Brief Definitive Report

Detection of Interleukin 8 and Tumor Necrosis Factor In Normal Humans after Intravenous Endotoxin: The Effect of Antiinflammatory Agents

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Summary

Interleukin 8 (IL-8), a potent activator of neutrophils, may be important in the early host response to serious Gram-negative infections. IL-8 was measured with other acute phase cytokines (tumor necrosis factor α [TNF-α], IL-6, and IL-1β) in 25 normal humans randomized to receive either intravenous endotoxin alone or endotoxin after oral administration of ibuprofen or pentoxifylline, agents that alter some of the inflammatory responses induced by endotoxin in vitro. TNF immunoreactivity was maximum at 1.5 h, and total TNF (area under the curve) was 4.2- and 4.5-fold greater in subjects given endotoxin/ibuprofen compared to subjects given endotoxin alone ($p = 0.026$) or endotoxin/pentoxifylline ($p = 0.004$), respectively. IL-6 levels were maximum at 2–3 h and did not differ among the three groups. No IL-1β was detected in any subject. IL-8 levels peaked at 2 h in subjects given either endotoxin alone or endotoxin/pentoxifylline, falling towards baseline by 5 h. Subjects given endotoxin/ibuprofen had a more sustained rise in IL-8 with peak levels 2.8- and 2.5-fold higher at 3 h compared to endotoxin alone ($p = 0.048$) or endotoxin/pentoxifylline ($p = 0.023$), respectively. Differences in total IL-8 release among groups approached statistical significance (ANOVA, $p = 0.07$). This trend reflected the increased release of IL-8 by the subjects receiving ibuprofen compared to pentoxifylline (1.9-fold higher; $p = 0.024$). This suggests that cyclooxygenase products may provide important negative feedback loops for cytokine production in vivo. Increases in circulating IL-8 are part of the acute inflammatory response of humans to endotoxin. Altered cytokine responses caused by antiinflammatory therapy may have important implications for both host defense and injury during septicemia.

Endotoxin activates a variety of host inflammatory responses and plays a role in septic shock and multiple organ failure (1). A model based on the administration of endotoxin to normal humans qualitatively reproduces several features of host responses which are similar to those occurring during the early stages of bacterial infection (2–4). After stimulation by endotoxin, IL-8 is expressed by several cell types, including monocyte/macrophages and endothelial cells. Neutrophil exposure to IL-8 in vitro results in proinflammatory effects, including neutrophil chemotaxis, degranulation, and expression of cell surface markers (5). We measured the release of IL-8 and its relationship to TNF-α, IL-1β, and IL-6 release after administration of endotoxin to normal humans. Additionally, the roles of two antiinflammatory agents, ibuprofen or pentoxifylline, on this acute response were evaluated. In animal and in vitro studies both of these agents have antiinflammatory and cytokine-modulating effects that suggest that they may be therapeutically beneficial in septic shock (6–9).

Materials and Methods

Subjects. 25 healthy volunteers (20 men, 5 women; 18–40 y old, mean 28 yr) were evaluated. The protocol was approved by our institutional review board and written consent was obtained from all subjects. The current investigation was performed simultaneously with evaluation of the effects of endotoxin and antiinflammatory agents on cardiovascular function (10). U.S. Standard Reference Endotoxin (Lot EC-5, *Escherichia coli* 0113, Food and Drug Administration) was administered intravenously (4 ng/kg of body wt) over 1 min and flushed with 10 ml of normal saline. Subjects were randomized into three groups: (*a*) no treatment before endotoxin ($n = 6$), (*b*) oral ibuprofen (Upjohn, Kalamazoo, MI) 800 mg 1.5 h before, at the time of, and 3 h after endotoxin ($n = 9$), and (*c*) oral pentoxifylline (Hoechst-Roussel, Somerville, NJ) 400 mg extended release tablets every 8 h for five doses before endotoxin ($n = 10$). Ibuprofen has antipyrogenic effects and alters some of the acute stress hormone responses after endotoxin administration to humans (2). The pentoxifylline dosing was chosen to achieve steady-state therapeutic drug levels during the first 4 h of the study. After an 8-h fast, the subjects were monitored as pre-

viously described (3). Arterial blood samples were clotted on ice, centrifuged for 10 min (500 *g*), and the serum was frozen at −70°C. Ibuprofen (Pharmakinetics Laboratory, Baltimore, MD) and pentoxifylline (Dr. M. Amantea, Pharmacy Development Service, National Institutes of Health, Bethesda, MD) plasma levels were measured using HPLC.

Cytokine Assays. Serum TNF-α, IL-1β, and IL-6 were measured in duplicate using double ligand immunoassays (R & D Systems, Minneapolis, MN). The limits of detection of these ELISAs were 31 pg/ml for IL-1β, and 15 pg/ml for IL-6 and TNF-α. Augmented TNF immunoreactivity seen in endotoxin/ibuprofen subjects was further evaluated in all three groups using a cytotoxicity assay (WEHI 164) (3). IL-8 was measured in heat-inactivated serum (56°C for 30 min) using a double ligand immunoassay (Sandoz Research Institute, Vienna, Austria) based on a mouse monoclonal anti–IL-8 and goat polyclonal anti–IL-8 linked to alkaline phosphatase. These antibodies react with endothelial and monocyte-derived IL-8 and had no measurable crossreactivity with high levels of other known cytokines or growth factors (11). The limits of detection were 20 pg/ml of rIL-8.

Statistical Analysis. Summary statistics are expressed as mean ± SEM. One-way ANOVA and unpaired *t* tests were used to assess differences among groups in both the total and temporal cytokine release. Total cytokine production was measured by calculating the area under the curve for each cytokine (12). Correlations were made using Pearson's *r* test. Nominal two-tailed *p* values are reported for each analysis.

Results

Systemic Responses. The subjects given endotoxin alone or endotoxin/pentoxifylline developed fever and constitutional symptoms (chills, myalgias, headache, and nausea) at 1 h that were diminished by 5 h. At 3 h, core temperature peaked from baseline (36.7 ± 0.07°C; all groups) in subjects given endotoxin (39.1 ± 0.15°C) or endotoxin/pentoxifylline (38.6 ± 0.17°C) and approached baseline by 5 h. The symptoms and the fever were attenuated by ibuprofen with a maximum temperature at 3 h (37.3 ± 0.17°C), which was less than subjects receiving endotoxin alone ($p < 0.001$) or endotoxin/pentoxifylline ($p < 0.001$). Pentoxifylline plasma levels at 0, 1.5, and 4 h were 403 ± 97, 257 ± 54, and 281 ± 77 pg/ml, respectively. Ibuprofen levels at 0, 1.5, and 3 h were 12.2 ± 4.0, 64.5 ± 21.5, and 38.9 ± 12.9 μg/ml, respectively. The total granulocyte count in all 25 subjects decreased from baseline (3,781 ± 219 × 10^9/L), to a nadir at 1 h (1,156 ± 82 × 10^9/L), rising by 6 h to 9,722 ± 822 × 10^9/liter granulocytes and approached baseline values by 24 h (6,897 ± 537 × 10^9/L). Neither ibuprofen nor pentoxifylline altered this response.

Cytokine Measurements. No TNF was detected in any subject at 0 h using either the immunoassay or cytotoxicity assay. The mean TNF immunoreactivity was maximum at 1.5 h in all three groups (endotoxin alone 812 ± 331, endotoxin/pentoxifylline 780 ± 247, endotoxin/ibuprofen 3,194 ± 1,065 pg/ml, Fig. 1 *A*). Total TNF immunoreactivity was 4.2-fold higher in subjects given endotoxin/ibuprofen (4,868 ± 1,721 pg/ml) compared to endotoxin alone (1,152 ± 470 pg/ml; $p = 0.026$) and 4.5-fold higher than subjects given endotoxin/pentoxifylline (1,077 ± 341 pg/ml; $p = 0.004$).

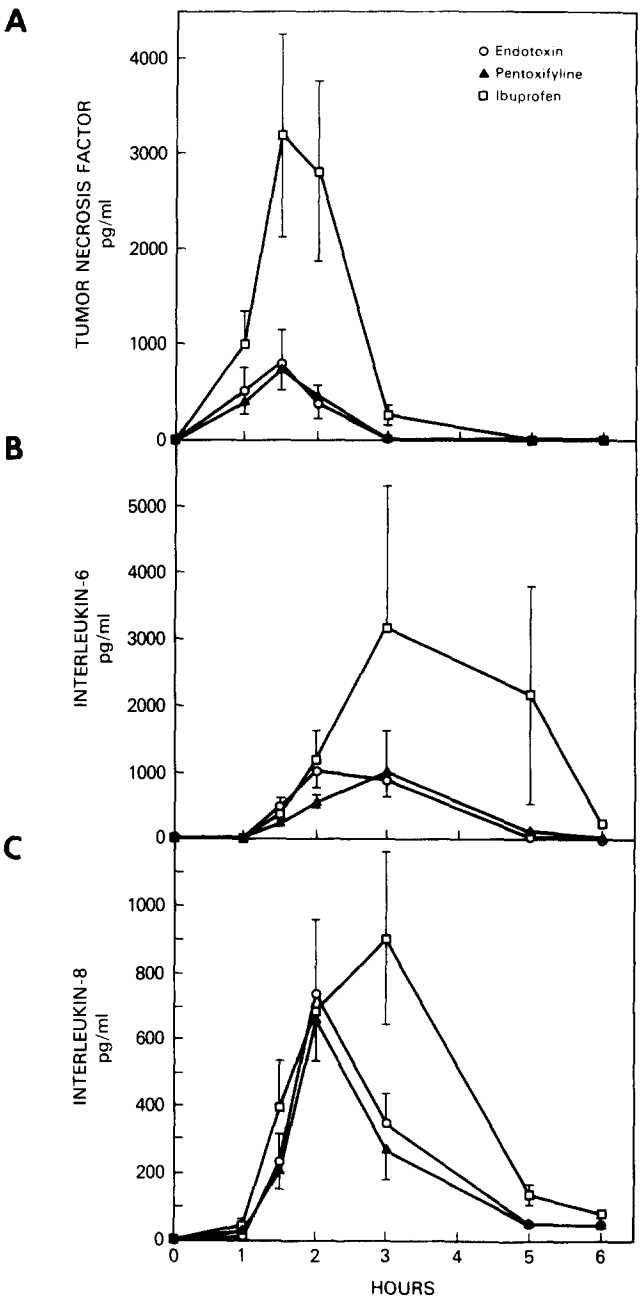

Figure 1. Cytokine response. Alterations in circulating immunoreactive TNF (*A*), IL-6 (*B*), and IL-8 (*C*) in normal humans given intravenous endotoxin alone, or given endotoxin after oral ibuprofen or oral pentoxifylline.

No difference in total TNF release occurred in subjects given endotoxin/pentoxifylline compared to subjects given endotoxin alone. Analysis of TNF cytotoxicity revealed a similar pattern of TNF release in all three groups. Total TNF cytotoxicity was higher in the endotoxin/ibuprofen subjects (1,636 ± 578 pg/ml) compared with the endotoxin group (582 ± 237 pg/ml; $p = 0.057$) or the endotoxin/pentoxifylline group (369 ± 123 pg/ml; $p = 0.017$).

No IL-6 was detected in any subject at 0 h. IL-6 levels were

maximum at 2 h in the endotoxin group (1,026 ± 250 pg/ml) and 3 h in the endotoxin/pentoxifylline group (1,009 ± 632 pg/ml) and endotoxin/ibuprofen group (3,176 ± 2,115 pg/ml) (Fig. 1 *B*). Total IL-6 response did not differ significantly among the three groups (endotoxin alone, 2,476 ± 1,107; endotoxin/ibuprofen, 11,292 ± 3,992; and endotoxin/pentoxifylline, 2,362 ± 835 pg/ml). No IL-1β was detected at any time point.

4 or 25 subjects had detectable IL-8 levels at 0 h (26–46 pg/ml). IL-8 levels were maximum at 2 h after endotoxin alone (641 ± 220 pg/ml) or after endotoxin/pentoxifylline (633 ± 196 pg/ml) and fell toward baseline values by 3 h in both groups. Levels of IL-8 continued to rise at 2 h in the endotoxin/ibuprofen group and peaked at 3 h (928 ± 213 pg/ml) (Fig. 1 *C*). Total IL-8 released was marginally different among the three groups (ANOVA, $p = 0.07$), with greater total IL-8 release after endotoxin/ibuprofen (2,671 ± 890 pg/ml) than endotoxin/pentoxifylline (1,409 ± 470 pg/ml; $p = 0.024$). IL-8 levels were higher at 3 and 5 h in the subjects given endotoxin/ibuprofen compared to endotoxin alone (3 h: $p = 0.048$; 5 h; $p = 0.027$) or endotoxin/pentoxifylline (3 h: $p = 0.023$; 5 h: $p = 0.006$). Total IL-8 after endotoxin alone (1,536 ± 627 pg/ml) was 1.7-fold lower than the endotoxin/ibuprofen group ($p = 0.10$) and did not differ from total IL-8 after endotoxin/pentoxifylline.

Total TNF immunoactivity correlated with total TNF cytotoxicity ($r = 0.599$, $p = 0.002$), total IL-6 ($r = 0.787$, $p < 0.001$), and total IL-8 ($r = 0.512$, $p = 0.009$). Total IL-6 correlated poorly with IL-8 activity ($r = 0.278$, $p = 0.178$).

Discussion

IL-8 has several properties that suggest that it plays a role in mediating some of the inflammatory responses of neutrophils induced by endotoxin, IL-1, and TNF (5). In addition to these proinflammatory responses, endothelial-derived IL-8 can inhibit neutrophil adhesion to IL-1-activated endothelial cells possibly limiting inflammatory damage from neutrophil-endothelial cell interaction (13). Our study demonstrates that IL-8 is part of the acute inflammatory response occurring in humans after the intravenous administration of endotoxin. IL-8 circulates in the blood and maximum levels occur after an earlier rise in TNF activity, parallel a similar rise in IL-6 activity, and then approach baseline values by 6 h. The peak levels of IL-8 after intravenous endotoxin are approximately fivefold less than those described in patients with septic shock (14). Thus, the nature, intensity, and duration of the primary stimulus for IL-8 production (i.e., bolus intravenous endotoxin vs. persistent bacteremia) may affect the absolute levels seen during the acute response.

In vitro data suggest that cyclooxygenase products serve an important function as part of an inhibitory feedback loop that limits TNF and other cytokine production (9, 15). The current study demonstrates that cyclooxygenase inhibitors in vivo result in an augmented release of TNF and IL-8. The implications of enhanced cytokine release in humans after endotoxin/ibuprofen remain to be clarified. Animal studies of septic shock using ibuprofen as a therapeutic adjunct show improvement in hemodynamic parameters and mortality, yet cytokine responses have not been reported (8). Additionally, the use of cyclooxygenase inhibitors can attenuate hypotension and death in animals given TNF (16, 17). Although TNF appears to be an important cause of organ damage and mortality in sepsis, a protective role of TNF in the normal host response to infection has been shown in mice challenged with live bacteria (18, 19). TNF levels measured by the cytotoxicity assay in the endotoxin/ibuprofen group are comparable to those reported during meningococcemia (20). The relatively brief exposure to high TNF levels and the absence of infection may explain why these levels of TNF were well tolerated in our subjects. Alternatively, cyclooxygenase inhibition may prevent the release of other mediators by TNF and thereby reduce morbidity despite higher TNF levels.

The levels of TNF after endotoxin/ibuprofen obtained in our study are higher than those reported by others using this model; variation in sample size and TNF assays may account for these differences (2). However, a recent report confirms an enhanced release of TNF in humans given ibuprofen and endotoxin (21). No IL-1β was detected in our human subjects after endotoxin administration. IL-1β has not been uniformly detected by others after endotoxin administration to humans, suggesting is serves as a local rather than circulating mediator of endotoxin-induced inflammatory responses (2, 21, 22).

Pentoxifylline inhibits TNF message transcription and translation in vitro (23). A concentration of 10^{-6} to 10^{-5} M results in 45–60% suppression of LPS-stimulated monocyte TNF mRNA (7). The plasma concentration of pentoxifylline in our study ranged from 10^{-6} to 10^{-7} M. This level of drug did not result in a significant diminution of any of the acute cytokine responses nor were the endotoxin-induced symptoms altered by the use of pentoxifylline. In contrast, patients with malignancies receiving oral pentoxifylline had decreased TNF mRNA in isolated mononuclear cells compared to pretreatment samples and this was associated with an increased sense of well being (24). Intravenous pentoxifylline given to humans before intravenous endotoxin from *Salmonella abortis equii* blocked the rise in circulating TNF (25). Thus, higher blood levels of pentoxifylline as well as the nature of the inflammatory stimulus may be integral to inhibition of the TNF response by pentoxifylline in vivo.

This human model of ibuprofen-enhanced cytokine release demonstrates that cyclooxygenase products serve as an important control mechanism that limits IL-8 and TNF release in vivo. The use of antiinflammatory agents during therapy of septic shock may result in altered cytokine responses, but whether manipulation of these responses will be detrimental or useful in the host response to infection needs further investigation.

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