain quality control of the capsular product.

C57BL/6 mice (Taconic Farms, Gaithersburg, Md.) between the ages of 6 and 8 weeks were used in these assays and were given chow and water ad libitum. In addition, the animals received proper housing, care, and use as outlined in both the National Institutes of Health guidelines and Harvard University provisions for proper care and use of laboratory animals. Murine resident peritoneal (MRP) cells from naive mice were collected aseptically by peritoneal lavage with ice-cold Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2-mercaptoethanol, and penicillin-streptomycin. Harvested cells were placed on ice, pelleted by centrifugation at 250 × *g* for 20 min at 4°C, resuspended in fresh medium, counted, and adjusted to 2.5×10^6 cells/ml with fresh medium.

Human peripheral blood (10 ml) was collected from healthy male donors into heparinized tubes during the morning of the experiment. A 3.5-ml sample of blood was layered over a bed of Mono-Poly resolving medium (ICN Biomedicals, Inc., Costa Mesa, Calif.) and centrifuged at 300 × *g* for 35 min at room temperature. Fractions containing monocytes or polymorphonuclear leukocytes (PMNL) were collected after centrifugation, washed three times with ice-cold DMEM, counted, checked for purity (>95% pure), and placed on ice. The cells were then adjusted to 2.5×10^6 cells/ml with fresh medium.

Following enumeration, 2.5×10^5 cells were placed into separate wells of a sterile, round-bottomed, 96-well culture plate. The cells were either left untreated in culture medium, cocultured with one of three polysaccharides, including *B. fragilis* CPC; poly-(1-6)-β-glucotriosyl-(1-3)-β-glucopyranose glucan (β-glucan), a yeast cell wall glucose polymer (Betafectin; Alpha Beta Technologies, Inc., Worcester, Mass.); or *Streptococcus pneumoniae* type III capsular polysaccharide (American Type Culture Collection, Rockville, Md.) at 0.01, 0.1, or 20 μg per well. Similar cultured MRP cells for TNF-α and IL-1α detection received either purified LPS from *B. fragilis* at 0.01, 0.1, or 20 μg per well or phenol-water-extracted *Escherichia coli* LPS (Sigma) at 0.005 or 0.025 μg per well (final volume, 100 μl per well).

Cell culture supernatants were collected, aseptically, at 4 or 24 h postculture from stimulated and unstimulated cells for TNF-α and IL-1α analysis and at 24 h for IL-10 analysis. Supernatants of purified naive human cells or those cocultured with CPC were collected at 1, 6, or 24 h postchallenge. Following collection, the supernatants were stored frozen at −20°C until each trial was complete. Prior to quantitation, supernatants were thawed and centrifuged at 5,000 × *g* for 10 min to remove cell debris. Samples were then tested in duplicate by enzyme-linked immunosorbent assay (ELISA) for murine TNF-α, IL-1α, and IL-10 (Endogen, Cambridge, Mass.) and human IL-8 (Bender, Vienna, Switzerland) in accordance with kit protocols. Developed ELISA tests were scanned with an EL 312E microplate reader (Biotek Instruments, Winooski, Vt.), and absorbance values were recorded. The concentration of each cytokine present in the culture medium was determined by averaging duplicate readings and comparing them with a standard curve. The results of all experiments are expressed as the average of three separate trials ± the standard deviation.

TABLE 1. Detection of TNF-α and IL-1α from MRP cells

Antigen and concn (μg/well)	Level of cytokine detected (pg/ml ± SD)			
	TNF-α		IL-1α	
	4 h	24 h	4 h	24 h
Medium	0 ± 0	16 ± 2.83	2 ± 2.12	27 ± 20.51
CPC[a]				
0.01	0 ± 0	128 ± 87.68	103 ± 4.24	26 ± 14.85
0.1	54 ± 1.41	152 ± 5.66	216 ± 26.16	38 ± 9.91
20	287 ± 51.27	25 ± 16.97	479 ± 7.07	1,363 ± 41.01
B. fragilis LPS				
0.01	37 ± 7.07	69 ± 14.85	163 ± 21.21	5 ± 7.07
0.1	68 ± 74.25	66 ± 77.78	257 ± 11.31	25 ± 7.07
20	166 ± 52.33	7 ± 9.19	162 ± 15.56	1,418 ± 105.36
E. coli LPS[b]				
0.005	126 ± 3.54	0 ± 0	319 ± 51.62	411 ± 43.84
0.025	127 ± 20.51	23 ± 31.82	391 ± 31.82	1,045 ± 31.11
S. pneumoniae type III[c]				
20	222 ± 23.07	ND[d]	383 ± 138.72	ND
β-Glucan[e]				
20	17 ± 18.63	ND	28 ± 31.16	ND

[a] CPC, *B. fragilis* NCTC 9343 capsular polysaccharide.
[b] Phenol-water extract.
[c] *S. pneumoniae* type III capsular polysaccharide.
[d] ND, not determined.
[e] β-Glucan, Betafectin.

The CPC and LPS of *B. fragilis* induced TNF-α and IL-1α in a dose-dependent fashion at 4 and 24 h, respectively, in trials using MRP cells (Table 1). Cells from animals cultured in medium did not secrete appreciable amounts of these monokines. Within 4 h postculture, IL-1α levels were slightly more elevated than TNF-α levels for CPC- and LPS-challenged cells. By 24 h, MRP cells cocultured with CPC or LPS produced TNF-α levels consistent with 4-h levels. Only those cells that received 20 μg of CPC secreted high levels of IL-1α. In addition to CPC and LPS, two other surface-expressed polysaccharides and an LPS control were tested for their ability to induce TNF-α and IL-1α from previously unstimulated MRP cells. These additional polysaccharides tested included the type III capsular polysaccharide of *S. pneumoniae*, β-glucan, and *E. coli* phenol-water-extracted LPS. β-Glucan has been reported to be incapable of eliciting production of TNF-α or IL-1 (14, 28). The soluble β-glucan used in our experiments did not elicit induction of TNF-α or IL-1α from MRP cells at 4 and 24 h postculture (Table 1). The pneumococcus type III capsule induced TNF-α and IL-1α from MRP cells. In these experiments, *B. fragilis* CPC induced a stronger response than did the type III capsule.

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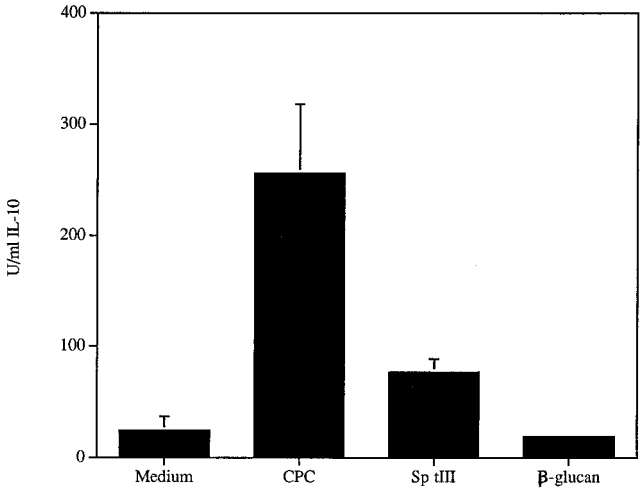

FIG. 1. Detection of IL-10 production from MRP cells by ELISA 24 h postculture. Cells were cultured in DMEM (medium) or in DMEM plus 20 μg of the polysaccharide *B. fragilis* CPC (CPC), *S. pneumoniae* type III capsule (Sp tIII), or β-glucan per well. The error bars represent standard deviations.

IL-10 is a potent immunoregulating cytokine which stimulates a Th_2 immune response, alternatively described as a humoral immune response. In addition, this molecule is important in the down regulation of Th_1, or cell-mediated immunity. Naive MRP cells cocultured with CPC for 24 h produced IL-10, while cells cocultured with the type III capsular polysaccharide of *S. pneumoniae* produced nominal levels of this cytokine (Fig. 1). β-Glucan failed to evoke production of IL-10.

IL-8 was detected from isolated mononuclear leukocytes (MNL) and PMNL cocultured with *B. fragilis* CPC (Fig. 2). Time course expression studies were used to define the kinetics of IL-8 production. Accumulation of IL-8 occurred rapidly from cells cocultured with 20 μg of CPC in vitro and was detected at maximal levels within 24 h postculture from each cell type. The responsiveness of PMNL to CPC was pronounced and occurred in a time-dependent fashion, with maximal levels occurring at 24 h compared with PMNL cultured in DMEM alone (Fig. 2). Only those PMNL cultured with polysaccharide produced high levels of IL-8. MNL produced unusual IL-8 profiles in response to the polysaccharides tested, as measured by ELISA. Cells cultured in DMEM alone yielded no response at the early time point (1 h). However, a nonspecific response was detected at 6 and 24 h postculture. This response occurred in all assays when MNL from each donor were cultured in culture medium. In contrast, MNL cocultured with CPC yielded increased IL-8 levels at 1 and 6 h postculture compared with the medium control. The response to CPC culture at the 24-h time point was comparable to that of the control (Fig. 2). However, MNL stimulated with CPC produced larger amounts of IL-8 at each assay point than those MNL challenged with the other saccharides.

The CPC of *B. fragilis* possesses many unusual biologic properties and represents the virulence factor responsible both for abscess induction in animal models of peritoneal sepsis and suppression of abscess formation associated with this disease process. The CPC comprises two constituent polysaccharides which each possess an unusual dual-charge motif (1, 26). Each constituent polysaccharide has positively and negatively charged groups that are critical for their ability to both promote and protect against abscess formation in animal models of sepsis (31, 33–35). Our previous studies have demonstrated that regulation of abscess formation by CPC is mediated by a T-cell-dependent form of immunity (30). We wanted to determine if CPC could elicit cytokines from host cells. The purpose of this study was to characterize the cytokine profile elicited by this unique polysaccharide structure in order to better understand its interaction with the host immune system.

Polysaccharides have exhibited various abilities to stimulate cytokine expression (14, 18, 28). The present studies demonstrate that cytokines are released from a variety of phagocytic cells in response to *B. fragilis* capsule challenge. TNF-α and IL-1α elicit classical physiologic changes at both the local and the systemic levels, including up regulation of cell adhesion molecule expression and tissue healing (29). The LPSs of many gram-negative bacteria elicit a proinflammatory cytokine response during host exposure. *B. fragilis* possesses LPS; however, the LPS of this organism is a relatively inert form, with little endotoxin activity (17). Despite this finding, recent work has shown that the LPS of *B. fragilis* can stimulate TNF-α from various murine cells (8). However, the preparations used in those studies were crude extracts containing both CPC and LPS (8). We have purified the CPC and LPS from crude phenol-water extracts and assessed the cytokine profile elicited by these isolated structures. TNF-α and IL-1α are secreted by MRP cells in response to each of these structures. The production of cytokines by MRP cells following coculture with CPC is similar to that following coculture with LPS; however, some differences are notable and indicate that the *B. fragilis* CPC contributes, at least in part, with the induction of cytokine production from various cells. In addition, we observed high levels of TNF-α in the 24-h sample treated with 0.01 and 0.1 μg of *B. fragilis* LPS per well, while those cells receiving the highest dose of LPS resembled that demonstrated by Delahooke et al. (8). We speculate that the differences observed between these studies may be due, at least in part, to the difference in cell types used, thioglycolate elicitation, and activation of peritoneal cells and antigen purity.

Our work centers on the biologic importance of the *B. fragilis* capsule in abscess formation; however, the understanding of the events immediately following exposure of the host to *B. fragilis* CPC is essential to the eventual goal of understanding abscess formation. Using purified antigens, we assessed TNF-α

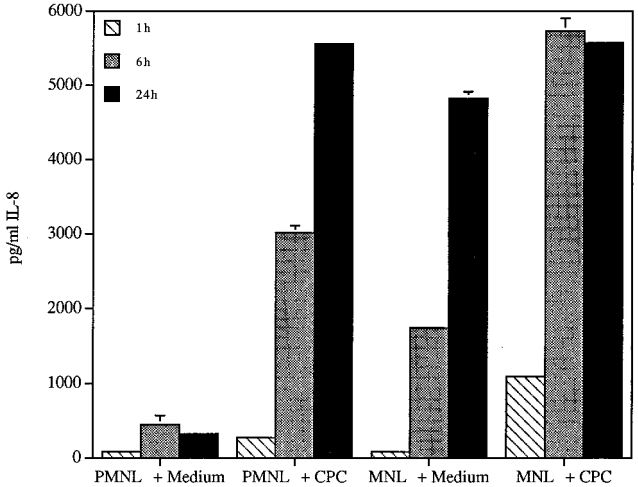

FIG. 2. ELISA detection of IL-8 production from human peripheral blood leukocytes. MNL or PMNL were cultured in DMEM (medium) or in DMEM plus 20 μg of *B. fragilis* CPC per well. The error bars represent standard deviations.

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and IL-1α production from MRP cells. Earlier work by Onderdonk et al. (23) delineated the cellular response within the peritoneal cavity of mice following *B. fragilis* whole-organism challenge. This response was characterized by an initial influx of lymphocytes, which was followed by a macrophage/monocyte response and culminated with a second lymphocyte influx 6 days later. We hypothesized that this cellular response within the peritoneal cavity to *B. fragilis* was mediated by cytokines elicited by the CPC.

IL-10 is produced by a variety of cells, including $CD4^+$ T cells, B cells, and macrophages (22, 37). This molecule possesses several characteristics, such as a potent down-modulator of the immune response; it profoundly influences the development of Th_1 and Th_2 forms of host immunity; and it reduces the sensitivity of cells to the effects of TNF-α (9). CPC elicits an IL-10 response from MRP cells. The detection of IL-10 in our system is interesting, since we have been able to detect TNF-α and IL-1α as well as IL-10 from MRP cells cultured in the presence of *B. fragilis* CPC. The presence of IL-10 may represent a cellular response to the increasing levels of TNF-α, since this has been described as an autoregulatory mechanism to decrease production of TNF-α (9). Secretion of IL-10 by macrophages is not fully understood as it relates to the progression of disease. This response may represent a key mechanism by which *B. fragilis* suppresses the immune system as it establishes a focus of infection. The induction of IL-10 synthesis may serve to make the host less sensitive to the presence of *B. fragilis* following contamination of the peritoneal cavity. Alternatively, the presence of IL-10 may reflect the humoral immune response to CPC that has been documented with this organism.

Abscesses are a localized collection of PMNL encased in a fibrinous capsule. Moser et al. (20) demonstrated that TNF-α and IL-1 are important in transendothelial migration of neutrophils. In conjunction with the observations of Onderdonk (23) regarding cell localization in the peritoneal cavity and the presence of PMNL in large numbers within abscesses, we were interested in looking for IL-8, a known attractant for neutrophils and an activator of neutrophil function (11). IL-8 was secreted from both isolated human MNL and PMNL in response to *B. fragilis* CPC. PMNL that received CPC responded quickly to the stimulus by producing high levels of IL-8. PMNL not receiving *B. fragilis* capsule secreted only low levels of this molecule. As neutrophils are the predominant immune cell associated with abscess formation, it is likely that one of the signals inducing the recruitment of these cells to the peritoneal cavity is IL-8. Interestingly, we consistently found that MNL cocultured with medium alone, β-glucan, or pneumococcal type III capsule yielded low levels of IL-8 at 1 and 6 h postculture. However, by 24 h, IL-8 levels had reached those obtained in experimental trials. Three different donors gave similar results, and this profile may be the result of handling of these cells prior to the assays performed. It was shown that IL-8 production was affected by CPC challenge. MNL assayed prior to the 24-h time point secreted higher levels of IL-8 than those MNL cultured in medium alone. We have demonstrated that IL-8 is produced by MNL as well as PMNL in response to CPC challenge; however, the responsiveness of monocytes to CPC challenge is less robust than the response of PMNL to CPC challenge. We believe the IL-8 produced by human PMNL in vitro coincides with the stimulus following animal challenge with CPC. As IL-8 plays a central role in accumulation of neutrophils at a site of infection by signaling extravasation during human disease, it seems likely that it is an important mediator of neutrophil attraction in the peritoneal cavity following challenge with CPC in animal models of sepsis.

This is the first report characterizing cytokine production elicited by the CPC of *B. fragilis* and is among the first studies to examine cytokine production by a purified bacterial polysaccharide. CPC is able to induce a complex profile of cytokines at detectable levels from cells of various origins. The importance of these secretory molecules during abscess formation is as yet undefined; however, understanding the host response to bacterial capsular polysaccharide such as the *B. fragilis* CPC may provide unique insight into the host immune interactions with this capsular polysaccharide and may also contribute to the overall understanding of the immunology between the host and other bacterial capsules.

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