Mechanisms of human complement factor B induction in sepsis and inhibition by activated protein C

Kim Goring,1,5* Yong Huang,1,5 Connie Mowat,1,5 Caroline Léger,4,5 Teik-How Lim,1,5 Raza Zaheer,1,5 Dereck Mok,1,5 Lee Anne Tibbles,1,5 David Zygun,1,3 and Brent W. Winston1,2,3,5

1Departments of Medicine, 2Biochemistry and Molecular Biology, and 3Critical Care Medicine; and 4Physiology and Biophysics and 5Immunology Research Group, University of Calgary, Calgary, Alberta, Canada

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Over recent years the coagulation cascade has gained some attention in determining the pathophysiology of sepsis. In fact, the only recognized specific therapy that has been shown to decrease the mortality associated with severe sepsis and septic shock is activated protein C (APC) (4, 41, 42). Unfortunately, the exact mechanism of its activity in lowering sepsis-related mortality is not known (22, 49) and is unlikely related to its anticoagulation effects alone (45). The anti-inflammatory effects of APC likely occur via interaction with endothelial protein C receptors on endothelial cells (36), neutrophils and monocytes (65), eosinophils (17), and epithelial cells (60) or may be a secondary effect from the inhibition of nuclear factor (NF)-κB activation (31).

In this study, we examined hBf gene expression in peripheral blood monocytes (PBMCs) from patients with septic shock compared with control intensive care unit (ICU) patients and we explored the mechanisms of hBf gene regulation by TNF-α, IFN-γ, and LPS in human monocytes/macrophages. TNF-α and IFN-γ or LPS stimulation of human monocytes showed a time- and dose-dependent induction of hBf mRNA expression.

SEPTIC SHOCK, a major cause of morbidity and mortality among hospitalized patients, is a clinical syndrome caused by the host response to invading microorganisms (1) through extensive activation of the inflammatory, complement, and coagulation systems (40). There is increasing evidence that activation of the alternative complement pathway may play an important role in the pathogenesis of septic shock (39, 61) especially because it can amplify all three pathways of complement following activation (15, 66). Complement factor B (Bf) is a serine protease that activates C3 convertase in the presence of factor D and properdin, thereby activating the alternative pathway of complement in the absence of antibodies. The alternative complement pathway is associated with inflammation, immunologic regulation, and bacterial cytotoxicity (16, 26). In addition to its role in activation of the alternative pathway and enhancing bacterial phagocytosis by macrophages, Bf may also play a role in B cell proliferation (51), monocyte cytotoxicity (37), macrophage spreading (5, 20), and apoptosis (69).

Whereas Bf is primarily synthesized in the liver (2, 44), it has been shown to be produced by a wide variety of extrahepatic cells, including monocytes/macrophages (27, 28, 71), fibroblasts (34), epithelial cells (64), and endothelial cells (55, 64). Macrophages have been shown to be an important extrahepatic site of Bf synthesis (28, 35) that may contribute significantly to the local concentration of Bf at sites of inflammation. Expression of inflammatory mediators such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS) at the site of inflammation induce Bf expression (27, 28, 35). In work done in animals and humans, these inflammatory mediators have also been shown to be pivotal in the pathogenesis of septic shock (24, 52, 74).

Recent work done in our laboratory has detailed the molecular regulation of murine Bf by TNF-α, IFN-γ, and LPS in murine macrophages (27, 28). Since Bf is a key component of the alternative pathway of complement, studying human Bf (hBf) gene regulation associated with sepsis may help us understand the pathophysiological mechanisms underlying the innate immune response to sepsis. Despite its potential importance, the exact mechanism of human Bf gene regulation is not clear.

* K. Goring Y. Huang contributed equally in the study of this paper.

Address for reprint requests and other correspondence: B. W. Winston, Depts. of Critical Care Medicine, Medicine and Biochemistry and Molecular Biology, Univ. of Calgary, Health Research Innovation Centre, Room 4C64, 3280 Hospital Dr., N.W., Calgary, Alberta, Canada, T2N 4N1 (E-mail: bwinston@ucalgary.ca).
A IFN-γ-activation site (GAS) and an NF-κB cis-binding site on the hBf promoter were required for IFN-γ and TNF-α, LPS-responsiveness, respectively. Additionally, treatment of human monocytes with recombinant human APC inhibited LPS-mediated hBf promoter activity as well as hBf protein expression, suggesting that the survival benefit conferred by APC in severe sepsis and septic shock may, in part, be due to effects on complement regulation.

**MATERIALS AND METHODS**

**Reagents.** TNF-α, IFN-γ, and macrophage-colony stimulating factor (M-CSF) were from R&D system; Pfu DNA polymerase was from Stratagene, and restriction enzymes were from New England Biolabs. Drotrecogin alfa (recombinant human activated protein C, APC) was from Eli Lilly Canada. pGL3-basic vector was from Promega and FuGENE6 was from Roche. LPS (0111:B4) was purchased from Sigma-Aldrich (Ontario, Canada).

Informed consent was obtained from all patients and volunteers according to the guidelines set out by the Conjoint Health Research Ethics Board of the University of Calgary and the Calgary Health Region. PBMCs were obtained from ICU patients with or without septic shock or from healthy volunteers. PBMCs were isolated from heparinized peripheral blood as previously described (47). Septic shock patients were identified according to the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference definitions (7). ICU nonseptic controls were composed of routine postoperative patients who were admitted to ICU for observation and had ≤2 systemic inflammatory response syndrome (SIRS) criteria. Blood was drawn within 12 h of entry into the ICU for both groups. U937 cells, a gift from Dr. Stephen Robbins, University of Calgary, were maintained in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (all from Invitrogen, Ontario, Canada).

**Isolation of total cellular RNA and RT-PCR.** Mononuclear cells were plated in culture dishes for 2 h; the total RNA was isolated using TRIzol (Invitrogen) as described by the manufacturer’s protocol. TRIzol was used to obtain the total RNA. The total RNA was isolated using TRIzol (Invitrogen) as described by the manufacturer’s protocol.

**hBf protein quantification.** PBMCs were collected as above and cultured in RPMI media supplemented with 10% FBS, 40 μg/ml M-CSF, 5 U/ml of penicillin, and 5 μg/ml of streptomycin. Cells were cultured for 4 days and then stimulated with LPS or TNF-α and IFN-γ for 48 h. Cell supernatant (50 μl) were used to quantify hBf protein expression using a microsphere-based flow cytometric assay developed in house. A monoclonal anti-Bf antibody (1379, a gift from Dr. Joshua Thurman, University of Colorado) was used as a capture antibody by coupling it to region 100 x-MAP beads (Luminex, Austin, TX) as previously described (67). Briefly, 1.25 × 10^6 beads were activated for 20 min in 80 μl of activation buffer (0.1 M NaH2PO4 at pH 6.2) containing 10 μl each of sulfo-NHS and EDC (each prepared fresh in ddH2O at 50 mg/ml). The beads were then incubated with 100 μg/ml anti-Bf capture antibody in coupling buffer (PBS pH 7.4) for 1 h, followed by a 30-min blocking step in PBS containing 1% bovine serum albumin (BSA) and 0.05% NaN3. The beads were resuspended in a final volume of 150 μl in the blocking solution, and 5 μl of beads were used per well. The secondary (detection) antibody was anti-hBf antiserum from Quidel (Santa Clara, CA), IgG-purified by SACRI (Sacramento, California), and biotinylated using EZ-Link Biotinylation kit (Pierce Biotechnology). Coupled beads were incubated with samples for 1.5 h, 7.5 μg/ml of secondary antibody for 1.5 h, and 1 mg/ml of Streptavidin-R-PE (Invitrogen) for 30 min. Each incubation was performed in the dark on an orbital shaker and followed by three washes with phosphate-buffered saline, pH 6.2. A minimum of 50 beads/well were read on a Luminex 200 System. Readings were acquired using StarStation 2.3 software, and a standard curve was generated using a five-parameter logistic curve-fitting algorithm.

**Isolation of human bF promoter.** 1270 bp of human Bf promoter were cloned from a human placenta genomic DNA library by PCR. The primers used for the cloning are 5'- GAGCGG- GACG-3' and 5'- TTCCCCGGGCACTAGGAGTTG-3'. The sequence of hBf promoter was confirmed by direct sequencing.

**Subcloning of pGL3-hb reporter gene and site-directed mutagenesis.** hBf-promoter-pGL3 chimeric reporter constructs were generated by restriction enzyme digestion followed by fragment ligation to the linearized pGL3 basic vector (Promega, WI). The GAS, interferon-stimulated response element (ISRE), and NF-κB binding elements on the hBf promoter were mutated using PCR site-directed mutagenesis as previously described (11, 57). Primers containing 5'- GAGCGG-CAACGGATTTGGTCGTAT-3' and 5'- TTCCCCGGGCACTAGGAGTTG-3' were designed to mutate the GAS, ISRE, NF-κB1, and NF-κB2 binding sites, respectively (mutated nucleic acids are underlined). The mutants were generated by PCR overlap extension method (27) using the hBf construct as template DNA. After PCR, the products containing the mutants were gel isolated, digested with appropriate restriction enzymes, and ligated into the pGL3-basic vector. The mutations were confirmed by direct sequencing.

**Transcriptional assay.** Transient transfection of U937 cells was performed using FuGENE6 according to the manufacturer’s protocol. The U937 cells were cotransfected with 1.5 μg of pGL3-hb reporter vector and 0.5 μg of LNC-Gal (from Dr. Stephen Robbins, University of Calgary). The cells were cultured for 20 h to allow expression of the transgenes. Transfectants were treated with media alone (control) or media in the presence of IFN-γ, TNF-α, or LPS for an additional 6 h. Cell lysates were analyzed for luciferase and β-galactosidase activities. Luciferase activity was measured using a luminometer (Molnlight 2010, Analytical Luminescence Laboratory). Measurements were taken for 30 s after mixing of the lysate and luciferin assay reagent (Promega). β-Galactosidase was assayed in cell lysates using a β-galactosidase enzyme assay kit (Promega). β-Galactosidase was used as an internal control for normalizing variability in reporter luciferase activity due to transfection efficiency or variability in the cell extract preparation. At least three independent experiments were done using each construct.

**Statistical analysis.** Descriptive statistics or box plots were used to analyze each variable separately. Analysis of continuous, normally distributed variables was undertaken using the Student’s t-test. Non-normally distributed continuous variables were analyzed using the Mann Whitney U test. A P-value < 0.05 was considered significant and all statistical tests were two-tailed.

**RESULTS**

Expression of hBf mRNA is increased in PBMCs isolated from patients with septic shock compared with control ICU patients. We first questioned whether hBf gene expression is upregulated in monocytes from septic shock patients compared with control ICU patients. There were 11 ICU patients in this study, 5 patients with septic shock and 6 control ICU patients (Table 1). The septic shock patients differed from the control ICU patients in the following parameters: the mean Acute Physiology and Chronic Health Evaluation II (APACHE II) score (28.6 ± 8.3 vs. 12.3 ± 6.0, P = 0.0043); maximal respiratory rate (32 ± 10 vs. 17 ± 9, P = 0.028); total Multiple Organ Dysfunction (MOD) score (12.4 ± 6.1 vs. 0.5 ± 0.5, P = 0.01); and minimum mean arterial pressure (MAP)
Table 1. **Patient characteristics**

<table>
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<tr>
<th>Characteristic</th>
<th>Septic Shock</th>
<th>Control</th>
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<tr>
<td>Number</td>
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<td>6</td>
</tr>
<tr>
<td>Age (Range), yr</td>
<td>62 (37–86)</td>
<td>57 (19–86)</td>
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<tr>
<td>Gender (F/M)</td>
<td>4/1</td>
<td>5/0</td>
</tr>
<tr>
<td>APACHE II</td>
<td>28.6±8.3</td>
<td>12.3±6.0</td>
</tr>
<tr>
<td>Maximum temp., °C</td>
<td>36.9±1.1</td>
<td>38.2±1.0</td>
</tr>
<tr>
<td>Minimum MAP, mmHg</td>
<td>53.4±10.6</td>
<td>80.2±6.5</td>
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<tr>
<td>Maximum heart rate, beats/min</td>
<td>32±10</td>
<td>17±9</td>
</tr>
<tr>
<td>Maximum creatinine, μM</td>
<td>275±162</td>
<td>73±26</td>
</tr>
<tr>
<td>Total MOD Score</td>
<td>12.4±6.1</td>
<td>0.5±0.5</td>
</tr>
</tbody>
</table>

Values are means ± SD. Blood was collected on study entry. F/M, female/male; APACHE II, Acute Physiology and Chronic Health Evaluation II; MAP, mean arterial pressure; MOD, multiple organ dysfunction.

(53.4 ± 10.6 mmHg vs. 80.2 ± 6.5 mmHg, P = 0.0006). The difference in maximal creatinine, maximal temperature, and maximal heart rate did not reach a level of statistical significance in these two populations. These data show that the septic shock patients have physiological characteristics compatible with septic shock and are different from the ICU control patients. To compare the level of hBf gene expression in both groups, we isolated PBMCs from these patients, purified total RNA, and assessed mRNA expression by RT-PCR. As shown in Fig. 1, the relative induction of hBf mRNA (as measured by densitometry corrected for GAPDH expression) was 2.5-fold higher in patients with septic shock compared with that of ICU control patients. These data show that hBf gene expression is increased in PBMCs from septic shock patients.

Inflammatory cytokines (TNF-α and IFN-γ) and LPS induce hBf mRNA expression in human PBMCs. We questioned whether IFN-γ and TNF-α, inflammatory mediators upregulated in septic shock (24, 74), or LPS would induce hBf mRNA expression in PBMCs ex vivo. TNF-α stimulation induced a low level of hBf mRNA expression by itself (Fig. 2), but TNF-α acted synergistically with IFN-γ to induce hBf mRNA expression in PBMCs. In the presence of IFN-γ, TNF-α produced a dose-dependent increase in the level of hBf mRNA (Fig. 2A). Similarly, in the presence of TNF-α, IFN-γ produced a dose-dependent increase in the level of hBf mRNA (Fig. 2B). A time course of hBf mRNA expression in the presence of TNF-α (10 ng/ml) and IFN-γ (1 ng/ml) is shown in Fig. 2C. Costimulation resulted in a detectable increase of hBf mRNA expression as early as 6 h after stimulation, which continued to increase through 24 h before beginning to decrease by 32 h.

LPS also showed a dose-dependent increase in the level of hBf mRNA expression (Fig. 2D). In addition, stimulation with 1 ng/ml LPS revealed a small detectable increase in hBf mRNA expression as early as 2 h, which continued to increase with time to peak at 6 h after stimulation (Fig. 2E). Thus costimulation with TNF-α and IFN-γ, or stimulation with LPS alone, results in dose- and time-dependent increases in hBf mRNA expression in human PBMCs.

The human Bf promoter is organized similar to that of murine Bf promoter. To examine the mechanism of hBf induction in human macrophages, we first cloned the hBf promoter from a human placenta genomic DNA library by PCR as described in the MATERIALS AND METHODS. Figure 3 compares the Bf promoter region from human and mouse genes and reveals that there is a significant amount of sequence identity between the two promoter regions. There are two potential NF-κB cis-binding sites [the first between −466 and −456 bp (N1) and the second located between −582 and −573 bp (N2)], a GAS binding site (−90 and −82 bp), and an ISRE binding site (−140 and −127 bp). The sequences of GAS and ISRE cis-binding sites in human and murine Bf promoters are identical. However, the sequences of the two NF-κB binding sites show I or 2 bp differences between the human and murine Bf promoters. These differences are highlighted with a double underline in Fig. 3.

We recently showed that the first −600 bp of the murine Bf promoter are critical for IFN-γ, TNF-α, and LPS responsiveness (27, 28). Therefore, we questioned whether this region of the human Bf promoter demonstrates similar responsiveness. The hBf promoter sequence that we cloned was identical to that present in the Gene Bank database (accession number NG_008191). 863 bp of the hBf promoter (−735 and +128) was subcloned into the pGL3-luciferase reporter vector [pGL3-hBf(−735)]. U937 cells, a human monocyte cell line, were transiently transfected with this reporter vector and then treated with media (control) or treated with TNF-α, IFN-γ, TNF-α-IFN-γ, or LPS in media for 6 h. The U937 cells were cotransfected with an LNC-Gal vector (constitutively expressing β-galactosidase). Cell lysates were analyzed 6 h after stimulation for luciferase activity as well as β-galactosidase activity [β-gal activity is used to control for transfection efficiency as previously described (27)]. Figure 4 shows that the transfected U937 cells treated with TNF-α, IFN-γ, TNF-α-IFN-γ, or LPS resulted in a 2.4-fold, 2.1-fold, 14.8-fold, or 2.4-fold increase in Bf promoter activity, respectively. These data are very similar to the hBf mRNA induction seen in stimulated PBMCs (Fig. 2). The synergistic response seen with IFN-γ and TNF-α costimulation has been well described with the murine Bf promoter (27).

The GAS cis element between −90 and −82 bp and activation of Stat1 are required for full expression of hBf in response to IFNγ. We examined which region of hBf promoter is responsible for IFNγ-mediated activation. A pGL3-hBf(−200) fragment, generated by restriction enzyme digestion of the hBf promoter, was transfected into U937 cells that were subsequently treated with IFN-γ (10 ng/ml). Figure 5A shows that

![Fig. 1. Human factor B (hBf) mRNA expression is increased in peripheral blood monocytes (PBMCs) isolated from patients with septic shock. A: induction of hBf mRNA in septic shock patients. PBMCs were isolated from ICU patients with septic shock (n = 5) and ICU control patients (n = 6). Total RNA was isolated and subjected to RT-PCR. Relative fold induction is shown on the y-axis. B: an example of RT-PCR from one control and one septic shock patient is shown. *Significant differences (P < 0.05) in mean relative hBf induction between the septic shock patient group and the ICU control patient group.](http://ajpcell.physiology.org/10.1152/ajpcell.00128.2009)
pGL3-hBf(-735) and pGL3-hBf(-200) resulted in 2.2- and 3.7-fold induction after IFN-γ treatment of U937 cells, respectively, indicating that the IFN-γ-responsive region is within the first −200 bp of the hBf promoter. This region contains potential GAS and ISRE binding sites. Since both ISRE and GAS cis-elements are involved in IFN-γ-stimulated Bf promoter activation in the mouse (28), we investigated whether the hBf promoter had both ISRE- and GAS-responsive cis-elements. Figure 3 reveals that both GAS (5′-TTCCGGGAA-3′; −90 to −82 bp) and ISRE (5′-AGTTTCGTTTCT-3′; −140 to −127 bp) cis-binding sites on the hBf promoter are identical in sequence to that of the murine promoter. To identify which of these sites may be involved in IFN-γ-responsiveness of the hBf promoter, the cis sequences of hBf-GAS and hBf-ISRE were mutated by overlapping PCR site-directed mutagenesis. The wild-type reporter plasmid pGL3-hBf(−735) was used to construct plasmids containing mutations in hBf promoter that mediates TNF-α-stimulated promoter activation in monocytes, we analyzed sequential 5′-hBf promoter deletion fragments. As shown in Fig. 6A, a series of hBf promoter deletion fragments ranging from −735 to −200 bp were cloned into the pGL3-luciferase reporter vector. Transiently transfected U937 cells were treated with media alone (control) or medium alone (control) for 6 h, and then cell lysates were analyzed for luciferase activity. Figure 6A shows that TNF-α treatment of U937 cells transfected with pGL3-hBf (−735) resulted in a 2.2-fold induction of luciferase activity when compared with control cells. The pGL3-hBf (−605) and pGL3-hBf (−571) constructs induced a 2.8-fold and 2.9-fold increase in luciferase activity, respectively, when stimulated with TNF-α. The construct containing −200 bp of the hBf promoter [pGL3-hBf (−200)] was unresponsive to TNF-α stimulation, indicating that the TNF-α-responsive region is located between −571 and −200 bp. Figure 4 shows potential NF-κB cis-binding sites within −735 bp of the transcription initiation site of the hBf promoter region. The first potential NF-κB cis-binding site (N1) is located between −466 and −456 bp and a second (N2) potential NF-κB cis-binding site is between −582 and −573. To further determine which of these sites is involved in TNF-α-responsiveness, the NF-κB cis-binding sequences were mutated by overlapping PCR site-directed mutagenesis. The wild-type construct and mutants were transiently transfected into U937 cells and subsequently stimulated with TNF-α (10 ng/ml) or medium alone (control) for 6 h. We found that the potential NF-κB binding site between −582 and −573 bp (N2) was not required for hBf induction by TNF-α (Fig. 6B). However, mutation of the NF-κB site between −466 and −456 bp (N1) abolished TNF-α-induced luciferase activity in transfected monocytes (Fig. 6B). These data indicate that the NF-κB cis-binding element at −466 to −456 bp, but not that between −582 to −573 bp, is required for hBf promoter activity by TNF-α.

The phosphorylation of Stat1 on tyrosine-701 and subsequent dimerization, translocation to the nucleus, and GAS cis element are required for the activation of hBf promoter by IFN-γ in human monocytes.

The NF-κB cis-element between −466 and −456 bp and activation of NF-κB are required for induction of hBf promoter activity in response to TNF-α. To identify the region of the hBf promoter that mediates NF-κB-stimulated promoter activation in monocytes, we analyzed sequential 5′-hBf promoter deletion fragments. As shown in Fig. 4A, a series of hBf promoter deletion fragments ranging from −735 to −200 bp were cloned into the pGL3-luciferase reporter vector. Transiently transfected U937 cells were treated with media alone (control) or medium alone (control) for 6 h, and then cell lysates were analyzed for luciferase activity. Figure 4A shows that TNF-α treatment of U937 cells transfected with pGL3-hBf (−735) resulted in a 2.2-fold induction of luciferase activity when compared with control cells. The pGL3-hBf (−605) and pGL3-hBf (−571) constructs induced a 2.8-fold and 2.9-fold increase in luciferase activity, respectively, when stimulated with TNF-α. The construct containing −200 bp of the hBf promoter [pGL3-hBf (−200)] was unresponsive to TNF-α stimulation, indicating that the NF-κB-responsive region is located between −571 and −200 bp. Figure 4 shows potential NF-κB cis-binding sites within −735 bp of the transcription initiation site of the hBf promoter region. The first potential NF-κB cis-binding site (N1) is located between −466 and −456 bp and a second (N2) potential NF-κB cis-binding site is between −582 and −573. To further determine which of these sites is involved in TNF-α-responsiveness, the NF-κB cis-binding sequences were mutated by overlapping PCR site-directed mutagenesis. The wild-type construct and mutants were transiently transfected into U937 cells and subsequently stimulated with TNF-α (10 ng/ml) or medium alone (control) for 6 h. We found that the potential NF-κB binding site between −582 and −573 bp (N2) was not required for hBf induction by TNF-α (Fig. 6B). However, mutation of the NF-κB site between −466 and −456 bp (N1) abolished TNF-α-induced luciferase activity in transfected monocytes (Fig. 6B). These data indicate that the NF-κB cis-binding element at −466 to −456 bp, but not that between −582 to −573 bp, is required for hBf promoter activity by TNF-α.
NF-κB is normally retained in the cytoplasm by IkB, the inhibitor of NF-κB. After cellular stimulation, the IkB proteins are phosphorylated and rapidly degraded by proteasomes, allowing the nuclear translocation of the NF-κB dimer (9, 19, 33, 73). We investigated whether the proteasome inhibitor MG132 affected TNF-α-induced hBf induction. pGL3-hBf (−735) transfected U937 cells were preincubated for 30 min with 1.0 μM of MG132 and then treated with TNF-α for 6 h. As shown in Fig. 6C, MG132 completely blocked the activation of hBf by TNF-α. We also used a dominant negative NF-κB [IkB-αS52A,S36A, NF-κB(DN)] construct to block IkB phosphorylation and subsequent proteasome-mediated degradation of IkB by TNF-α. We used a human and murine 5′ untranslated promoter region of the Bf gene. A: comparison of human (hBf) and murine (mBf) promoter sequence shows significant sequence homology. B: schematic of the Bf promoter shows the position of the cis-binding elements of a number of potential trans-acting factors (NF-κB, ISRE, GAS) on the Bf promoter. The relative position of the cis-binding element on the Bf promoter is indicated as base pair (bp) distance from the transcription initiation site. The exact binding sequences of potential NF-κB, ISRE, and GAS cis-binding elements are indicated. Double underlined bases identify where the human sequence differs from the mouse sequence in the transcription factor cis-binding site. Arrowhead denotes transcription initiation site.

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Fig. 3. Human and murine 5′ untranslated promoter region of the Bf gene. A: comparison of human (hBf) and murine (mBf) promoter sequence shows significant sequence homology. B: schematic of the Bf promoter shows the position of the cis-binding elements of a number of potential trans-acting factors (NF-κB, ISRE, GAS) on the Bf promoter. The relative position of the cis-binding element on the Bf promoter is indicated as base pair (bp) distance from the transcription initiation site. The exact binding sequences of potential NF-κB, ISRE, and GAS cis-binding elements are indicated. Double underlined bases identify where the human sequence differs from the mouse sequence in the transcription factor cis-binding site. Arrowhead denotes transcription initiation site.
gene regulation by TNF-α, cytes from septic shock patients and the mechanisms of hBf

reduced (that LPS-stimulated hBf protein expression was significantly
PBMCs induce Bf protein expression (Fig. 8)

DISCUSSION

tion of hBf promoter activity by LPS. In addition, NF-κB(DN) also completely abolished LPS-induced hBf promoter activity (Fig. 7B). These data demonstrate that LPS-mediated hBf pro-
moter activation depends on the phosphorylation and degrada-
tion of IkB and NF-κB binding to the cis element at −466 to −456 bp on the hBf promoter.

APC inhibits LPS-stimulated hBf promoter activity in human monocytes. APC has been shown to be effective in the treatment of patients with severe sepsis (4). APC is believed to have both anti-inflammatory and anticoagulant activities (53). However, the exact mechanism by which APC treatment improves the outcome in sepsis is still not clear. We therefore examined the effect of APC on LPS-mediated hBf promoter activity in U937 cells. As shown in Fig. 8A, pretreatment of pGL3-hBf (−735) promoter:reporter-transfected U937 cells with APC (9 nM for 18 h) completely blocked the activation of hBf promoter activity by LPS (10 ng/ml). To test this inhibition ex vivo, PBMCs from healthy volunteers were cultured in media containing FBS and M-CSF as described in the MATERIALS AND METHODS. We show that LPS-stimulated PBMCs induce Bf protein expression (Fig. 8B). We also show that LPS-stimulated hBf protein expression was significantly reduced (P = 0.018) in PBMCs pretreated with APC (Fig. 8B). Therefore, blocking LPS-mediated hBf induction by APC may be one mechanism by which APC confers its protective effect in severe sepsis.

In this study, we investigated hBf gene expression in monocytes from septic shock patients and the mechanisms of hBf gene regulation by TNF-α, IFN-γ, and LPS in human mono-
cytes/macrophages. PBMCs isolated from ICU patients with septic shock showed increased hBf mRNA expression com-
pared with control ICU patients. The increased levels of hBf in septic shock patients, along with previous evidence showing cleavage (activation) of Bf and activation of the alternative complement pathway in sepsis (39), strongly suggest that Bf and the alternative complement pathway play an important role in sepsis and septic shock. Costimulation with TNF-α and IFN-γ or stimulation with LPS demonstrated a time- and dose-dependent induction of hBf mRNA expression in PBMCs isolated from human volunteers.

We cloned the human Bf promoter region and showed that the region between −735 and +128 bp mediated IFN-γ, TNF-α, and LPS responsiveness as well as the synergistic effect of IFN-γ/TNF-α on hBf promoter activity. Site-directed
mutagenesis of a GAS cis-binding element (−90 to −82 bp),
but not a potential ISRE site (−140 to −127), abrogated IFN-γ responsiveness. A Stat1 dominant negative mutant blocked hBF induction by IFN-γ indicating the importance of Stat1 phosphorylation (activation) on IFN-γ signaling and activation of the hBF promoter. Mutagenesis of a NF-κB cis-binding element at −466 to −456 bp, but not that between −582 and −573 bp, abrogated TNF-α and LPS responsiveness of the hBF promoter. A NF-κB dominant negative mutant and the proteasome inhibitor MG132 blocked hBF promoter activation by TNF-α and LPS. These findings show that hBF gene expression is induced in monocytes from septic shock patients and that the induction of hBF by IFN-γ, TNF-α, and LPS is through GAS and NF-κB cis-binding sites on the hBF promoter. Most interestingly, recombinant human APC inhibited LPS-stimulated hBF promoter activity and hBF protein induction suggesting that APC may block hBF expression in sepsis as a potentially new mechanism of its therapeutic effect in severe sepsis.

Many studies provide evidence that the complement system is activated in patients with sepsis and septic shock (21), and activation of the complement system is at least partially responsible for the proinflammatory effects seen in sepsis (16, 33). Moreover, activation of the complement system alone can induce septic shock-like symptoms (58). Previous reports have shown that the alternative pathway of complement contributes significantly to the innate immune response in sepsis (38, 39, 61). The active form of Bf (its cleavage fragment, Bb) is elevated in patients with septic shock (39), indicating a potential role for the alternative pathway in the development of septic shock. There are, however, few reports showing hBF gene expression in septic shock. To our knowledge, this is the first report to demonstrate that hBF gene expression was in-

Fig. 6. The nuclear factor (NF)-κB cis-binding element (−466 to −456 bp) and NF-κB are involved in induction of hBF promoter activity in response to TNF-α in U937 cells. U937 cells were cotransfected with hBF-pGL3 constructs and an LNC-Gal plasmid and then rested for 20 h before treatment. Transfected cells were treated for 6 h before analyzing cell lysates for luciferase and β-galactosidase activities. A: U937 cells were transfected with pGL3-hBF constructs of different promoter fragment lengths (as indicated) and treated with media alone (C) or 10 ng/ml of TNF-α (T). B: U937 cells were transfected with either wild-type (−735 to +128 bp) or mutated constructs and treated with media alone (C) or 10 ng/ml of TNF-α (T). N = NF-κB site (N1 for site at −456, N2 for −573); X over any site indicates it was mutated). C: U937 cells transfected with pGL3-hBF (−735) and LNC-Gal plasmid and cotransfected with NF-κB(DN) or pretreated with MG132 (1 μM) and then treated with media alone (C) or 10 ng/ml of TNF-α (T). * and **Significant difference (P < 0.05) of mean values between TNFα-treated groups and between groups with and without MG132 or NF-κB(DN) pretreatment, respectively. N = 3 for each experimental group. Mean relative values ± SE are shown.

Fig. 7. NF-κB cis-binding element (−466 to −456 bp) and NF-κB are involved in induction of the hBF promoter activity in response to LPS in U937 cells. U937 cells were cotransfected with pGL3-hBF promoter constructs and a control LNC-Gal plasmid and then rested for 20 h before treatment. Transfected cells were treated for 6 h before analyzing cell lysates for luciferase and β-galactosidase activities. A: U937 cells were transfected with either wild-type (−735 to +128 bp) or mutated hBF promoter constructs and treated with media alone (C) or 10 ng/ml of TNF-α (T). N = NF-κB site (N1 for site at −456, N2 for −573; X over any site indicates it was mutated). * and **Significant difference (P < 0.05) of mean values between LPS-treated groups and between groups with and without MG132 or NF-κB(DN) pretreatment, respectively. N = 3 for each experimental group. Mean relative values ± SE are shown.
Monocytes/macrophages play an important role in initiating and regulating immune and inflammatory responses (73). Monocytes/macrophages produce proinflammatory cytokines including TNF-α and IFNs, which in turn induce Bf production (27, 28, 35). Studies in animals and humans have shown that TNF-α (52), IFN-γ (24), and LPS (74) are pivotal mediators in the pathogenesis of septic shock. Therefore, monocytes/macrophages may provide a source of localized Bf induction through the localized actions of TNF-α, IFN-γ, and LPS in septic shock. In this report, we show the upregulation of Bf gene expression by IFN-γ, TNF-α, and LPS in human monocytes as well as the upregulation of Bf protein expression in PBMCs upon LPS stimulation. Since complement may be an important factor in the development of shock in sepsis (12, 29, 30, 54), determining the mechanism of Bf gene expression by IFN-γ, TNF-α, and LPS is highly relevant. This is the first step in attempting to clarify the role of Bf in sepsis and septic shock.

IFN-γ stimulates the production of Bf and influences the effects of other proinflammatory cytokines such as TNF-α (27). The induction of Bf by IFN-γ in PBMCs is consistent with previous reports (63); however, the mechanism has never been detailed. We have recently shown that Stat1 and IRF-1 are involved in IFN-γ signaling and that both GAS and ISRE cis elements are required for the induction of Bf in murine macrophages by IFN-γ (28). The sequences of GAS and ISRE cis-elements are identical in both human and murine Bf promoters. However, here we show that Stat1 signaling to the GAS cis-element (~90 to ~82) of the human Bf promoter is necessary and sufficient for Bf induction by IFN-γ in human monocytes, suggesting species-specific gene regulation of hBf. Nonaka et al. (48) described a region in the hBf promoter between ~260 and +19 bp that was not sufficient to confer IFN-γ responsiveness in HepG2 cells, which may suggest cell-specific gene regulation as well. Although the liver plays a key role in Bf induction systemically, monocytes/macrophages may well play an important role in localized Bf induction in sepsis. Our data on hBf gene regulation in monocytes/macrophages may not be generalizable to Bf gene regulation in human hepatocytes.

Like the murine Bf promoter, the human Bf promoter contains two potential NF-κB cis-binding elements. By using site-directed mutagenesis of potential NF-κB sites, we found that a NF-κB cis-binding element at ~466 to ~456 bp, but not the site between ~582 and ~573 bp, is necessary and sufficient for TNF-α-mediated hBf promoter responsiveness. This is in agreement with our previous study conducted on the murine Bf promoter activity in macrophages, where the NF-κB site closest to transcriptional start site is responsible for TNF-α-mediated promoter activity (27). We have found that hBf induction by TNF-α was inhibited by the proteasome inhibitor MG132 and a dominant negative NF-κB. This supports the concept that Bf induction by TNF-α is dependent on the activation of NF-κB, a process that requires IkB phosphorylation and ubiquitin-mediated proteasomal degradation. When PBMCs were treated with TNF-α and IFN-γ together, we were able to show a synergistic response as well as dose- and time-dependent increases in gene expression of hBf. Mutation of both the GAS and NF-κB cis-binding sites on the hBf promoter completely blocked the induction of hBf by TNF-α and IFN-γ (data not shown), suggesting that both GAS and NF-κB cis-element binding are necessary for the synergistic effect. The synergistic effect may reflect the importance of cytokine interactions in amplifying their biological effects in sepsis and septic shock. Interestingly, TNF-α and IFN-γ augment Bf promoter activity to a greater extent on the human Bf promoter than on the murine Bf promoter (28). The difference between them may be due to a small difference in promoters, species dependence, or cell type difference.

In addition to IFN-γ and TNF-α, LPS is known to induce Bf (27, 34). LPS has been considered to be a key inflammatory factor in Gram-negative sepsis and septic shock (10). The effect of LPS is through initiating multiple intracellular signaling events, including the activation of NF-κB (27), which leads to synthesis and release of proinflammatory mediators, including TNF-α (59). The presence of increased LPS and TNF-α is clearly associated with a poor clinical outcome in patients with septic shock (18). In the experiments presented here, we show a dose- and time-dependent induction of hBf by LPS in PBMCs, predominantly mediated via the NF-κB signaling pathway. We found that mutation of the NF-κB cis-binding site between ~466 and ~456 bp blocked hBf promoter activation by LPS, as did the proteasome inhibitor MG132 and cotransfection of the dominant negative NF-κB. It is possible that LPS...
may induce other mediators such as TNF-α and IFNs, which subsequently act on the hBF promoter to potentiate Bf induction.

It is worth noting that hBF promoter responsiveness to IFN-γ, TNF-α, and LPS was performed using a U937 cell line, an early transformed monocyte cell line. The transfected hBF promoter responsiveness of the cell line was similar to the relative Bf induction seen in isolated PBMC-derived macrophages in vitro. Because there are potential limitations in generalizing the data from a transformed cell line to the effects seen in vivo in human monocytes/macrophages, we showed that LPS-stimulated human PBMCs induced hBF mRNA and protein.

To date, the only effective therapy for severe sepsis or septic shock has been found to be recombinant human APC (4). In a randomized, double-blind, placebo-controlled trial it reduced mortality by 6.1% in severe sepsis (4). We show here that treatment of human monocytes with APC inhibited LPS-mediated upregulation of hBF promoter activity and protein expression, suggesting a potential mechanism of action for APC treatment in severe sepsis and septic shock. Further investigation is required to understand the mechanism(s) mediating the inhibition by APC. It is possible that APC blocks LPS-mediated hBF induction via inhibition of cytokine production such as TNF-α (31, 32, 45) or by inhibiting NF-κB activation (31, 72). In fact, we have determined that APC does inhibit TNF-α protein induction in LPS-stimulated PBMCs (data not shown). Thus the effects of APC on LPS-stimulated hBF induction in PBMCs may be direct or indirect, through the inhibition of cytokine induction.

Inflammation-mediated Bf upregulation may contribute to the genesis of shock in sepsis by augmenting complement activation. Sepsis has been shown to activate complement (38, 39, 61), leading to the formation of C5a and C5a, which have been shown to contribute to the pathogenesis of septic shock (21, 23). Excessive production of C5a can lead to an unregulated pro-inflammatory response, ultimately causing tissue injury and multiorgan failure (29). Recent data indicate that blockade of either C5a or C5aR with antibodies or antagonist substantially improves survival in septic animal models (12, 29, 30). In other animal models of *Streptococcus pneumoniae*-induced septic shock, Saeland et al. (56) showed that spleens isolated from wild-type and C1q−/− mice manifested hyperemia and thrombotic vessel occlusion. However, no thrombus formation was seen in spleens of C2/Bf−/− mice. Thus Bf and the alternative pathway of complement may contribute to shock-induced intravascular coagulation. Since Bf may contribute to the amplification of complement activation, we would hypothesize that Bf−/− mice would be protected from LPS-mediated complement overactivation and endotoxic shock. However, Matsumoto et al. (43) showed that Bf−/− mice and wild-type mice have similar endotoxin sensitivities after intraperitoneal injection with 0.75 or 1 mg of *Salmonella typhosa* LPS. Since complement activation in sepsis occurs by all three pathways, using a large sepsis stimulus in a Bf−/− knock-out model may overwhelm the effect of Bf deficiency. In several other models of inflammatory disease, Bf−/− mice have been shown to be protected from injuries resulting from ischemia-reperfusion (66), allergic encephalomyelitis (45), collagen induced (24) and passively transfused arthritis (3), and autoimmune disease (25, 46, 68, 70). Therefore, the role of Bf in sepsis and septic shock needs further detailed investigation.

Understanding the pathway from cytokines (TNF-α, IFN-γ, and IL-1β) and LPS to Bf induction and subsequent complement activation may help clarify the mechanism of complement-mediated host damage seen in septic shock. Septic shock is a complicated disorder that is initiated by excessive and unregulated inflammatory cytokine production and release of bacterial cell wall components like LPS (6). Although activation of complement pathways has beneficial effect through lysis or opsonization of invading microorganisms, excessive activation could have harmful effects through activation of several pro-inflammatory pathways (54). Therefore selective inhibition of the alternative pathway may be more advantageous than inhibition of all three complement pathways because it would maintain the other two complement pathways intact against pathogens. Furthermore, because Bf serves as a focal point involved in the amplification of active complement by all three complement pathways (16, 39), inhibition of Bf activation may have added protection from excessive complement activation.

In summary, we demonstrated that Bf gene expression is induced in PBMCs from septic shock patients. Costimulation with TNF-α and IFN-γ, or stimulation with LPS demonstrated a time- and dose-dependent induction of hBF mRNA expression in PBMCs isolated from healthy human volunteers. The induction of Bf by IFN-γ, TNF-α, and LPS is through specific GAS and NF-κB cis-binding sites on the human Bf promoter. TNF-α and IFN-γ synergistically induce hBF promoter activation in monocytes/macrophages. Interestingly, LPS-stimulated hBF promoter activation and protein induction is inhibited by APC suggesting a new potential mechanism of the beneficial action of APC in severe sepsis.

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