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Anti-Endotoxin Therapy in Primate Bacteremia with HA-1A and BPI

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Objective

The *in vivo* neutralizing activities of an anti-lipopolysaccharide (LPS) antibody HA-1A (Centoxin [Centocor, Malvern, PA]), a human immunoglobulin M monoclonal antibody, and of bactericidal/permeability-increasing protein (BPI), an endogenously produced human LPS-neutralizing protein, were studied in a primate model of lethal *Escherichia coli* bacteremia.

Summary Background Data

HA-1A has been used with variable success against LPS activity in some animal models and in a recently reported clinical trial. However, no data assessing the efficacy of this agent in subhuman primates is available. Bactericidal/permeability-increasing protein is a product of polymorphomononuclear cells (PMNs) that is stored in azurophilic granules and exhibits LPS-neutralizing activity *in vitro* and in some *in vivo* models.

Methods

Immediately after *E. coli* infusion and in a blinded fashion, three baboons were treated with BPI (5 mg/kg bolus infusion and 95 μg/kg/min infusion over 4 hr). Three animals received 3 mg/kg BW of HA-1A, whereas another three baboons received a placebo treatment.

Results

The BPI-treated animals demonstrated significantly ($p < 0.03$) lower circulating LPS-limulus amoebocyte lysate (LAL) activity compared with the control animals, but this reduction in LPS-LAL activity was not associated with improved survival. HA-1A treatment did not reduce LPS-LAL activity. However, both BPI and HA-1A treatment did attenuate the pro-inflammatory cytokine response.

Conclusion

The current data suggests that incomplete neutralization of endotoxin activity does not alter mortality from severe bacteremia. Given the diversity of mediator production under such circumstances, a strategy of combination therapy in the form of anti-lipopolysaccharide and anti-cytokine treatment may be necessary to achieve optimal survival.

The acute systemic and tissue-specific sequalae of gram-negative infections are initiated by endotoxin (lipopolysaccharide [LPS]), a compound of the outer membrane of the gram-negative bacterial cell wall.[1-3] The physiologic derangements seen in sepsis are caused by the reaction of LPS with responsive immune competent cells, resulting in excessive production of inflammatory mediators.[4-7] Our previous work demonstrated that successful blockade of one or more pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, results in significantly higher survival rates in a primate model of *Escherichia coli* septic shock.[8,9]

Much recent interest has focused on blocking LPS activity as the proximal signal of the inflammatory mediator cascade. Several approaches directed toward neutralization of LPS activity have been pursued vigorously, including direction of monoclonal antibodies (mAbs) toward structures on the core glycolipid of LPS.[10-13] One such mAb (HA-1A) is a human immunoglobulin M generated from a hybridoma cell line that purportedly binds specifically to an epitope within the lipid A domain of bacterial endotoxin. This antibody has been used with variable success against LPS activity in some animal models,[14-16] as well as in a recently reported clinical trial.[17] In this clinical study, administration of HA-1A was associated with decreased 28-day all-cause mortality in a subgroup of patients with documented gram-negative bacteremia.

Alternative therapeutic approaches also are possible, including one that uses an endogenously derived LPS-neutralizing protein.[18] The neutralization efficacy of one such protein, the bactericidal/permeability-increasing protein (BPI), a ~55 kd protein, has been reported *in vitro*[19,20] and *in vivo*.[21] Bacterial/permeability-increasing protein is a product of polymorphomononuclear cells (PMNs) that is stored in azurophilic granules and is specifically cytotoxic for many gram-negative bacteria.[22,23] The target-cell specificity of the cationic BPI is attributable to its strong affinity for the negatively charged LPS.[24] Binding of BPI to susceptible bacteria, such as *E. coli*, is followed rapidly by discrete cell wall alterations, resulting in increased outer membrane permeability to hydrophobic substances, inhibition of growth, and ultimately, loss of bacterial cell viability.[25]

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Because a proposed mechanism of action of BPI and HA-1A is to bind and neutralize LPS, this study tested the ability of each protein to neutralize LPS activity after the infusion of live *E. coli* in subhuman primates. Compared with placebo treated animals, BPI significantly ($p < 0.03$) neutralized LPS. In contrast, HA-1A did not neutralize LPS activity as assessed by the chromogenic limulus lysate assay. Both BPI and HA-1A attenuated the TNF-α and IL-6 cytokine response to bacteremia; neither agent prevented the animals from progressing into septic shock and consequent mortality in this model.

MATERIAL AND METHODS

The bactericidal/permeability-increasing protein was provided by Incyte Pharmaceuticals, Inc. (Palo Alto, CA). The LPS content of recombinant BPI is less than 1 EU/mg. The protein was supplied as a 5 mg/mL-solution in phosphate-buffered saline (PBS). HA-1A was purchased from Centocor B.V. (Leiden, The Netherlands) as a 5 mg/mL-solution containing 5% human serum albumin.

Frozen aliquots of *E. coli (086:B7)* were grown initially in 0.2 L-flasks of trypticase-soy broth at 37 C overnight, and subsequently, were cultured at 37 C for 18 hours on trypticase-soy broth agarose slants. Slants were washed with normal saline, and cells were washed, pelleted, and reconstituted in physiologic saline. To assure uniform mortality with simultaneous antibiotic therapy (gentamicin sulfate, 2 mg/kg), *E. coli* were diluted with saline to an optical density of 2.80 absorbance units at 550 nm, corresponding to 2×10^{11} CFU/kg BW, or approximately twice the quantities reported previously.[8,9,26] In every case, the number of viable bacteria administered was confirmed by serial log dilution and culturing on sheep blood agarose pour-plates at 37 C overnight.

Prior studies defined the influence of gentamicin sulfate (Elkins-Sinn, Inc., Cherry Hill, NJ) on the *in vivo* appearance of endotoxin during *E. coli* infusion.[27] This was undertaken to both mimic the clinical setting in which antibiotic therapies routinely are employed and potentially increase the amount of bacterial lipopolysaccharide released from the infused *E. coli*. Pharmacokinetic studies indicated that a loading dose of BPI (5 mg/kg), followed by an continuous infusion dose of 95 μg/kg/min would achieve the projected circulating level of 2 μg/mL of blood.[27]

HA-1A was administered as 3 mg/kg BW over 15 minutes, which is a slightly higher dosage than used in a human study reported by Ziegler et al.[17]

Nine baboons (Papio anubis), weighing 14 to 21 kg were purchased from Southwest Foundation for Bio-

medical Research (San Antonio, TX). All the animals were quarantined for a minimum of 2 weeks at the Research Animal Resource Center of Cornell University Medical College (CUMC) to confirm their good health and lack of disease communicable to humans. The experiment protocol was approved by the Institutional Animal Care and Use Committee at CUMC. The baboons were studied in groups of three, and one animal in each study group was treated with placebo. Three BPI-treated animals, three HA-1A-treated animals, and three placebo-treated animals were studied. Preliminary results of the BPI treated animals are reported elsewhere.[27]

Study Protocol

After an overnight fast, animals were anesthetized with ketamine (10 mg/kg intramuscularly), and the cephalic vein was cannulated percutaneously. Thereafter, anesthesia was maintained by intravenous administration of sodium pentobarbital. The upper airway was controlled by placement of a cuffed endotracheal tube, and the animals maintained spontaneous respirations. An arterial catheter and a pulmonary artery catheter were placed percutaneously via the femoral artery and vein, respectively. These permitted repeated systemic arterial and pulmonary arterial blood sampling, as well as continuous monitoring of heart rate, systemic blood pressure, pulmonary capillary wedge pressures, cardiac output, and core temperature. An indwelling urinary catheter was placed to allow urine collection and monitor urine output. All animals received 0.9% NaCl (3 mL/kg/hr) as maintenance intravenous fluid.

After baseline blood sampling and a waiting period of at least 1 hour to allow equilibration, the *E. coli* was administered via the femoral vein over 15 minutes, followed immediately by 2 mg/kg of gentamicin. Then the animals were administered either the placebo, HA-1A, or BPI. The control group received a saline solution as placebo. Arterial blood samples were obtained at 30, 60, 90, 120, and 150 minutes, and at hourly intervals thereafter through 8 hours. The treatment regimens were assigned randomly, and investigators caring for the animals were blinded to treatment.

Length of the Study

All animals were monitored over a period of 8 hours after the *E. coli* administration. In case of a drop in blood pressure of > 30% from baseline together with an increase of heart rate of > 30%, animals received the bolus administration of 10 mL/kg of lactated Ringer's solution. After 8 hours, the animals were brought back to their cages after removal of all monitoring lines. The animals were checked frequently until their death, or if necessary, were killed by the intravenous administration of 65 mg/kg of sodium pentobarbital at 48 hours.

Analysis

Concentrations of TNF-α, soluble TNF receptor-I (sTNFR-I), IL-1β, IL-1 receptor antagonist (IL-1ra), and IL-6 were analyzed from serial blood samples as described previously.[8,9] Tumor necrosis factor-α cytotoxicity was assessed using the WEHI 164 clone 13 fibroblast bioassay; the sensitivity of the assay is 15 to 30 pg/mL.[28] To determine the total concentrations of the sTNFR-I, an affinity-purified polyclonal goat antibody was used as the capture protein. Rabbit polyclonal antisera raised against human sTNFR-I (rabbit no. A 8081) were purified by affinity chromatography using Affi-Gel 10 (Bio-Rad, Rockville Centre, NY) columns coupled with sTNFR-I. Aliquots of the affinity-purified antibodies were biotin conjugated with NHS-LC-biotin (Pierce, Rockford, IL) according to the manufacturer's specifications and were used as the second antibody in the enzyme-linked immunosorbent assays (ELISAs). The concentration of sTNFR-I was calculated according to a standard curve generated with human sTNFR-I. The sensitivity of the assay is 0.2 ng/mL.

Interleukin-1β was determinate by ELISA. The sandwich ELISA uses two monoclonal antibodies, one biotinylated, and signal amplification is achieved through a biotin/streptavidin-conjugated horseradish peroxidase system. The sensitivity of the ELISA is 10 pg/mL.

For IL-1ra determination, a sandwich ELISA using a mouse monoclonal antibody raised against nonglycosylated recombinant human IL-1ra was used (R&D Systems, Inc., Minneapolis, MN). A goat polyclonal antibody conjugated with horseradish peroxidase was used to visualize the captured protein. Recombinant human IL-1ra was used as the standard, and samples were assayed in the presence of equivalent quantities of control human plasma depleted of IL-1ra.

Interleukin-6 activity was determined using the B.9 hybridoma cell proliferation assay.[29] One unit of activity is defined as the quantity of IL-6 required to produce half-maximal proliferation (lower limit of detection, 200 B.9 U/mL). Samples were heat inactivated at 56 C for 30 minutes before analysis and were assayed at a dilution of 1:100 or greater.

Limulus Amoebocyte Lysate Assay

Endotoxin levels were determined using an endotoxin-specific chromogenic limulus test, the Endospecy test (Seikagaku Corporation, Tokyo, Japan).[30] Plasma

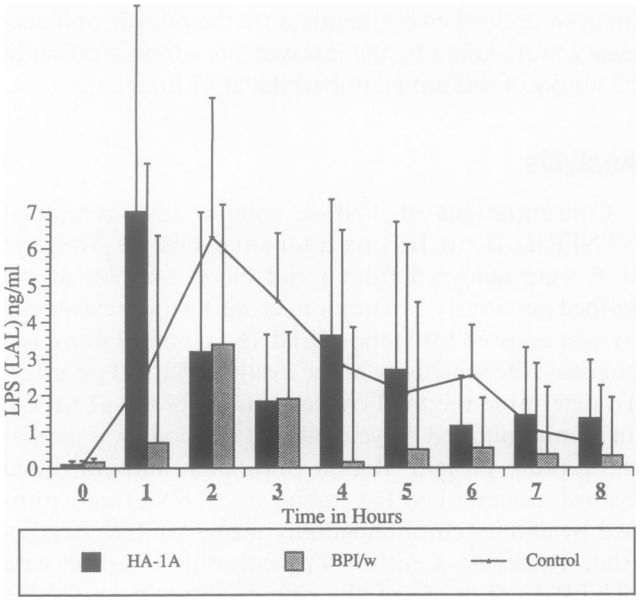

Figure 1. Endotoxin (LPS) plasma levels after *E. coli* administration of 2 × 10^{11} CFU/kg at time 0 hours. Whereas BPI treatment demonstrated a 70% decrease in circulating LPS activity (ANOVA $p < 0.03$), HA-1A did not neutralize LPS activity.

was pretreated by the perchloric acid method (PCA method) to remove factors interfering with the limulus test. Plasma samples were diluted 500-fold. An aliquot of 50 μL of this diluted plasma was then used. For quantitative measurement, a synthetic chromogenic substrate (n-tert-butoxycarbonyl-L-leucy-L-glycyl-L-arginine-p-nitroaniline) was added to the lysate to act as the substrate for the clotting enzyme. A 50 μL-aliquot of limulus reagent was added to 50 μL of treated plasma, mixed for several seconds, and incubated at 37 C for 30 minutes. The amount of p-nitroaniline (pNA) released from the substrate was detected after diazo-coupling. The absorbance of the solution was measured at 545 nm, and a standard curve was plotted on a bilogarithmic scale. The sensitivity of this assay is 2 pg/mL.

Bactericidal/permeability-increasing protein analyses for pharmacokinetic purposes were assayed, by sandwich ELISA, as reported previously.[19] The capture antibody was polyclonal (rabbit) anti-BPI. The reporter antibody was biotinylated rabbit anti-BPI. Unknowns were determined by comparison to standards of full-length recombinant human BPI. Results are presented as ng/mL plasma.

Statistics

Changes in LPS and cytokine concentrations were evaluated by analysis of variance (ANOVA) and Newman-Keuls' multiple range tests. Correlation coefficients were obtained using least squares. Significance was determined at the 95% confidence level.

RESULTS

One hour after *E. coli* administration, blood LPS-LAL activity in the control animals showed a mean value of 2.20 ± 0.36 ng/mL, and peak levels at 2 hours of 6.86 ± 3.22 ng/mL. Animals treated with BPI had markedly lower LPS-LAL activity at 1 hour of 0.69 ± 0.57 ng/mL, and peak LPS-LAL activity of 3.39 ± 2.1 ng/mL. The levels for the HA-1A treated animals were 7.00 ± 5.66 ng/mL at 1 hour and 3.18 ± 3.8 ng/mL at 2 hours. In contrast to HA-1A, BPI decreased the amount of LPS-LAL activity significantly, $p < 0.03$ (Fig. 1).

Tumor necrosis factor-α bioactivity (WEHI) peaked 2 hours after *E. coli* administration in both the control and BPI-treated animals, whereas HA-1A-treated animals demonstrated peak TNF-α activity at 2.5 hours. All animals demonstrated a characteristic monophasic pattern of TNF-α activity; the peak levels for the control animals were 35.7 ± 10.5 ng/mL, for the BPI-treated animals, 28.2 ± 16.7 ng/mL, and for the HA-1A-treated group, 27.3 ± 6.9 ng/mL (Fig. 2).

Interleukin-1β levels increased gradually over an 8-hour period after the *E. coli* infusion in both the placebo-treated and the HA-1A-treated animals. In contrast, BPI-treated animals demonstrated peak plasma IL-1β levels of 1757 ± 1048 pg/mL after 2.5 hours. The HA-1A-treated animals demonstrated peak levels of 826 ± 433 pg/mL at 8 hours, whereas the control animals displayed peak IL-1β plasma levels of 1157 ± 425 pg/mL 8 hours after the *E. coli* administration (Fig. 3).

Plasma IL-6 bioactivity (B.9) increased between 1.5 and 2.5 hours in all animals. In contrast to both the BPI

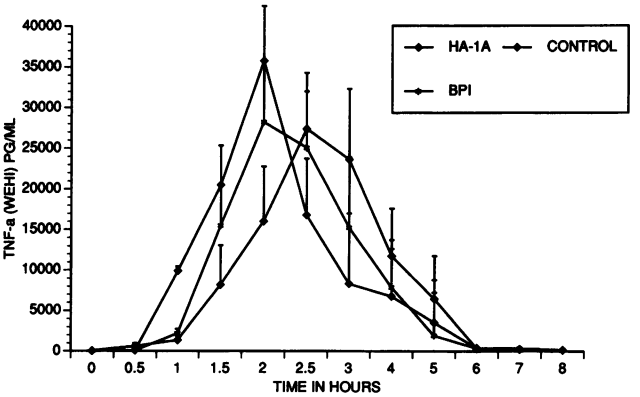

Figure 2. Tumor necrosis factor-α activity after both HA-1A and BPI treatment; p = NS. In the HA-1A group, a shift to the right was demonstrated.

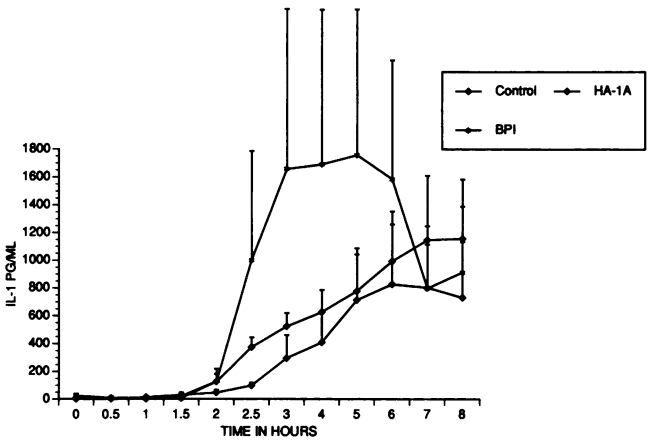

Figure 3. HA-1A treatment resulted in lower IL-1β cytokine response over the 8-hour study period, compared with control animals. In contrast, BPI stimulated IL-1β during the first 6.5 hours; p = NS.

and the HA-1A-treated animals, the control animals sustained higher levels of IL-6 bioactivity over the 8-hour period after the *E. coli* administration. After a peak level of 20975 ± 13346 U/mL at 5 hours and a drop to 10436 ± 2340 U/mL, a second peak of 20357 ± 8638 U/mL at 8 hours was demonstrated. Interleukin-6 bioactivity in the BPI and HA-1A-treated animals was attenuated, albeit not significantly, compared with controls throughout the 8-hour evaluation period. Peak levels of IL-6 bioactivity for the BPI group were 4836 ± 2909 U/mL at 4 hours, and for the HA-1A group, 13011 ± 2008 U/mL at 8 hours (Fig. 4).

Plasma IL-1ra levels increased 2.5 hours after the *E. coli* administration and peaked at 8 hours. The lowest circulating levels at 8 hours were observed in the BPI group, at 512 ± 154 ng/mL. The HA-1A group demonstrated 1034 ± 278 ng/mL, and the control group demonstrated at 1031 ± 544 ng/mL. The BPI group showed lower levels (p = 0.06) throughout the evaluation period (Fig. 5).

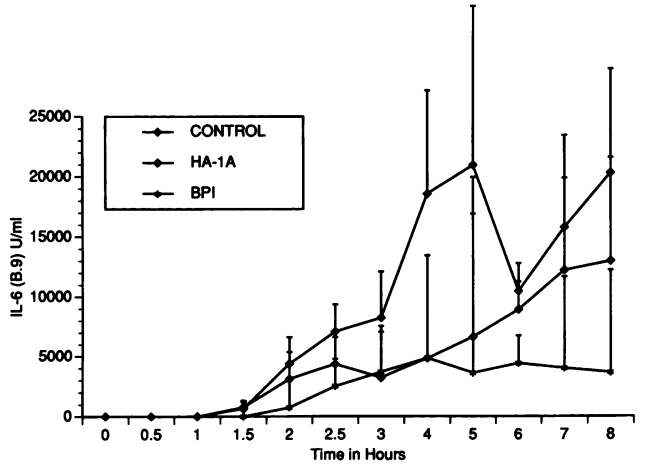

Figure 4. Both BPI and HA-1A treatment decreases IL-6 activity throughout the evaluation period; p = NS.

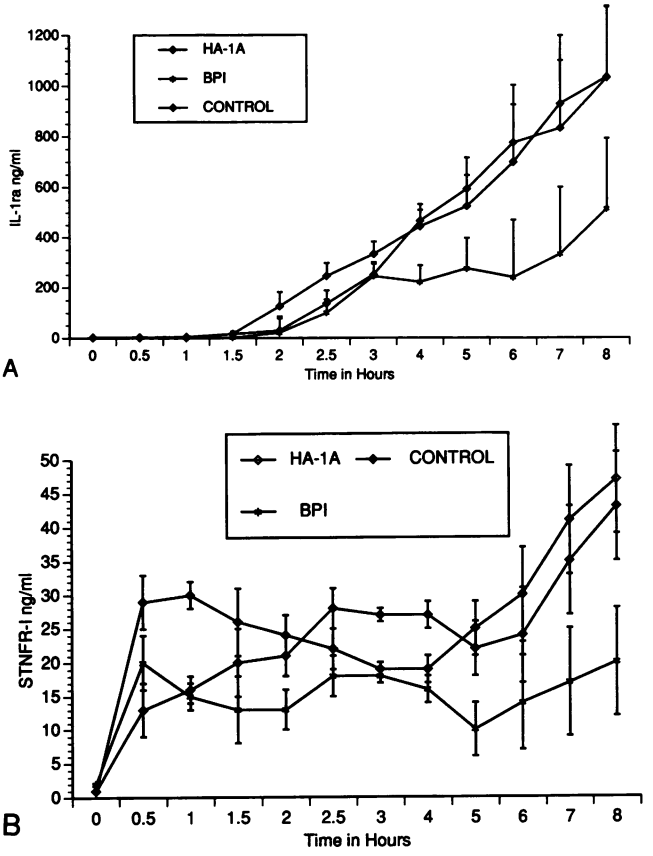

Figure 5. (A) Bactericidal/permeability-increasing treatment partially attenuated IL-1ra levels; p = 0.06. The pro-inflammatory cytokine inhibitory response appeared at ~2 hours after the *E. coli* administration and increased over the evaluation period. (B) Circulating endogenous cytokine antagonist sTNFR-I (p55) was attenuated by BPI treatment. In contrast to the respective proinflammatory cytokine, the attenuation over the entire study period was statistically significant; ANOVA p < 0.05.

Circulating sTNFR-I levels were elevated within 30 minutes after *E. coli* administration; the BPI group demonstrated levels of 20 ± 5 ng/mL, the HA-1A group had levels of 29 ± 4 ng/mL, and the control animals demonstrated levels of 13 ± 2 ng/mL. Thereafter, BPI-treated animals exhibited significantly (p < 0.05) lower sTNFR-I levels compared with the group control and HA-1A treated animals. Soluble TNFR-I levels peaked after 8 hours in the control animals at 43 ± 15 ng/mL, and levels of 47 ± 8 ng/mL were observed in the HA-1A group. BPI-treated animals displayed circulating sTNFR-I levels of 20 ± 6 ng/mL at 8 hours (Fig. 5A).

Hemodynamic Response

All baboons demonstrated hemodynamic instability within 30 to 60 minutes after the *E. coli* administration.

Table 1. HEMODYNAMICS IN PRIMATE BACTEREMIA WITH SEPTIC SHOCK

Hemodynamics	Time/Hr	BPI (n = 3)	HA-1A (n = 3)	Control (n = 3)
Blood pressure (%)	0	100	100	100
	2	74 ± 7	62 ± 9	81 ± 5
	4	59 ± 4	70 ± 15	69 ± 4
	6	79 ± 2	79 ± 13	80 ± 7
	8	86 ± 3	67 ± 4	78 ± 15
Heart rate (%)	0	100	100	100
	2	153 ± 18	139 ± 4	154 ± 3
	4	162 ± 13	131 ± 7	153 ± 7
	6	152 ± 9	135 ± 4	141 ± 7
	8	150 ± 13	141 ± 5	117 ± 26
Cardiac output (%)	0	100	100	100
	2	130 ± 8	103 ± 12	121 ± 4
	4	136 ± 17	112 ± 27	128 ± 11
	6	101 ± 11	88 ± 16	117 ± 13
	8	95 ± 7	75 ± 14	86 ± 12

Data are expressed in mean ± SEM as a percentage of baseline values at time 0 hours = 100%.

E. coli administration resulted in tachycardia of 145 ± 13 heart beats per minute and a decline in mean arterial blood pressure within 3 hours in the control animals from 113 ± 7 mm Hg to 64 ± 9 mm Hg, and in the BPI-treated animals, from 107 ± 8 mm Hg to 51 ± 3 mm Hg. The HA-1A group experienced a drop in mean arterial blood pressure from 121 ± 12 mm Hg to 66 ± 14 mm Hg. A hyperdynamic state of septic shock was observed in the BPI group and the control groups. Thirty minutes after *E. coli* administration, cardiac output increased from 1.95 ± 0.2 L/min to 2.55 ± 0.3 L/min, whereas the BPI group displayed an increase from 2.09 ± 0.3 L/min to 2.86 ± 0.4 L/min. Thirty minutes after the *E. coli* administration, the cardiac output in the HA-1A group was merely stable, between 1.9 ± 0.1 L/min to 2.0 ± 0.4 L/min (Table 1).

Survival at 48 hours after the *E. coli* administration was unaffected by either BPI or HA-1A treatment. One animal in the BPI group (at 18 hours) and one animal in the control group (at 15 hours) died before 24 hours. All of the remaining animals died within 48 hours.

DISCUSSION

Recent clinical studies have demonstrated the difficulty in achieving fully effective anti-endotoxin therapy during human sepsis. Although the confounding clinical variables influencing such therapies have been discussed extensively,[15–17] most of these approaches were never subjected to reported evaluation in large animal models. Indeed, the results of a trial of anti-LPS treatment with HA-1A in a canine model[14] were not published until well after the results of human trials[17] were reported widely.

Our study demonstrated partial LPS neutralization by the administration of BPI. By contrast, HA-1A has no LPS neutralizing effect in this septic baboon model. Although the LPS neutralizing capacity of BPI is well documented *in vitro*,[18,19,22] the epitopic specificity of HA-1A has been more problematic.[15,31–33] A recent study demonstrated that HA-1A binds to endotoxin via an epitope in the lipid A domain of LPS by an interaction with the V region of the antibody.[34] However, the aforementioned trial of HA-1A in a canine model of gram-negative septic shock did not alter the magnitude of bacteremia or endotoxemia, and actually decreased survival.[14] The authors of the latter study concluded that HA-1A therapy in humans should be limited until the conditions under which the beneficial or deleterious effects of this monoclonal antibody were defined more completely.

One purported mechanism of LPS clearance by HA-1A has been suggested by Siegel et al.,[35] who demonstrated that HA-1A may lower bioavailability of endotoxin by mediating binding of LPS to the complement receptor-I (CR-I) on erythrocytes and subsequent clearance by phagocytosis of the red blood cells via the reticuloendothelial system or direct internalization by peripheral blood neutrophils. In our study, we did not find any evidence of reduced LPS activity in the baboons treated with HA-1A, compared with both the placebo or the BPI-treated groups.

In a recent published study, Kelly et al.[36] demonstrated the bactericidal activity of BPI in a murine model; in this model, BPI enhanced significantly bacterial clearance from the vascular and pulmonary systems. Bactericidal/permeability-increasing protein shares sequence homology with the serum LPS binding protein (LBP).[37] However, BPI has divergent effects because LBP lacks bactericidal activity and, as an acute phase protein, LBP markedly potentiates the ability of LPS to activate inflammatory cells. Indeed, recombinant LBP promotes LPS-stimulated production of TNF-α by LPS responsive THP-1 cells, particularly at low LPS concentrations.[38] To reduce LPS binding to inflammatory cells, BPI must compete successfully with serum LBP for binding to LPS and gram-negative bacteria. In fact, Trown et al. recently demonstrated a significantly higher binding affinity to LPS for BPI than for LBP.[39] Bactericidal/permeability-increasing protein also was found to have a significantly higher affinity (15–35-fold) than LBP in binding to *E. coli J5* bacteria.

Currently, it is widely presumed that several classes of endogenous pro-inflammatory mediators, such as cytokines, are central to the sequalae of the sepsis syndrome. Compelling experimental evidence supports a pivotal role for the pro-inflammatory cytokines TNF-α and IL-1β as mediators of the early hemodynamic collapse during conditions of endotoxemia or bacteremia.[4–6,40] It has been demonstrated in experimental studies that the infusion of TNF-α can precipitate both systemic and organ-specific responses reminiscent of those observed during severe clinical infection, and recent evidence demonstrates a state of cardiovascular compromise during the administration of IL-1α.[8] Furthermore, the presumptive treatment of sepsis with specific cytokine antagonists, such as soluble receptors to TNF or an IL-1ra, appears to abrogate many of the adverse consequences of the bacteremic state.[8,9]

Results of two clinical studies with anti-cytokine treatment in septic patients with either an anti-TNF-α murine mAb, or the IL-1ra have been reported recently.[41,42] Survival estimates in each study failed to demonstrate any advantage with either therapy. However, subgroup analyses of those patients with increased TNF-α levels at the beginning of the study did show a trend toward improved survival with a high dose of anti-TNF mAb (10mg/kg).[41] A similar observation was reported in the IL-1ra study, in which the more critically ill patients also benefited from anti-cytokine therapy.[42]

The host cytokine response after the live *E. coli* challenge was attenuated, although not significantly, in the HA-1A-treated animals. This might explain some positive outcome of an earlier study,[17] in which cytokine levels of the investigated patients were not reported. In this study, survival was improved in patients with bacteremia and shock, while bacteremic patients without shock did not benefit from the HA-1A treatment.[17,43] This observation may be a result of the interference with the host cytokine response by HA-1A, as displayed in our study.

The BPI-treated animals also appeared to influence the pro-inflammatory cytokine inhibitors, IL-1ra and the sTNFR-I. Unlike the HA-1A group, in the BPI group, sTNFR-I and IL-1ra were attenuated compared to the placebo-treated animals, $p < 0.05$ and $p = 0.06$, respectively. Previously, we reported that both sTNFR-I and IL-1ra are induced by endotoxin administration in normal subjects and are detected consistently in critically ill patients.[7,9]

Interleukin-1ra, unlike sTNFR, which binds to the circulating ligand, binds directly to the IL-1 receptor, thereby preventing the agonist influence of either IL-1β or IL-1α. Consequently, IL-1ra is a true receptor antagonist, and the binding of IL-1ra to its receptors does not result in signal transduction or any known agonist properties.[44]

The sTNFR-I detected in the circulation represents the extra cellular domain of the TNF type I receptor. *In vitro* studies have demonstrated that this soluble receptor binds to TNF and competitively inhibits binding to cellular receptors on U-937 cells.[45] We observed that sTNFR-I—TNF complexes are biologically inactive and are not detected with some immunoassays,[9] such as those used in this report, for free TNF activity. In a recent study of critically ill patients, we observed that soluble TNF receptor-I levels were significantly lower ($p < 0.001$) in survivors as compared with nonsurvivors.[7] This observation, together with results from Van der Poll et al.,[46] who demonstrated a significant correlation between TNF-α immunoreactivity and circulating soluble TNF receptor-I levels, also confirm data from the aforementioned clinical trial.[41]

Although there was a significant decrease in LPS levels in BPI-treated animals compared with controls, all animals developed the sequalae of septic shock. This included a fall in mean arterial pressure, tachycardia, and acutely increased cardiac output, and all animals ultimately died. These changes are consistent with the results from our earlier studies of *E. coli* septic shock in primates.[8,9,26] As discussed previously,[27] the current model varies slightly from that used previously to evaluate anti-cytokine therapies. Nevertheless, cytokine levels achieved in response to bacteremia are comparable among control groups in these studies. The most notable differences between our prior studies with anti-cytokine therapy and the present study are observed in the magnitude of hemodynamic changes and the survival of treated animals. In fact, prior studies demonstrated a significant improvement in acute survival by a blockade of either TNF-α or IL-1.[8,9]

The magnitude of LPS neutralization achieved in the current study still does not appear to attenuate sufficiently the pro-inflammatory cytokine release to achieve improved survival. The extent of LPS neutralization necessary to diminish this otherwise adverse level of cytokine activation has yet to be established. Our current study suggests that a greater than 70% LPS neutralization (< 2 ng LPS/mL) is necessary for a beneficial response.

In addition to the anti-LPS agents used in this study, other therapies targeting LPS are in the early stages of development.[47] The LPS-binding protein and the CD14 receptor[48] that transmits signals from LPS-LBP complexes, also may become targets for direct immunotherapy. Macrophage activation and cytokine production in response to endotoxin are diminished significantly by either depletion of LBP in serum or by blocking the CD14 receptor with specific antibodies.[49,50]

In addition to stimulating the mediator cascade through the increased activity of TNF-α,[51] LPS can elicit a variety of different pathways leading to the deleterious host response. For instance, the generation of arachidonic acid by phospholipase A_2 in cell membranes is an important early event in the activation of most mediator cells. Subsequent metabolism of the arachidonic acid so generated by either the cyclooxygenase pathway, leading to the production of prostaglandins, or the lipooxygenase pathway, leading to the production of leukotrienes, is critical to the development of host inflammatory responses.[52] There is abundant evidence that LPS can elicit the production of leukotrienes and prostaglandins.[53,54]

Taking into account the aforementioned results of two multicenter trials with different anti-cytokine monotherapies in critically ill patients[42,43] and the pluripotent stimulation activity of LPS, our data suggest that a strategy of combination therapy in the form of anti-LPS and anti-cytokine treatment may alter survival. Opal et al.[55] have demonstrated previously in a rodent model of *Pseudomonas* sepsis that this immunotherapeutic approach does provide greater protection than the monotherapy modality.

References

1. Morrison DC, Ulevitch RJ. The effects of bacterial endotoxins on host mediation systems. Am J Pathol 1978; 93:526–617.
2. Brandtzaeg P, Kierulf P, Gaustad P, et al. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J Infect Disease 1989; 159:195–204.
3. Morrison DC, Ryan JL. Endotoxin and disease mechanisms. Ann Rev Med 1987; 38:417–432.
4. Hesse DG, Tracey KJ, Moldawer LL, et al. Cytokine appearance in human endotoxemia and primate bacteremia. Surg Gynecol Obstet 1988; 166:147–153.
5. Spinas GA, Bloesch D, Kaufmann M-T, et al. Induction of plasma inhibitors of interleukin-1 and TNF-α activity by endotoxin administration to normal humans. Am J Physiol 1990; R993–R997.
6. Michi HR, Manogue KR, Spriggs DR, et al. Detection of circulating tumor necrosis factor after endotoxin administration. N Engl J Med 1988; 318:1481–1486.
7. Rogy MA, Coyle SM, Oldenburg HSA, et al. Persistently elevated soluble TNF receptor and IL-1 receptor antagonist levels in critically ill patients. J Am Coll Surg 1994;178:132–138.
8. Fischer E., Marano MA, Van Zee KJ, et al. Interleukin-1 receptor blockade improves survival and hemodynamic performance in *Escherichia coli* septic shock, but fails to alter host responses to sublethal endotoxemia. J Clin Invest 1992; 89:1551–1557.
9. Van Zee KJ, Kohno T, Fischer E, et al. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor-α *in vitro* and *in vivo*. Proc Natl Acad Sci U S A 1992; 89:4845–4849.
10. Miner KM, Manyak CL, Williams E, et al. Characterization of murine monoclonal antibodies to Escherichia coli J5. Infect Immunol 1986; 52:56–62.
11. Greenman RL, Schein RMH, Martin MA, et al. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of gram-negative sepsis. JAMA 1991; 266:1097–1103.
12. Young LS, Gascon R, Bermudez LEM. Monoclonal antibodies for treatment of gram-negative infections. Rev Infect Dis 1989; 11: S1564–S1569.
13. Dunn DL, Bogard WC Jr, Cerra FB. Efficacy of type-specific and cross reactive murine monoclonal antibodies directed against endotoxin during experimental sepsis. Surgery 1985; 98:283–290.
14. Quezado ZMN, Natanson C, Alluig DW, et al. A controlled trial of HA-1A in a canine model of gram-negative septic shock. JAMA 1993; 269:2221–2227.
15. Baumgartner JD, Hermann D, Glauser MP. The HA-1A monoclonal antibody for gram-negative sepsis. N Engl J Med 1991; 325: 281–282.
16. Teng NNH, Kaplan HS, Herbert JM, et al. Protektion against gram-negative bacteremia and endotoxemia with human monoclonal antibodies. Proc Natl Acad Sci USA 1985; 82:1790–1794.
17. Ziegler EJ, Fisher CJ, Sprung CL, et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. N Engl J Med 1991; 324:429–436.
18. Ooi CE, Weiss J, Doerfler ME, et al. Endotoxin-neutralizing properties of the 25 kD N-terminal fragment and a newly isolated 30 kD C-terminal fragment of the 55–60 kD bactericidal/permeability-increasing protein of human neutrophils. J Exp Med 1992; 174: 649–655.
19. Marra NM, Wilde CG, Griffith JE, et al. Bactericidal/permeability-increasing protein has endotoxin neutralizing activity. J Immunol 1990; 144:662–666.
20. Marra MN, Wilde CG, Collins MS, et al. The role of bactericidal/permeability-increasing protein as a natural inhibitor of bacterial endotoxin. J Immunol 1992; 148:532–537.
21. Opal SM, Fisher CJ, Cross AS, et al. Bactericidal/permeability-increasing protein as an anti-endotoxin therapeutic agent: comparisons with anti-core glycolipid polyclonal antibody therapy. (abstract) Clin Res 1992; 40:231.
22. Weiss J, Elsbach P, Shu C, et al. Human bactericidal/permeability-increasing protein and a recombinant NH_2-terminal fragment cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria. J Clin Invest 1992; 90:1122–1127.
23. Weersink AJL, van Kessel KPM, Van den Tol ME, et al. Human granulocytes express a 55-kDa lipopolysaccharide-binding protein on the cell surface that is identical to the bactericidal/permeability-increasing protein. J Immunol 1993; 150:253–263.
24. Shafer WM, LE Martin, Spitznagel JK. Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. Infect Immun 1984; 45:29–34.
25. Mannion BA, Weiss J, Elsbach P. Separation of sublethal and lethal effects of the bactericidal/permeability-increasing protein on *Escherichia coli*. J Clin Invest 1990; 86:631–641.
26. Fong J, Tracey KJ, Moldawer LL, et al. Antibodies to cachectin/tumor necrosis factor reduce IL-1β and IL-6 appearance during lethal bacteremia. J Exp Med 1989; 170:1627–1633.
27. Rogy MA, Oldenburg HSA, Calvano SE, et al. The role of bactericidal/permeability-increasing protein in the treatment of primate bacteremia and septic shock. J Clin Immunol 1994; 14:120–133.
28. Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods 1986; 95:99–105.
29. Aarden LA, De Groot ER, Schaap OL, Lansdorp PM. Production of hybridoma growth factor by human monocytes. Eur J Immunol 1987; 17:1411–1416.

30. Iheda T, Hirata K, Tabuchi K, et al. Quantitative measurement of endotoxin in canine plasma using the new endotoxin-specific chromogenic test. Circ Shock 1987; 23:263–269.
31. Bogard WC Jr, Daminano AO, Leone AO, et al. The human monoclonal antibody HA-1A: studies on the epitope location within the endotoxin molecule and epitope exposure on the surface of viable gram-negative bacteria. (abstract) Circ Shock 1991; 34: 119.
32. Baumgartner JD. Immunotherapy with antibodies to core lipopolysaccharide: a critical appraisal. Infect Dis Clin North Am 1991; 5:915–927.
33. Warren HS, Amato SF, Fitting C, et al. Assessment of ability of murine and human anti-lipid A monoclonal antibodies to bind and neutralize lipopolysaccharide. J Exp Med 1993; 177:89–97.
34. Bogard WC Jr, Siegel SA, Leone AO, et al. Human monoclonal antibody HA-1A binds to endotoxin via an epitope in the lipid A domain of lipopolysaccharide. J Immunol 1993; 150:4438–4449.
35. Siegel SA, Fearon DT, Neblock DS, et al. CB 410 HA-1A (Centoxin) a human monoclonal IgM anti-endotoxin mAb mediates immune adherence of *E. coli* J5 LPS via CRI of human red blood cells and neutrophils. (abstract) J Cell Biochem 1992; 16C:172.
36. Kelly CA, Cech AC, Argenteanu M, et al. Role of bactericidal permeability-increasing protein in the treatment of gram-negative pneumonia. Surgery 1993; 114:140–146.
37. Tobias PS, Soldau K, Ulevitch RJ. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. J Biolog Chem 1989; 264:10867–10873
38. Pugin J, Schürer Maly CC, Leturco D, et al. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide binding protein and soluble CD14. Proc Natl Acad Sci USA 1993; 90:2744–2748.
39. Trown PW, Gazzano-Santoro H, Mezaros K, et al. $rBPI_{123}$ outcompetes LBP for binding to LPS and gram-negative bacteria. Presented at the International Conference on Endotoxins IV, Amsterdam, August 17–20, 1993; (abstract) 8:4.
40. Girior BP. Mediators of septic shock: new approaches for interrupting the endogenous inflammatory cascade. Crit Care Med 1993; 21:780–786.
41. Fisher CJ, Opal SM, Dhainaut JF, et al. Influence of an anti-tumor necrosis factor monoclonal antibody on cytokine levels in patients with sepsis. Crit Care Med 1993; 21:318–327.
42. Fisher C, Dhainaut J-FA, Opal SM, et al. An evaluation of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) in the treatment of patients with sepsis syndrome: results from a randomized, double-blind placebo-controlled trial. JAMA 1994 (in press).
43. Luce JM. Introduction of new technology into critical care practice: a history of HA-1A human monoclonal antibody against endotoxin. Crit Care Med 1993; 21:1233–1241.
44. Dripps DJ, Brandhuber BJ, Thompson RC, Eisenberg SP. Interleukin-1 (IL-1) receptor antagonist binds to the 80-kDa IL-1 receptor but does not initiate IL-1 signal transduction. J Biol Chem 1991; 266:10331–10336.
45. Kull FC, Jacobs S, Cuatrecasas P. Cellular receptor for ^{125}I-labeled tumor necrosis factor: specific binding, affinity labeling, and relationship to sensitivity. Proc Natl Acad Sci U S A 1985; 82:5756–5759.
46. Van der Poll T, Janson J, van Leenen D, et al. Release of soluble receptors for tumor necrosis factor in clinical sepsis and experimental endotoxemia. J Infect Dis 1993; 168:955–960.
47. Lynn WA, Golenbock DT. Lipopolysaccharide antagonists. Immunol Today 1992; 13:271–276.
48. Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; 249:1431–1433.
49. Schumann RR, Leong SR, Flaggs GW, et al. Structure and function of lipopolysaccharide binding protein. Science 1990; 249: 1429–1431.
50. Wright SD, Ramos RA, Hermanowski-Vosatka A, et al. Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14. J Exp Med 1991; 173:1281–1286.
51. Fong Y, Moldawer LL, Marano M, et al. Endotoxemia elicits increased circulating β_2-IFN/IL-6 in man. J Immunol 1989; 142: 2321–2324.
52. Larsen GL, Henson PM. Mediators of inflammation. Ann Rev Immunol 1983; 1:335–360.
53. Morrison, DC, Ryan JL. Endotoxins and disease mechanisms. Ann Rev Med 1987; 38:417–432.
54. Riedo FX, Munford RS, Campbell WB, et al. Deacylated lipopolysaccharide inhibits plasminogen activator inhibitor-1, prostacyclin, and prostaglandin E_2 induction by lipopolysaccharide but not by tumor necrosis factor-α. J Immunol 1990; 144:3506–3512.
55. Opal SM, Cross AS, Sadoff JC, et al. Efficacy of anti-polysaccharide and anti-tumor necrosis factor monoclonal antibodies in a neutropenic rat model of pseudomonas sepsis. J Clin Invest 1991; 88: 885–890.