Brief Definitive Report

C5a STIMULATES SECRETION OF TUMOR NECROSIS FACTOR FROM HUMAN MONONUCLEAR CELLS IN VITRO

Comparison with Secretion of Interleukin 1β and Interleukin 1α

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TNF-α/cachectin, produced by mononuclear phagocytes and other cells, is thought to be a critical mediator of the septic shock syndrome by inducing hypotension, activating leukocytes, promoting endothelial coagulant activity, and causing metabolic acidosis and acute renal failure (1–3). Although endotoxins from Gram-negative bacteria are thought to produce septic shock in part by inducing the synthesis and release of TNF (3), Gram-positive organisms, fungi, and acute antigen-antibody reactions (such as transfusion reactions) can also produce a shock-like state (4).

Human C5a is an 11-kD glycopeptide generated by the cleavage of C5 by either the classical or alternative complement pathway convertases, or by other proteases (5). An anaphylatoxin, C5a is chemotactic for phagocytic leukocytes; it stimulates the generation of LTB_4 and oxygen radicals, release of lysosomal enzymes, degranulation of mast cells, and induces vasodilation and vascular permeability (5–7). C5a also enhances lymphocyte function (8).

C5a is generated in the course of sepsis, trauma, and other inflammatory states (5) associated with the acute phase response. Many of the acute phase responses are due to the actions of the cytokines, TNF-α and IL-1 (9), and both may play important roles in the septic shock syndrome (10).

Goodman et al. (11) reported that the murine macrophage line, P388D1, secreted IL-1 in response to C5a. We have recently reported that human C5a and C5a des Arg stimulated IL-1 release from freshly obtained human mononuclear cells (MNC), in the absence of endotoxin (12).

In this study, we compared human mononuclear cell secretion of immunoreactive IL-1α, IL-1β, and TNF-α in response to stimulation by purified human C5a. Our results indicate that C5a is a potent stimulus of mononuclear cell secretion of these

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cytokines, and does so in a dose-dependent fashion. With increasing C5a concentrations, there was increased secretion of TNF-α, IL-1β, and IL-1α. There was no statistically significant difference between the amount of TNF-α or IL-1β secreted in response to a given C5a stimulus. Both TNF-α and IL-1β were secreted in significantly greater concentrations than IL-1α in response to C5a stimulation. Our results suggest that complement activation may influence hemodynamic, hematologic, and metabolic functions by the induction of these cytokines.

Materials and Methods

Materials. C5a and C5a des Arg were isolated by immunoadsorbent column chromatography, ion exchange, and gel filtration, as previously described (13). Endotoxin was not detected in C5a by the Limulus Amoebocyte Lysate (LAL) assay (sensitivity of 20 pg/ml; Associates of Cape Cod, Woods Hole, MA). A positive control was used in which 20 pg/ml of *Escherichia coli* endotoxin (Sigma Chemical Co., St. Louis, MO) was mixed with C5a (250 ng/ml).

Human MNC Incubations. Human MNC were isolated from heparinized (5 U/ml) venous blood from four healthy donors as previously described (12). MNC were suspended at 5×10^6 cells/ml in Eagle's MEM (Gibco, Grand Island, NY). To examine the effect of C5a on cytokine production, 100 μl of various concentrations of the purified C5a diluted in MEM were incubated with 100 μl of MNC suspension in 96-well flat bottomed microtiter wells and then incubated for 24 h in serum-free conditions at 37°C. Because stimulated MNC produce PGE_2 in response to C5a, indomethacin (final concentration, 0.5 μg/ml) was added to the media to inhibit cyclooxygenase (12). Although the endotoxin levels of the C5a samples were undetectable (<20 pg/μg C5a), endotoxin might be capable of stimulating IL-1 or TNF production at levels below the sensitivity of the LAL test (14). Polymyxin B has been shown to block many of the in vitro effects of endotoxins present in a wide variety of biologically active substances. Therefore, each sample of C5a was preincubated with 5 μg/ml of polymyxin B (PMB, clinical grade; Pfizer Inc., New York, NY) for 1 h at room temperature in the absence of serum. Reports from other laboratories and our previous studies demonstrate that PMB neutralizes the ability of exogenously added LPS to induce cytokine production; PMB had no effect on C5a-induced IL-1 production (12). This further confirmed the absence of significant endotoxin contamination of these C5a preparations.

The effect of human C5a on MNC TNF-α and IL-1 production was examined over a broad range of concentrations, from 1–500 ng/ml. After 24 h of incubation at 37°C, the supernatants of the MNC were diluted and assayed for TNF-α and IL-1α and IL-1β levels by using the respective RIAs.

RIAs. 3 RIAs were used to measure TNF-α (15), IL-1β (16), and IL-1α (17). These RIAs have been shown to detect <100 pg/ml of each cytokine in human MNC supernatants. Detailed studies, described elsewhere (15–17), demonstrate that there is no crossreactivity amongst each of these cytokine RIAs, nor do these RIAs detect human IFN-α, IFN-γ, granulocyte-macrophage CSF, lymphotoxin, or C5a. In addition, each RIA correlated with functional bioassays (cytotoxicity, T cell proliferation), with correlation coefficients of >0.8 ($p < 0.05$ by paired student's t test). Bioassay for TNF-α used the L929 cytotoxicity assay, as described in our report detailing the RIA method for TNF-α (15).

Results

Comparison of Human MNC Production of TNF-α, IL-1β, and IL-1α in Response to C5a. A dose-dependent effect of C5a on TNF-α, IL-1β, and IL-1α secretion was clearly observed in the MNC of all donors (Fig. 1). With increasing concentrations of C5a, increased cytokine secretion was observed. Due to greater variability for TNF-α production between individuals, IL-1β and TNF-α levels at varying C5a concentrations did not differ significantly ($p > 0.05$ by paired student's t test and anal-

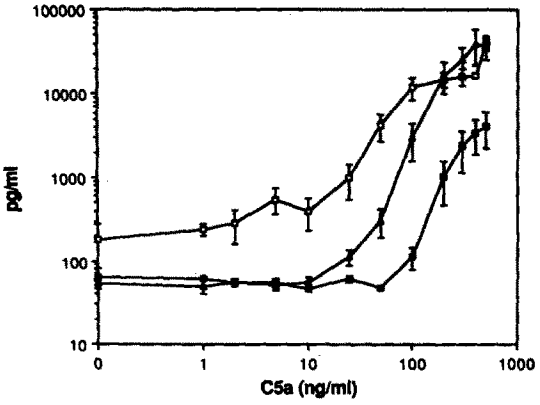

FIGURE 1. Effects of C5a on human MNC secretion of TNF (□), IL-1β (▲), and IL-1α (■), measured by RIA. The data are expressed as the mean ± SE for four donors.

ysis of variance [ANOVA]), while secreted IL-1β levels were significantly higher than IL-1α levels over a broad range of C5a concentrations (25–500 ng/ml C5a; $p <$ 0.05–0.01 by paired student's t test and ANOVA). Significant ($p < 0.05$) MNC cytokine production over control levels required a C5a concentration of 25 ng/ml for IL-1β, 50 ng/ml for TNF-α, and 200 ng/ml for IL-1α. We also compared the response to both C5a and purified C5a des Arg. TNF-α and IL-1β secretion in response to C5a des Arg was approximately one-tenth to one-fifth that seen for C5a over a biologically relevant range of anaphylatoxin concentrations from 30–100 ng/ml (data not shown). The addition of C5a to 20% normal human serum gave results intermediate between C5a des Arg and C5a (data not shown).

When the same supernatants were assayed for TNF bioactivity using the L929 cell cytotoxicity bioassay, standardized against human TNF-α, similar patterns were observed. When the data from both the bioassay and RIA were compared, a strong linear correlation was observed ($r = 0.92$).

Effect of Polymyxin B Preincubation on C5a-stimulated TNF-α Production. Endotoxin in our C5a preparations was undetectable (<20 pg/μg). The preincubation of C5a with polymyxin B had no influence on TNF-α production by MNC ($p > 0.05$); when endotoxin (100 ng/ml) was preincubated with PMB (5 μg/ml), MNC TNF-α production was effectively blocked. Identical results were seen when IL-1α and IL-1β were assayed.

Discussion

Human C5a has been reported to stimulate IL-1 production in $P388D_1$ cells, a murine macrophage cell line (11), and we have recently shown that purified human C5a stimulates normal human peripheral blood MNC to produce IL-1β (12). In the present studies, we report that C5a induces TNF-α production in human PBMC, demonstrated by both RIA and bioassay. In addition, we compared the in vitro secretion of TNF-α with IL-1β and IL-1α. C5a increases the production of these cytokines in a dose-dependent fashion. There were no statistically significant differences between the increases seen for both TNF-α and IL-1β in response to clinically achievable concentrations of C5a (ranging from 25–100 ng/ml). Both TNF-α and IL-1β were secreted in significantly greater concentrations in response to C5a than IL-1α.

When purified C5a des Arg was compared with C5a, we found that TNF-α and IL-1β secretion in response to C5a des Arg was approximately one tenth to one fifth as great as that for C5a. Addition of C5a to 20% normal human serum gave results intermediate between C5a des Arg and the more potent C5a. This is not surprising, as serum carboxypeptidase B rapidly converts C5a to C5a des Arg, reducing its potency as a stimulus, while additional serum factors such as C3 cleavage products (C3a, iC3b) and clotting products may be stimulatory. It is for these reasons that we chose, in these initial investigations, to study purified C5a in serum-free media.

In these studies, the C5a was free of any detectable endotoxin, and potential endotoxin effects were additionally controlled by the inclusion of polymyxin B in appropriate incubations. The effectiveness of PMB in blocking detectable endotoxin stimulation of cytokine production was documented in our investigation.

Recently, it has been appreciated that polypeptide factors, elaborated by the host in response to stimuli such as infection mediate responses such as fever, acute phase protein synthesis, abnormal metabolism, and shock. For example, the C3H/HeJ mouse is resistant to the effects of LPS, presumably due to genetic defect in the post-translational production of TNF and IL-1 (18). TNF-α and IL-1 are both mediators of the acute phase response; both are capable, administered individually, of inducing fever and the shock syndrome, and share many of the same biological activities (1–3, 10, 19). Together, these cytokines act synergistically to mediate the local Schwartzman reaction (20), as well as the shock syndrome (10). Since C5a induces these cytokines together, their synergism may be relevant in a number of pathologic situations. In several syndromes in which TNF-α has been shown to play a role, complement activation also occurs. Endotoxemia in humans, meningococcal sepsis, and malaria are examples, and in each situation, complement activation also has been reported (21, 22).

Receptors for C5a are present on neutrophils and monocytes, as well as other cells, and its binding leads to cellular activation (5, 7). The occurrence of complement activation with various shock syndromes such as septic and endotoxic shock, trauma, and "shock lung," or the adult respiratory distress syndrome (ARDS), led to the hypothesis that C5a caused activated neutrophils to adhere to and injure capillary endothelial cells, leading to hypovolemia, shock, and ARDS (23). More recent evidence calls into question the necessary participation of the "activated granulocyte" in the pathogenesis of these syndromes, as they have been described in severely neutropenic patients (24). With the demonstration of C5a as a potent stimulus to TNF-α and IL-1 secretion, C5a again emerges as a potentially important factor in the pathogenesis of this syndrome, independent of a requirement for neutrophil participation. It is thus possible that clinical syndromes as diverse as sepsis, trauma, cardiopulmonary bypass, or immune complex diseases, all associated with complement activation, could result in fever, hypotension, coagulopathy, and disordered metabolism. These effects of TNF-α and IL-1 may in turn have resulted from the generation of C5a.

Summary

We have demonstrated that purified C5a is a potent stimulus to human PBMC secretion of TNF-α, IL-1β, and IL-1α, which proceeds in a dose-dependent fashion. At a given concentration of C5a, TNF-α and IL-1β secretion did not differ significantly;

both were secreted in significantly greater quantity than IL-1α. Clinical conditions such as Gram-positive and Gram-negative bacterial infections, trauma, and immune complex diseases activate complement. Through the mediation of TNF and IL-1 secreted in response to C5a, these diverse disorders can share common features of fever, coagulopathy, acute phase protein production, and disordered metabolism.

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