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Bacterial Lipoprotein Delays Apoptosis in Human Neutrophils through Inhibition of Caspase-3 Activity: Regulatory Roles for CD14 and TLR-2

Colm P. Power, 1 Jiang H. Wang, Brian Manning, Malcolm R. Kell, Noel F. Aherne, Qiong D. Wu, and H. Paul Redmond

The human sepsis syndrome resulting from bacterial infection continues to account for a significant proportion of hospital mortality. Neutralizing strategies aimed at individual bacterial wall products (such as LPS) have enjoyed limited success in this arena. Bacterial lipoprotein (BLP) is a major constituent of the wall of diverse bacterial forms and profoundly influences cellular function in vivo and in vitro, and has been implicated in the etiology of human sepsis. Delayed polymorphonuclear cell (PMN) apoptosis is a characteristic feature of human sepsis arising from Gram-negative or Gram-positive bacterial infection. Bacterial wall product ligation and subsequent receptor-mediated events upstream of caspase inhibition in neutrophils remain incompletely understood. BLP has been shown to exert its cellular effects primarily through TLR-2, and it is now widely accepted that lateral associations with the TLRs represent the means by which CD14 communicates intracellular messages. In this study, we demonstrate that BLP inhibits neutrophil mitochondrial membrane depolarization with a subsequent reduction in caspase-3 processing, ultimately leading to a significant delay in PMN apoptosis. Pretreatment of PMNs with an anti-TLR-2 mAb or anti-CD14 mAb prevented BLP from delaying PMN apoptosis to such a marked degree. Combination blockade using both mAbs completely prevented the effects of BLP (in 1 and 10 ng/ml concentrations) on PMN apoptosis. At higher concentrations of BLP, the anti-apoptotic effects were observed, but were not as pronounced. Our findings therefore provide the first evidence of a crucial role for both CD14 and TLR-2 in delayed PMN apoptosis arising from bacterial infection. The Journal of Immunology, 2004, 173: 5229–5237.

The innate immune system recognizes microbial pathogens via highly conserved pattern recognition receptors that can associate with bacterial structures and ultimately convey warning signals to host cells. Effective coordination of this inflammatory response involves identification of disparate bacterial cell wall products. These molecules synthesized exclusively by microbes include LPS, peptidoglycans, lipoteichoic acids, and bacterial lipoproteins (BLP). 2 Each of these molecules possesses specific active moieties, and BLP in particular is characterized by a unique NH2-terminal lipo- amino acid, N-acetyl-S-diacylglyceryl cysteine. We and others have shown that lipoproteins from diverse bacterial sources activate a variety of host defense cell types, including monocytes, macrophages, neutrophils, etc., and stimulate their proinflammatory cytokine production (1, 2). BLP is restricted to Gram-negative organisms, whereas BLP is produced by the complete spectrum of bacterial pathogens (3). In fact, it has been shown that lipoproteins are the most abundant protein in the cell wall of certain Gram-negative bacteria (~700,000 molecules/cell) and may outweigh LPS as a cell wall component by as much as 4-fold (4, 5). It follows therefore that BLP represents a ubiquitous.

Ag that may contribute to the dysregulation of the immune response that is typical of the sepsis syndrome arising from bacterial infection of both Gram-negative and Gram-positive origin.

Three cloned families of molecules on the surface of leukocytes are known to bind the toxic lipid A moiety of LPS. These include CD14, the macrophage scavenger receptors (SR-A family), and the β2 integrins (CD11b/CD18) (6). Although an avid ligand for LPS, CD14 has no intrinsic signaling properties, as it is deficient in an intracytoplasmic transducing domain and must exercise its effects through significant lateral associations with other receptors. The long awaited identification of a transmembrane coreceptor for CD14 would appear to have been realized in the family of TLRs. Two of which (TLR-4 and TLR-2) demonstrate a marked pertinence to mammalian immune responses. It is now widely accepted that CD14 (in concert with LPS-binding protein) presents LPS to its high affinity transducer TLR-4, thereby precipitating the molecular adjustments and cytokine profiles associated with bacterial-induced systemic inflammation (7, 8). CD14 also enhances cellular responses of phagocytes to bacterial wall products that activate cells via TLR-2 (5, 9). BLP has been shown to trigger host defense mechanisms primarily through TLR-2. This is evidenced by BLP-mediated TLR-2-dependent IL-12/NO production by monocytes/macrophages and BLP-induced monocyte apoptosis, again regulated through TLR-2 (3, 10).

There is limited information pertaining to BLP and its effects on neutrophil (polymorphonuclear cell (PMN)) function. PMNs represent a subset of leukocytes with potent phagocytic, proteolytic, and oxidative potential. They play a pivotal role in host defense by engulfing and removing pathogenic microorganisms. Their ability to exit blood vessels and migrate rapidly to extravascular sites is crucial for the successful resolution of bacterial infections. PMN

Department of Academic Surgery, National University of Ireland, Cork University Hospital, Cork, Ireland

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1 Address correspondence and reprint requests to Dr. Colm P. Power at the current address: Department of Vascular Surgery, Mater Misericordiae Hospital, Eccles Street, Dublin, Ireland. E-mail address: cjppower@yahoo.com

2 Abbreviations used in this paper: BLP, bacterial lipoprotein; ΔΨm, mitochondrial membrane depolarization; cyt-c, cytochrome c; mCD14, membrane CD14; PMN, polymorphonuclear cell; sCD14, soluble CD14.
activation is therefore a prerequisite for optimal recovery from Gram-positive and Gram-negative infection. The release of cytotoxic substances in response to microbes by PMNs can, however, also have deleterious effects on host tissue. PMN-mediated bystander injury to normal tissues is an inescapable feature of the local inflammatory response, and may also contribute to tissue damage in organs distant from the site of infection such as the lung (11) and liver (12) as well as the generalized increase in microvascular permeability that accompanies systemic inflammation (13). The autotoxic potential of the PMN is compounded by the fact that, unlike many cell types that undergo apoptosis in response to bacterial wall products, the constitutive apoptotic rate of the PMN is delayed as a consequence of cell activation. The net effect, therefore, is the persistence of PMNs at inflammatory foci with a concomitant prolongation of PMN-mediated tissue injury. LPS has a pronounced inhibitory effect on PMN apoptosis, as demonstrated by Colotta in 1992 (14) and many others since, but the influence of BLP on PMN apoptosis remains unexplored. We, and others, have demonstrated that BLP is capable of PMN activation with the attendant up-regulation of CD11b/CD18, enhanced respiratory burst, and increased reactive oxygen species production associated with an inflammatory PMN phenotype (15, 16). In addition, Soler-Rodriguez and colleagues (5) have shown that PMN activation by BLP is partially dependent on CD14. It has recently been demonstrated that PMNs express TLRs (17); however, their functional significance in PMNs has not been investigated, and although TLRs are implicated in bacterial product-apoptotic signaling in some cells, this has not yet been shown in human neutrophils.

In this study, we describe the profoundly antiapoptotic effect of BLP on human PMNs, and furthermore, we illustrate how TLR-2 and CD14 contribute to the regulation of this phenomenon. In confirming the proinflammatory nature of BLP, we delineate its effects on a number of key PMN apoptotic parameters, namely, mitochondrial membrane depolarization (Δψm) and caspase-3 activity.

Materials and Methods

Reagents

RPMI 1640, FCS, penicillin, streptomycin sulfate, and glutamine were purchased from Invitrogen Life Technologies (Paisley, Scotland, U.K.). Dextran, Percoll, SDS, sodium citrate, sodium chloride, sodium orthovanadate, calcium chloride, glyceral, bromphenol blue, 2-ME, EDTA, HEPES, N-lauroylsarcosine, PMSE, Tris, Triton X-100, and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO). Pepstatin A and aprotinin were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). BLP, a synthetic bacterial lipopeptide (Pam3 Cys-Ser-Lys4-OH) derived from the immunologically active N terminus of bacterial lipoproteins, was obtained from Endosafe, Charleston, SC. A mouse anti-human CD14-neutralizing mAb (M5E2) was obtained from BD Pharmingen (San Diego, CA), and a mouse anti-human CD14-neutralizing mAb (2392) was a gift from Genentech (San Francisco, CA). Ficoll-Paque and Annexin V FITC were obtained from PharMingen (San Diego, CA). A mouse anti-human TLR-2-blocking mAb (mAb 2392, 25 μg/ml) and Annexin V FITC (final concentration 0.6 μg/ml) diluted in binding buffer (pH 7.4, 10 mM HEPES, 140 mM sodium chloride, 2.5 mM calcium chloride) for 5 min at room temperature. Untreated PMNs were used as control cells. To identify roles for CD14 and TLR-2, PMNs were incubated with neutralizing mAbs specific for membrane CD14 (mAb CD14) (mAb M5E2, 10 μg/ml) and TLR-2 (mAb 2392, 25 μg/ml) for 1 h at room temperature before exposure to BLP. FITC-conjugated isotype control IgG1 and IgG2a mAbs were similarly used. PMNs were analyzed on a FACScan flow cytometer equipped with CellQuest software (BD Biosciences, Mountain View, CA) with excitation at 488 nm and emission collected through a 530/30 band pass filter for Annexin V FITC in fluorescence channel 1 (FL1-H) and a 585/42 band pass filter for propidium iodide in fluorescence channel 2 (FL2-H). Ten thousand events were collected while gating on physical parameters to exclude cell debris.

Measurement of Δψm

To detect variations in Δψm at the single cell level, we used the cytometric technique developed by Cossarizza and colleagues (19), using the lipophilic cationic probe JC-1. JC-1 is more advantageous over rhodamines and other carbocyanines, capable of entering selectively into mitochondria, because it changes reversibly in color from green to orange as mitochondrial potential increases (over values of ~80–100mV). This property is due to the reversible formation of JC-1 aggregates upon membrane polarization that causes shifts in emitted light from 530 nm (i.e., emission of JC-1 monomeric form) to 590 nm (i.e., emission of J-aggregate) when excited at 488 nm. Both color forms can be detected using flow cytometric filters in such a way that green emissions are analyzed in FL1-H and orange emissions in FL2-H. Briefly, PMNs (0.5 × 10^6/ml) that had been exposed to 100 ng/ml concentrations of BLP were incubated with 5 μg/ml JC-1 for 15 min at 37°C in the dark. The suspension was agitated until the dye was fully dissolved, giving a uniform red-violet color. Data pertaining to the Δψm of PMNs after exposure to BLP were then acquired on a FACScan flow cytometer equipped with CellQuest software (BD Biosciences) with excitation at 488 nm and emission collected through a 530/30 band pass filter for FL1-H and a 585/42 band pass filter for FL2-H. We routinely collected 10,000 events per sample and excluded debris again by forward versus side scatter gating.

Colorimetric measurement of caspase-3 activity

Following incubation of PMNs (2 × 10^6/ml) with either culture medium or BLP (100 ng/ml) for 0, 3, 6, and 9 h, cells were collected by centrifugation at 250 × g for 10 min. To identify roles for CD14 and TLR-2, PMNs were incubated with neutralizing mAbs specific for mCD14 (mAb M5E2, 10 μg/ml) and TLR-2 (mAb 2392, 25 μg/ml) for 1 h at room temperature before exposure to BLP. Isotype control mAbs were similarly used. The pellet was then lysed with cell lysis buffer (25 μl per 1 × 10^6 cells) as provided in the caspase-3 colorimetric assay kit (R&D Systems, Minneapolis, MN). The cell lysate was incubated on ice for 10 min and then centrifuged at 10,000 × g for 1 min. As we were using a specific number of PMNs in each experiment, it was unnecessary to calculate protein content. The reactions were calculated using 50 μl of cell lysate and 50 μl of 2 × reaction buffer in each well of a 96-well flat-bottom microplate. A further 5 μl of caspase-3 colorimetric substrate (DEVD-pNA) was added to
Ab (R&D Systems), followed by incubation with alkaline phosphatase–caspase-3 protein, the membranes were probed with caspase-3 polyclonal Schuell Microscience, Dassel, Germany). For immunodetection of the SDS-PAGE, and transblotted onto nitrocellulose membranes (Schleicher & 10% glycerol, 5% 2-ME, 0.01% bromphenol blue). Aliquots containing tured at 95° protein assay reagent kit (Pierce, Rockford, IL). The proteins were dena-
determined with a colorimetric reaction using a microbicinchoninic acid 137 mM sodium chloride, 1.0 mM PMSF, 2.0 mM sodium orthovanadate, or BLP (100 ng/ml) for 0, 3, 6, and 9 h, cells were extensively washed with cold PBS and lysed in ice with lysis buffer (1% Triton X-100, 20 mM Tris, 173 mM sodium chloride, 1.0 mM PMSF, 2.0 mM sodium orthovanadate, 10 μg/ml pepstatin A, and 2.0 μg/ml aprotinin). Protein concentration was de-
termined with a colorimetric reaction using a microbicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). The proteins were dena-
tured at 95°C for 10 min in loading buffer (60 mM Tris-HCl, 2.5% SDS, 10% glycerol, 5% 2-ME, 0.01% bromphenol blue). Aliquots containing equal amounts of total protein from each sample were separated by 15% SDS-PAGE, and transblotted onto nitrocellulose membranes (Schleicher & Schuell Microscience, Dassel, Germany). For immunodetection of the caspase-3 protein, the membranes were probed with caspase-3 polyclonal Ab (R&D Systems), followed by incubation with alkaline phosphatase-
conjugated anti-goat secondary mAb (Promega, Madison, WI). Caspase-3 protein (pro- and active forms) was visualized using 5-bromo-4-chloro-3-indolyl-phosphate/NBT (Promega).

**Statistical analysis**

All data are presented as the mean ± SE. ANOVA was used to identify initial statistically significant differences in apoptotic rates, and post hoc analysis was performed using the Tukey wholly significant difference method for comparisons between mean apoptotic rates in respective groups. Additional adjustment for multiple comparisons across all time points and concentration values used Bonferroni correction analysis. Results were analyzed using Stata-8.2. Differences were judged statistically significant when p values were <0.05.

**Results**

**BLP delays the constitutive rate of PMN apoptosis**

Over a 24-h period, up to ~80% of isolated human PMNs demonstrate evidence of apoptosis. The complement of PMNs undergoing apoptosis increases in a time-dependent fashion. We observed that BLP in 1–1000 ng/ml concentrations was capable of inhibiting the normal progression of the PMN apoptotic demise (Fig. 1). This was more pronounced at higher doses of BLP, but reached a plateau at very high concentrations (100–1000 ng/ml). We found that the effects of BLP in this series of experiments were identical with those of LPS (data not shown). We subsequently confirmed the antiapoptotic effects of BLP, as evidenced by the disparity in the morphological appearance of control and BLP-treated neutrophils (Fig. 2).

**TLR-2 and CD14 regulate the antiapoptotic effects of BLP**

We proceeded to assess the effects of BLP on PMN apoptosis in the presence of blocking mAbs directed at CD14 and TLR-2. We found that CD14 blockade with mAb M5E2 (10 μg/ml for 1 h) partly inhibited the delay in PMN apoptosis mediated by BLP in 1 and 10 ng/ml concentrations. At early time points (6 h), there was no difference in the apoptotic rates of any group in both the 1 and 10 ng/ml BLP experiments (Fig. 3, A and B). By 12 h, the differences were quite marked. Increasing BLP concentrations (100 and 1000 ng/ml), however, did elicit a response at 6 h, as can be seen in Fig. 3, C and D. At these same doses of BLP, the preincubation of PMNs with mAb M5E2 did not influence the antiapoptotic effects of BLP.

The preincubation of PMNs with mAb 2392 (25 μg/ml) for 1 h before exposure to BLP had more pronounced effects. Blockade of TLR-2 resulted in attenuation of BLP-induced apoptotic delay at all concentrations of BLP (1–1000 ng/ml) (Fig. 4). Although this did not completely abrogate the effects of BLP across all

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**FIGURE 1.** Rates of neutrophil apoptosis over a 24-h time period in the presence and absence of BLP. At 6 h: there is no statistical difference between the apoptotic rates of control PMNs and those exposed to 1 ng/ml BLP. BLP in 10 ng/ml concentration delayed PMN apoptosis significantly as compared with the control rate (*, p < 0.01); however, higher concentrations of BLP (100 and 1000 ng/ml) delayed PMN apoptosis to a greater degree (**, p < 0.001). At 12 h: all concentrations of BLP were observed to retard PMN apoptosis at this time point, once again higher concentrations of BLP having a more statistically pronounced effect. At 18 and 24 h: a similar pattern is seen. Using this assay, we have consistently identified that ~80% of control PMNs are apoptotic by 24 h. At no time point did the effect of 100 ng/ml differ from that seen with 1000 ng/ml BLP. More rigorous analysis with Tukey and Bonferroni correction still detected significant differences between the effect of these higher concentrations and 1 and 10 ng/ml doses on PMN apoptosis at 12, 18, and 24 h (p < 0.05).

**FIGURE 2.** We confirmed that BLP delayed apoptosis in human neutrophils by identifying characteristic morphological appearances of PMNs under light microscopy (×1000). PMNs undergoing the constitutive rate of apoptosis are illustrated in A, with the attendant cell shrinkage and cytoplasmic and nuclear condensation associated with apoptosis. BLP delays apoptosis, and this is demonstrated by the absence of such stigmata (B), and PMNs are seen to retain their trilobed nuclei with no nuclear condensing (12 h).
FIGURE 3. The effect of mAb CD14 on the BLP-mediated delay in PMN apoptosis. A, At 6 h, the 1 ng/ml dose of BLP did not influence PMN apoptosis; CD14 blockade subsequently did not alter this finding. At 12 h, blockade with mAb CD14 did restore the rate of PMN apoptosis toward the control rate (p < 0.05); however, BLP continued to delay PMN apoptosis to a greater degree (p < 0.01). B, Using the 10 ng/ml concentration of BLP, we found that the results depicted in A were replicated. C and D, At more concentrated doses, BLP once again had a profoundly retardative effect on PMN apoptosis; however, the use of mAb CD14 did not alter this in any way. We found that apoptotic rates were significantly higher (p < 0.05) after PMN incubation with mAb CD14 in the presence of BLP at 1 and 10 ng/ml concentrations at 12, 18, and 24 h. After Bonferroni correction for multiple testing across the 16 dose-time groups, only the 6-h 10 ng/ml comparison with control apoptotic rate lost significance.

FIGURE 4. The effect of mAb TLR-2 on the BLP-mediated delay in PMN apoptosis. A, Once again, BLP (1 ng/ml) significantly delayed PMN apoptosis (p < 0.01), and in this series of experiments did so at 6 h as well. Blockade with mAb TLR-2 abolished this effect at 6 and 12 h and attenuated the effect of BLP (reduction from p < 0.01 to p < 0.05) at 18 and 24 h. B, The 10 ng/ml concentration of BLP produced similar effects to those seen in A; however, the higher dose of BLP overcame TLR-2 blockade at 12 h, and this persisted to 18 and 24 h. TLR-2 blockade did abrogate the effects of BLP with a statistically significant reduction once again from p < 0.01 to p < 0.05 at these time points. C and D, BLP in 100 and 1000 ng/ml concentrations once again produced a profound effect on PMN apoptosis (p < 0.001) at all time points. TLR-2 blockade reduced this effect and rendered the BLP-mediated delay in PMN apoptosis significant only at p < 0.01. After additional Bonferroni adjustment, however, some statistically significant differences were lost, namely between control PMN apoptotic rates and mAb TLR-2 effects at 1 and 10 ng/ml BLP doses at the 6-h time point.
time points, we did note that mAb 2392 restored the constitutive rate of apoptosis at 6 and 12 h in the 1 ng/ml BLP samples (Fig. 4A) and did similarly at 6 h in the 10 ng/ml BLP samples (Fig. 4B). At higher concentrations of BLP, we once again noted that the effects of TLR-2 blockade were incompletely compromised (Fig. 4, C and D).

FIGURE 5. The effect of combined CD14 and TLR-2 blockade on the BLP-mediated delay in PMN apoptosis. A and B, Combined blockade with mAb CD14 (mAb M5E2, 10 μg/ml) and TLR-2 (mAb 2392, 25 μg/ml) completely abrogated the effect of BLP (1 and 10 ng/ml) on PMN apoptosis at all time points. C, This effect was also seen at 6 and 12 h when we investigated how combined blockade affected the apoptotic influence of 100 ng/ml BLP and also reduced the effect of BLP on PMN apoptosis (p < 0.001) at 18 and 24 h (p < 0.01). D, Blockade of both receptors could not abrogate the effects of 1000 ng/ml BLP completely, but restored the apoptotic rate toward control levels in a statistically similar fashion to that seen at 18 and 24 h in Fig. 4C. After Bonferroni correction, we observed that statistically significant differences arose between control apoptotic rate and dual blockade in the 10 ng/ml BLP experiment at 24 h and the 100 ng/ml BLP experiments at 6 and 12 h, indicating that dual blockade lost some potency at these times and concentrations.

FIGURE 6. Effects of BLP (100 ng/ml) on PMN Δψm after 12 h of coincubation. Data pertaining to the Δψm of PMNs after exposure to BLP were acquired on a FACScan flow cytometer equipped with CellQuest software (BD Biosciences) with excitation at 488 nm and emission collected through a 530/30 band pass filter for FL1-H and a 585/42 band pass filter for FL2-H. A, On FL-1, depolarization is represented by a shift in the histogram to the right. BLP inhibited this depolarization at concentrations and time points similar to those displayed in previous graphs, but we present the findings of 100 ng/ml at 12 h. There is a substantial difference (p < 0.01) in the degree of depolarization evident at 0 and 12 h in control PMNs. The effects of BLP are easily recognized in that this shift to the right is not as pronounced, indicating a lesser degree of Δψm accrues. The double peak illustrates that at 12 h, BLP has not fully attenuated all depolarization and that apoptotic and nonapoptotic (BLP-affected) cells exist in all cell populations. B, On FL-2, the depolarization is represented by a shift to the left. We display the peaks evident at 0 and 12 h as controls illustrating the normal constitutive rate of membrane depolarization, and we illustrate how BLP attenuates this shift to the left at 12 h by stabilizing the mitochondrial membrane. We have included this histogram to demonstrate that BLP exerted the appropriate effects when both emissions are measured.
Combination blockade of TLR-2 and CD14 provided the greatest attenuation of the antiapoptotic effect of BLP. At 1 and 10 ng/ml concentrations of BLP, there was no demonstrable delay in apoptosis in the dual-blockade group (Fig. 5, A and B) except at the 24-h time point in the 10 ng/ml BLP sample. This illustrates the significant synergism of TLR-2 and CD14 in combination. As we had previously remarked, higher concentrations of BLP were antiapoptotic (Fig. 5, C and D), but to a lesser degree than observed in the single-blockade experiments.

BLP attenuates neutrophil Δψm

An orderly progression in the depolarization of the PMN mitochondrial membrane is a normal characteristic of constitutive PMN apoptosis. We measured this time-dependent phenomenon in both the presence and absence of BLP in 1–1000 ng/ml concentrations. We chose similar time points (6, 12, 18, and 24 h) and found that depolarization was significantly inhibited at all time points ($p < 0.01$) using all concentrations of BLP. This correlated well with the degree of inhibition of apoptosis, and was in fact a more sensitive test of PMN function, as we observed disparities in depolarization even at 6 h. The flow cytometrical histogram depicted in Fig. 6 is representative of the effects of BLP in the 100 ng/ml concentration and illustrates the degree of depolarization apparent at 12 h.

Bacterial lipoprotein delays PMN apoptosis through inhibition of caspase-3

A colorimetric assay was used to assess the contribution of caspase-3 activity to the perceived delay in PMN apoptosis attributable to BLP. Caspase-3 activity in test cells was unaltered at 0 and 3 h, but significantly inhibited at both 6 and 9 h ($p < 0.01$) as compared with control cells (Fig. 7A). A similar pattern was observed when we assessed how BLP might alter caspase-3 protein expression. The Western immunoblot measured the conversion of the proform of caspase-3 to the active form, a prerequisite for the ordered progression of apoptosis. As we would expect, there is a concomitant absence of active caspase-3 in PMNs treated with BLP across the 9-h time period.

FIGURE 7. Effect of BLP on PMN caspase-3 activity and protein expression. A, At 0 and 3 h, BLP exerted no appreciable effect on the degree of PMN caspase-3 activity, as measured by colorimetric reaction. At 6 and 9 h, however, a statistically significant difference in caspase-3 activity (+, $p < 0.01$) was clearly seen. Control PMNs demonstrated an increasing amount of caspase-3 activity correlating with the constitutive rate of apoptosis. The presence of BLP markedly inhibited caspase-3 activity, a phenomenon that translated into delayed PMN apoptosis. B, Similar effects were observed when we assessed how BLP might alter caspase-3 protein expression. The Western immunoblot measured the conversion of the proform of caspase-3 to the active form, a prerequisite for the ordered progression of apoptosis. C refers to control PMNs; B refers to those PMNs exposed to BLP. The numbers 0, 3, 6, and 9 represent hours in culture. At 0 h, the proform of caspase-3 exists to a large degree, and as time progresses there is a plainly evident reduction in the amount of caspase-3 present in its proform, as indicated by the C9 blot. This translates into a more pronounced presence of the active form of caspase-3 as we approach the 9-h mark. The presence of BLP prevented the processing of the proform of caspase-3 to its active form across all time points. B9 depicts a much greater amount of procaspase-3 than can be seen at 9 h in control PMNs (C9). As we would expect, there is a concomitant absence of active caspase-3 in PMNs treated with BLP across the 9-h time period.

FIGURE 8. Effects of CD14 and TLR-2 blockade on BLP-mediated PMN caspase-3 inhibition. In all experiments, BLP inhibited PMN caspase-3 activity at 6 and 9 h ($p < 0.05$). A, At 6 h, mAb M5E2 had no effect on BLP-mediated caspase-3 inhibition. At 9 h, however, pretreatment of PMNs with mAb M5E2 reduced this inhibition in a statistically significant fashion. B, At 6 h, mAb 2392 had no effect on BLP-mediated caspase-3 inhibition. At 9 h, however, pretreatment of PMNs with mAb 2392 had significantly lessened this inhibition to a greater degree than that seen with mAb M5E2. C, Dual blockade had more profound effects as pretreatment with both mAb 2392 and mAb M5E2 significantly prevented the BLP-mediated caspase-3 inhibition at both 6 and 9 h. These pairwise analyses were performed according to Tukey, but after Bonferroni correction the statistically significant difference observed at the 9-h time point mentioned in A above was lost. All other statistically significant observations were retained.
observed when we assessed PMN caspase-3 protein expression by Western immunoblotting after exposure to BLP. This was reflected in the degree of protein expression observed after 0, 3, 6, and 9 h in the presence and absence of BLP at a 100 ng/ml concentration. We observed the constitutive conversion of the proform of caspase-3 to the active form over this time period in control cells, a process that was significantly attenuated in the presence of BLP. This translated into more pronounced proform protein expression in BLP-treated cells over the same time period (Fig. 7B).

**BLP inhibition of PMN caspase-3 activity is attenuated by use of mAbs to CD14 and TLR-2**

To establish how blockade of CD14 and TLR-2 would contribute to the BLP-mediated inhibition of caspase-3 activity, we repeated the colorimetric experiments outlined above. PMNs were incubated with neutralizing mAbs specific for CD14 (mAb M5E2, 10 μg/ml) and TLR-2 (mAb 2392, 25 μg/ml) for 1 h at room temperature before exposure to BLP. We used the 10 ng/ml concentration of BLP in these experiments, as the effects of the mAbs (on PMN apoptotic rate) were most evident at lower concentrations of BLP. The 10 ng/ml concentration of BLP significantly inhibited caspase-3 activity at 6 and 9 h (p < 0.05). The observed effects on PMN caspase-3 activity correlated with those seen when we assessed PMN apoptotic rate. The presence of mAb CD14 did not effect a significant change in BLP-treated PMN caspase-3 activity at 6 h, but at 9 h there was a statistically significant difference between the caspase-3 activity of BLP-treated PMNs and those preliminarily incubated with M5E2. A similar pattern of caspase-3 activity in mAb 2392-treated cells was seen after exposure to BLP at 6 and 9 h. Dual blockade demonstrated the greatest impact on caspase-3 activity as it attenuated the BLP-mediated inhibition at both 6 and 9 h with respect to both control and other test groups (see above) with p < 0.05 at both time points after Bonferroni correction (Fig. 8).

**Discussion**

In this study, we present data illustrating the potent inhibitory effects of the ubiquitous bacterial cell wall component, BLP, on PMN apoptosis. In conditions such as the systemic inflammatory response syndrome, levels of known antiapoptotic mediators such as GM-CSF and IL-2 are raised, and this similarly pertains to LPS (20). The clinical progression of systemic inflammatory response syndrome from sepsis to severe sepsis and septic shock has been documented (21), as has the absolute impact of bloodstream infection on mortality. An overall estimate of attributable mortality is 25% when all organisms are considered, implying that the majority of deaths arise from infection rather than the underlying disease process (22). Quantitatively, BLP is the predominant immunoregulatory component common to the outer membrane of both Gram-negative and Gram-positive organisms, a fact that logically must translate to both blood-borne and site-specific sepsis arising from polymicrobial infection. Zhang et al. (23) have shown that growing bacteria release significant amounts of BLP, and during midlogarithmic growth ~1–1.5 μg of BLP/ml was detected in culture supernatants from different bacteria of the *Enterobacteriaceae* family. Spirochaetal organisms that lack LPS initiate considerable host injury presumably by lipoproteins that exist in generous quantities on their cell surfaces (24), and there is recent evidence that these molecules act as major proinflammatory agonists, influencing both innate and adaptive immune responses during bacterial infection (1, 2). Collectively, these data implicate BLPs in the pathogenesis and promulgation of infectious diseases in humans.

A characteristic feature of systemic inflammation of bacterial origin is delayed neutrophil apoptosis with its associated PMN-mediated local tissue damage. Our results indicate that BLP is capable of delaying PMN apoptosis to a similar degree, as has been previously observed with LPS (14). We performed side-by-side experiments comparing BLP and LPS and noted no differences in the antiapoptotic effects of either mediator (data not shown). A time- and concentration-dependent relationship between BLP and apoptotic rate was evidenced. In vitro concentrations of LPS <100 ng/ml are thought to represent physiological levels in vivo. We have shown that at 1 ng/ml, BLP significantly retards PMN apoptosis, and as dosages increase the effect becomes more pronounced until a plateau is reached at the 100–1000 ng/ml range. CD14 has been shown to play a role in PMN activation, as measured by CD11b/CD18 expression (5, 16), and for this reason we sought to elucidate its relative contribution to BLP-induced apoptotic retardation. We noted a partial, but significant restoration toward the constitutive rate of PMN apoptosis when PMNs were treated with M5E2 (a specific mCD14-blocking mAb) before BLP exposure. The restorative/blocking effect, however, was not perceived when higher concentrations of BLP were used (100–1000 ng/ml). This suggests that at high concentrations mCD14 is bypassed by BLP, a phenomenon noted by our group and Soler-Rodriguez et al. (5, 16), in Mac-1 expression and by others regarding the activating spectrum of LPS (25). It is entirely conceivable that at higher doses of BLP the soluble form of CD14 (sCD14) facilitates the delay in apoptosis by adopting the role of M5E2-blocked mCD14. As we are primarily concerned with membrane-bound receptors in this study (TLR-2 has not yet been shown to exist as a soluble protein in serum), we did not pursue this less sensitive signaling pathway. Furthermore, exclusion of FCS from experimental conditions as a method of investigating the role of sCD14 precludes accurate apoptotic study by depriving cells of growth factors constitutively present in serum.

BLP-mediated apoptotic delay in PMNs has not previously been shown, and that this effect is mCD14 dependent only at low doses prompted us to investigate the role of TLR-2 as the more crucial membrane transducer of its antiapoptotic effects. Lipoprotein signaling is thought to be primarily dependent upon TLR-2, and in monocytes induction of apoptosis is mediated through this receptor (3). Because BLP can activate macrophages from LPS-hyporesponsive mice (C3H/HeJ) (26), TLR-4 does not appear to be necessary for BLP activation of cells. We found that the mAb directed at TLR-2 (mAb 2392) was capable of inhibiting the antiapoptotic effects of BLP at all concentrations used (1–1000 ng/ml). The blocking potential of mAb 2392 was far more in evidence at lower concentrations of BLP. These findings therefore identified TLR-2 as the predominant receptor mediating the antiapoptotic effects of BLP. Combined blockade of TLR-2 and CD14 completely prevented BLP from modulating PMN apoptosis at 1 and 10 ng/ml concentrations. At higher concentrations (100–1000 ng/ml), we witnessed the retardative effects of BLP that we had previously observed in the individual blocking mAb experiments. A logical assumption in light of these results is that TLR-2 and CD14 are the most sensitive receptors to BLP particularly when it is present in low doses. At higher concentrations, CD11b/CD18 or sCD14 may facilitate BLP-induced alterations in PMN apoptotic rate, but this at most is a secondary mechanism and probably not directly pertinent to the physiological situation, except in overwhelming sepsis.

Intracellularly, apoptosis is governed by cysteine proteases known as caspases. The regulation of caspase activity is influenced by a number of key molecular events occurring early in the apoptotic process that dictate both the cast of caspases involved and
cytochrome c (cyto-c) from mitochondria. During apoptosis, cyto-c is released from mitochondria, allowing it to associate with other proapoptotic molecules in the cytosol. This free cyto-c forms an essential part of the vertebrate apoptosisosome, which is composed of cyto-c, Apaf-1, and procaspase-9 (28). Resultant activation of caspase-9 after formation of this complex initiates further cascade down to the executioner caspase-3. Other apoptotic mediators are also released from mitochondria. Some cells possess mitochondria containing procaspase-3 (29), and yet others release the caspase-activating molecule apoptosis-inducing factor that can process procaspase-3 in vitro (30). Current thinking suggests that mitochondrial depolarization involves collapse of the inner transmembrane potential, allowing the development of a permeability transition (31). Permeability transition pore opening results in a volume dysregulation of mitochondria due to matrix hyperosmolality with subsequent expansion. This expansion eventually causes outer membrane disruption, releasing caspase-activating proteins located within the intermembrane space into the cytosol. A number of proapoptotic stimuli such as the Bel-2 family member Bax, oxidants, ceramide, etc., promote release of cyto-c from mitochondria, and it has been shown that certain antiapoptotic mediators such as cyclosporins and bongkrekic delay apoptosis by stabilizing the mitochondrion (32). It has been demonstrated that LPS inhibits PMN apoptosis preferentially through stabilization of the mitochondrial membrane and subsequent inhibition of caspase-3 (33). As another bacterial wall product, we felt BLP would exert its effects in a similar fashion, and this was evidenced by attenuated PMN mitochondrial depolarization in the presence of BLP. We did not investigate the effects of BLP on caspase-8, as this caspase has been shown to be inhibited by LPS independently of mitochondrial depolarization and represents a lesser phenomenon. The particular limb of the apoptotic pathway primarily modulated by bacterial products (28, 33) necessarily incorporates caspase-9 (as part of the apoptosisome) en route to caspase-3 activation, so we similarly deferred investigating this component of the pathway. BLP had a pronounced effect on both the activity and protein expression of PMN caspase-3. We found that BLP prevented effective caspase-3 processing; the colorimetric assay illustrated a marked difference in the Western blot by decreased detection of the caspase-3 fragments (~20, 18, and 16 kDa) produced during constitutive apoptosis. We also established how blockade of TLR-2 and CD14 affected BLP-mediated inhibition of caspase-3. The inhibitory effects of BLP on caspase-3 activity mirrored its effects on apoptotic rate; we witnessed a similar pattern regarding caspase-3 activity and apoptosis when we used M5E2 and mAb 2392. It can be assumed, therefore, that blockade of either CD14 and/or TLR-2 inhibits the antiapoptotic activity of BLP through downstream communications with caspase-3. As LPS has been shown to inhibit PMN apoptosis preferentially through stabilization of the mitochondrial membrane and subsequent inhibition of caspase-3 (33), we reasoned that any downstream effects on caspase-3 by BLP would also reflect effects occurring at a mitochondrial level (as we have shown). Our results are similar to those found in LPS-mediated retardation of PMN apoptosis, and although this is only the second bacterial product investigated in this regard, our findings suggest that PMN apoptosis is modified in a similar fashion by all bacterial wall components irrespective of the membrane receptor they primarily recruit (TLR-2, TLR-4, etc.).

We have previously delineated how a variety of mediators (oxidants, heat shock responses, etc.) can accelerate cellular apoptosis (34–36); the most interesting of these, however, is enhanced neutrophil apoptosis via their direct ingestion of Escherichia coli. From a teleological perspective, phagocytosis of whole intact bacteria by neutrophils should trigger apoptotic death as it represents a successful mission and implies the cessation of further inflammatory activity. Bacterial lysis in contrast causes dissemination of bacterial wall components, and it is an intriguing facet of PMN bioactivity that ligation of both bacterial forms has such disparate functional implications. It would appear the factor governing this dichotomy is the presence of specific receptors capable of distinguishing the manner in which systemic bacterial presence is manifest. CD14 and TLR-2 are two such receptors, and their function in modulating PMN apoptosis in response to bacterial lipoprotein has been highlighted in this study.

Our study provides some valuable insights into PMN activity on a number of levels. Although we have previously shown that BLP tolerance results in overexpression of complement receptor type 3 and FcγIII/II in murine neutrophils (1), this is the first demonstration of a functional significance for any member of the TLR family in human PMNs. We have shown the pertinence of TLR-2 to the novel antiapoptotic effects of bacterial lipoprotein. Furthermore, we have delineated a role for CD14 in the delayed PMN apoptosis associated with these bacterial wall components. Our findings therefore provide evidence of a crucial role for both CD14 and TLR-2 in delayed PMN apoptosis arising from bacterial infection.

References


