Recombinant C5a Stimulates Transcription Rather Than Translation of Interleukin-1 (IL-1) and Tumor Necrosis Factor: Translational Signal Provided by Lipopolysaccharide or IL-1 Itself

By Ralf Schindler, Jeffrey A. Gelfand, and Charles A. Dinarello.

We investigated the effects of recombinant C5a (rC5a) on gene expression and synthesis of interleukin-1β (IL-1β) and tumor necrosis factor (TNF) in fresh human peripheral blood mononuclear cells (PBMC). Total (cell-associated and secreted) cytokine synthesis was measured. In the strict absence of endotoxin (lipopolysaccharide [LPS]), rC5a resulted in a small but statistically insignificant increase in immunoreactive IL-1β and TNF, as well as in IL-1 and IL-6 bioactivity. On the other hand, rC5a induced marked transcriptional activation of IL-1β and TNF in a dose-dependent fashion with an optimal concentration of 50 ng/mL. The rC5a-induced cytokine messenger RNA (mRNA) was not spontaneously translated into protein. At 50 ng/mL, rC5a induced the same levels of mRNA for IL-1β and TNF as 1 ng/mL of LPS, whereas LPS induced 12 times more IL-1β protein and 70 times more TNF protein than rC5a alone. The C5a-induced mRNA half-life was the same as that induced by LPS. Formyl-Meth-Leu-Phe (fMLP) did not induce cytokine transcription. Pretreatment with rC5a enhanced cytokine synthesis induced by other stimuli. After 2 hours of preincubation with rC5a, PBMC synthesized 3 to 10 times more IL-1β and TNF on subsequent stimulation by LPS or IL-1 itself. We conclude that rC5a provides primarily a transcriptional but not translational signal for IL-1β and TNF; the half-life of the untranslated mRNA is the same as that of translated message; rC5a-induced transcription upregulates PBMC for enhanced synthesis of these cytokinins; and a translational signal can be provided by LPS or IL-1 itself.

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IL-6-dependent hybridoma cell line B9, a generous gift from Dr L.A. Aarden (Blood Transfusion Laboratory, Amsterdam, the Netherlands). The B9 assay was performed as described previously. Three different dilutions of the sample were assayed and results expressed in units per milliliter. One unit was defined as the concentration resulting in half-maximal proliferation of B9 cells and was equivalent to 0.3 pg of rIL-6.

Preparation of PBMC. PBMC were separated from freshly obtained heparinized human blood by centrifugation through Ficoll/Hypaque (Ficoll Type 400, Sigma; Hypaque-M, 90%, Winthrop Breon Lab, New York, NY). The water used in the preparation of Ficoll/Hypaque was subjected to ultrafiltration using polysulfone. PBMC were washed twice with normal saline and resuspended in RPMI 1640 culture medium (Whittaker M.A., Walkersville, MD), supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The RPMI was also subjected to ultrafiltration to remove microbial products. PBMC were counted using a Coulter counter (Coulter Electronics, Hialeah, FL) and were resuspended at 5 x 10^6 cells/mL with 2% heat-inactivated human AB serum. An aliquot of 0.4 mL of cells was added to wells of a 24-well tissue culture plate (Falcon 3047, Oxnard, CA). An equal volume of RPMI alone or RPMI containing rC5a or LPS was added and incubated with the cells at 37°C in a humidified atmosphere containing 5% CO₂. All incubations were performed in the presence of 0.5 mg/mL indomethacin (final concentration). Cytokine production was determined 24 hours after stimulation unless otherwise indicated. Total cytokine synthesis was assayed after subjecting the PBMC cultures to three freeze-thaw cycles, which has been previously established as the optimal method for extracting the cell-associated compartment.

RNA isolation and analysis. For experiments studying gene expression, 20 to 30 x 10⁶ PBMC were incubated at 2.5 x 10⁶ cells/mL in 50 mL polypropylene tubes at 37°C (Becton Dickinson, Lincoln Park, NJ). In some experiments, the tubes were rotated at 10 rpm. After an incubation period with the stimulus (LPS, fMLP, or rC5a) in the presence of 0.5 μg/mL indomethacin, cells were pelleted by centrifugation at 500g and lysed in guanidine isothiocyanate for isolation of RNA. For detection of protein, a 1-mL aliquot of the same cell suspension was transferred to a 24-well tissue culture plate (Falcon 3047), incubated for a total period of 24 hours, and then subjected to RIA. Total cellular RNA was obtained by centrifugation through 5.7 mol/L cesium chloride in 0.1 mol/L EDTA. For each Northern blot, equal amounts of RNA (20 μg/lane unless otherwise indicated) were subjected to gel electrophoresis using 1.2% agarose (International Biotechnology Inc, New Haven, CT) gels containing 6.6% formaldehyde as a denaturing agent. The RNA was visualized by staining with ethidium bromide (Sigma) added to the loading buffer. Ethidium bromide staining did not interfere with the subsequent transfer or hybridization. The RNA was transferred by capillary action to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) and fixed by short wave UV radiation.Dot blots were performed using a manifold apparatus (Schleicher & Schüll, Dassel, FRG). Seven twofold serial dilutions of 20 μg total RNA were blotted onto nylon membranes; thus, the

Fig 1. Effect of rC5a on immunoreactive IL-1β and TNF synthesis. PBMC from 10 different subjects were incubated with different concentrations of rC5a or LPS at 10 ng/mL. Total cytokine production was determined after 24 hours. The horizontal lines represent the detection limit of the RIA.
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Effect of recombinant C5a on the synthesis of immunoreactive IL-1β and TNF. As shown in Fig 1, unstimulated PBMC did not produce detectable IL-1β, except in 2 of 10 donors where IL-1β was detected at 50 to 100 pg/mL (the detection limit of the RIA is 40 to 80 pg/mL). PBMC incubated with 200 and 50 ng/mL rC5a produced IL-1β in 4 of 10 donors with maximal levels of 250 pg/mL. The mean difference between the stimulated and unstimulated cells was not statistically significant. TNF production was detected in unstimulated PBMC in 8 of the 10 cases; again, rC5a stimulated small but statistically insignificant levels of TNF above unstimulated controls. When the same cells were incubated with LPS at 5 ng/mL, large amounts of IL-1β and TNF were produced. We next measured IL-1 and IL-6 bioactivity using D10S and B9 cells. These cells are highly sensitive to the respective cytokine and proliferate in response to femtomolar concentrations of IL-1 or IL-6. IL-1 bioactivity was found in unstimulated PBMC in the range of 2 to 50 pg/mL. Bioactivity was higher in PBMC incubated with 200, 50, and 10 ng/mL rC5a (Fig 2) compared with unstimulated controls; however, this difference did not reach significance. Similar results were obtained measuring IL-6 bioactivity on B9 cells (Fig 2).

Recombinant C5a but not fMLP stimulate transcription of IL-1β and TNF. We next investigated the induction of transcription of IL-1β and TNF by complement. As shown in
Fig 4. Time course of rC5a-induced IL-1β mRNA. PBMC from two different donors were incubated in the absence or presence of 50 ng/mL rC5a and RNA was extracted at the indicated time points. RNA from PBMC stimulated with LPS for 4 hours is shown as a control. Tubes were either kept stationary or were rotated during the incubation period. Cytokine production 24 hours after rC5a stimulation was determined by RIA in the same cell cultures.

Fig 3. rC5a induced dose-dependent accumulation of IL-1β and TNF messenger RNA (mRNA) after 4 hours compared with unstimulated PBMC. A concentration of 50 ng/mL rC5a appears optimal for induction of cytokine transcription. However, the same cells did not produce IL-1β or TNF when incubated for an additional 20 hours. Incubation of unstimulated PBMC in stationary culture tubes can result in the appearance of small amounts of IL-1β mRNA at 4 and 8 hours (Fig 4); again, rC5a increased mRNA accumulation for IL-1β. The time course of mRNA appearance was similar with or without rC5a. When the culture tubes were rotated during the incubation period, mRNA for IL-1β was barely detectable in unstimulated cells (Fig 4), suggesting that adherence of PBMC to plastic or to each other was responsible for transcription of IL-1β. rC5a also increased IL-1β mRNA in PBMC incubated in rotating tubes.

We next incubated PBMC with different concentrations of LPS or rC5a and compared mRNA and protein levels induced by these stimuli. As shown in Fig 5, rC5a at 200 ng/mL stimulated approximately the same amount of mRNA for IL-1β and TNF as 1 ng/mL of LPS; however, LPS induced 12 times more IL-1β protein and 70 times more TNF protein than rC5a.

fMLP, another chemotactic peptide that shares many of the agonist properties with C5a, did not stimulate synthesis of IL-1β or TNF. Unlike rC5a, fMLP did not induce transcription of IL-1β even at high (100 nmol/L) concentrations. Figure 6 shows the results of two Northern blots using different donors.

rC5a primes PBMC for enhanced production of IL-1β and TNF. PBMC were preincubated with 50 ng/mL rC5a for 2 hours and then stimulated with either LPS, IL-1α, or PMA. This resulted in greater amounts of cytokines synthesized compared with PBMC preincubated in medium alone. The range of increased cytokine synthesis varied from 300% to 900% (Table 1). This effect of rC5a was best observed at lower concentrations of LPS. Shorter preincubation periods with rC5a were less effective for enhancing cytokine production induced by LPS or IL-1α. rC5a also increased IL-1β synthesis when PMA (10 ng/mL) was used as a second stimulus (3.0 ± 0.83 ng/mL without rC5a v 9.2 ± 1.8 ng/mL with rC5a; n = 7, P < .01). Preincubation of PBMC with fMLP did not result in enhanced cytokine synthesis after LPS or IL-1α (data not shown).

In the case of IL-1α being used as a second stimulus, this effect was also evident at the level of transcription. As shown in Fig 7, preincubation of PBMC with rC5a followed by stimulation with IL-1α resulted in higher IL-1β and TNF mRNA levels than incubation with IL-1α alone. The kinetics...
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Fig 6. fMLP does not induce transcription of IL-1β. fMLP at 10 and 100 nmol/L was incubated with PBMC from two different donors for 4 hours. RNA was extracted, and 15 μg/lane were subjected to Northern analysis.

of accumulation of IL-1–induced IL-1β mRNA were similar regardless of preincubation with rC5a (data not shown).

To elucidate whether the accumulation of IL-1β mRNA after rC5a was due to transcriptional activation or an effect on mRNA stability, we studied the half-life of IL-1β mRNA induced by rC5a or LPS (Fig 8). Levels of IL-1β mRNA decreased with time and were only hardly detectable at 12 hours after addition of actinomycin D regardless of whether mRNA was induced by rC5a or by LPS.

DISCUSSION

Conflicting results have been reported regarding the induction of cytokines by complement components. Early studies showed that purified human C5a induced IL-1 bioactivity in murine P388D1 cells and that C5a, C5a deArg, and C3a purified from human serum are capable of stimulating IL-1 and TNF production from human PBMC. However, a subsequent study failed to demonstrate increased IL-1 production from adherent human blood monocytes after stimulation with various complement fragments, including C3a, C5a, C3b, and factor B. This report was consistent with the earlier studies of Gekowski and Atkins who failed to stimulate endogenous pyrogen activity from rabbit blood monocytes incubated with human C5a or C3a. In the former study, IL-1 activity was determined on murine thymocytes, an assay that could be influenced by other cytokines such as IL-2, IL-4, IL-6, and IL-7, as well as by IL-1 inhibitors present in culture supernatants. The results of our studies indicate that despite the ability of rC5a to trigger superoxide production and act as a chemoattractant, rC5a is a poor inducer of the synthesis of IL-1, TNF, or IL-6 from fresh human PBMC. Although there was a small increase of cytokine synthesis at 50 ng/mL rC5a, this difference did not reach statistical significance and was a full order of magnitude less than the concentrations previously reported.

However, we did observe accumulation of IL-1β and TNF after rC5a. Because rC5a- and LPS-induced mRNA for IL-1β had the same half-life, the effect of rC5a appears to be an increase in transcription of the IL-1β gene. Comparing the amount of transcriptional activation by different concentrations of LPS, it was clear that the rC5a-induced mRNA for IL-1β was not being efficiently translated into protein (Fig 5). However, when an additional stimulus such as LPS, PMA, or IL-1α itself was provided, rC5a-primed PBMC produced more IL-1β and TNF than cells incubated with LPS, PMA, or IL-1α alone. The phenomenon of transcriptional activation without protein synthesis was also observed by us and other groups. Transcriptional rather than translational activation has also been reported for TNF.

The small increase of IL-1β and TNF synthesis after rC5a cannot be attributed to failure to detect secretion and/or different processing of these cytokines because we measure total cytokine synthesis (cell-associated plus secreted) and the RIAs detect both the precursor and the mature forms.

Donor 1

Donor 2

Table 1. Priming of IL-1β and TNF by C5a and Translational Signal by IL-1α or LPS

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IL-1β (pg/mL)</th>
<th>TNF (pg/mL)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No rC5a</td>
<td>+ rC5a</td>
</tr>
<tr>
<td>RPMI</td>
<td>75 ± 29</td>
<td>81 ± 26</td>
</tr>
<tr>
<td>IL-1 α (100 ng/mL)</td>
<td>498 ± 134</td>
<td>1,744 ± 601*</td>
</tr>
<tr>
<td>LPS (250 pg/mL)</td>
<td>346 ± 145</td>
<td>2,423 ± 430*</td>
</tr>
</tbody>
</table>

PBMC were preincubated for 2 hours in the absence or presence of 50 ng/mL rC5a and subsequently stimulated with IL-1 α (n = 11) or LPS (n = 6). IL-1 β and TNF were determined by RIA after 24 hours. Results are mean ± SEM.

*P < .01.
On the other hand, IL-1β mRNA induced by rC5a was found to have the same half-life as LPS-induced mRNA, i.e., rC5a appears not to influence IL-1β mRNA stability substantially. Thus, it appears that rC5a lacks a translational signal for IL-1β synthesis. Besides conferring mRNA instability, an AU-rich sequence in the 3' untranslated region of many cytokines has been shown to directly inhibit translation of interferon β mRNA in oocytes. In a similar fashion, IL-1β synthesis may be regulated to a substantial degree at the level of translation of its mRNA. Agents such as LPS, PMA, or IL-1 itself deliver both transcriptional as well as translational signals.

The recombinant C5a used in the present study was expressed in E. coli and therefore is not glycosylated. The lack of IL-1 and TNF induction by rC5a could be explained by a requirement for the sugar moieties triggering a translational signal. However, the recombinant material is capable of replacing natural C5a from its receptor and has similar biologic activities regarding superoxide generation and chemotaxis. However, receptor binding of fMLP also provides these same postreceptor signals, but they are not sufficient to activate cytokine transcription. It appears that there may be an independent requirement for glycosylation of the C5a molecule for full translation, and it is possible that the carbohydrate moieties provide such a translational trigger. For example, the N-terminal amino acids bind the C5a receptor activating transcription whereas the carboxyl end is glycosylated and may cross-link an adjacent receptor. Thus, two signals may be delivered by natural C5a.

Several observations suggest that the transcriptional activation of IL-1β and TNF is indeed caused by C5a and not by contaminating LPS. First, rC5a induced mRNA for IL-1β and TNF in a dose-dependent fashion with an optimal concentration of 50 ng/mL. Higher concentrations consistently induced less transcription of these cytokines. A bell-shaped dose-response curve with an optimal concentration of 10 to 100 ng/mL (10^-8 to 10^-9 mol/L) of C5a has also been observed by other groups regarding the chemotactic effects of C5a. Decreased neutrophil migration is observed at higher concentrations of C5a and is a well-known phenomenon termed deactivation. C5a-induced prostaglandins, which suppress cytokine synthesis, play no role in our studies because cyclooxygenase-inhibiting concentrations of indomethacin were included in all incubations.

Unlike rC5a, another potent chemoattractant, fMLP, did not induce transcription of IL-1β, nor did fMLP prime PBMC for enhanced cytokine production at concentrations...
that are chemotactic and increase intracellular calcium in monocytes. The same preparation of fMLP used in the present study is chemotactic on neutrophils. The binding of C5a to its receptor also increases intracellular calcium, whereas IL-1 does not. Because C5a and LPS activate gene expression for IL-1β, whereas fMLP does not, the pathway for induction of IL-1 transcription seems to be independent on changes in G-protein coupling, activation of phospholipase A or increased intracellular calcium, and separated from the chemotactic effects of C5a and fMLP.

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Recombinant C5a stimulates transcription rather than translation of interleukin-1 (IL-1) and tumor necrosis factor: translational signal provided by lipopolysaccharide or IL-1 itself

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