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Effect of Human C-Reactive Protein on Chemokine and Chemotactic Factor-Induced Neutrophil Chemotaxis and Signaling

Wangjian Zhong, Qin Zen, Julie Tebo, Klaus Schlottmann, Mark Coggeshall, and Richard F. Mortensen

C-reactive protein (CRP) is a unique serum pentraxin and the prototype acute phase reactant. CRP is a ligand for specific receptors on phagocytic leukocytes, and mediates activation reactions of monocytes/macrophages, but inhibits the respiratory burst of neutrophils (PMN). Since CRP selectively accumulates at inflammatory sites in which IL-8 is also produced, we tested the effects of CRP on the responsiveness of PMN to IL-8 and the bacterial chemotactic peptide, FMLP-phenylalanine (FMLPP). Purified human CRP inhibited the chemotactic response of PMN to IL-8 and FMLPP. A mouse IgM mAb that was generated against the leukocyte CRP receptor (CRP-R) also inhibited the chemotactic response. Incubation of purified CRP with activated PMN generated CRP-derived peptides that also inhibited chemotaxis. A synthetic CRP peptide (residues 27–38) that binds to the CRP-R had weak chemotactic activity, whereas two other CRP synthetic peptides (residues 174–185 and 191–205) inhibited chemotaxis of PMNs to both IL-8 and FMLPP. CRP did not alter receptor-specific binding of IL-8, but exerted its effect at the level of signaling. CRP augmented both IL-8- and FMLPP-induced mitogen-activated protein kinase (extracellular signal-regulated kinase-2) activity. CRP at acute phase levels increased both agonist-induced and noninduced phosphatidylinositol-3 kinase activity. The results suggest a role for CRP as a regulator of leukocyte infiltration at inflammatory sites. The Journal of Immunology, 1998, 161: 2533–2540.
neutrophil chemotactic response, but augments phosphatidyl-
inositol-3 kinase (PI-3K) signaling and the mitogen-activated protein kinase (MAPK) pathway, suggesting that CRP influences PMN responses via a distinct signaling pathway.

Materials and Methods

Reagents

FMLP, FMLPP, PMA, DMSO, and fericytochrome C (horse heart) were obtained from Sigma (St. Louis, MO). The PMA and FMLPP were stored concentrated in DMSO at −20°C and diluted with buffer just before use. The p-aminophenyl-PC-Sepharose for CRP purification was purchased from Pierce (Rockford, IL). HL-8 isoforms of both 72 and 77 amino acids were purchased from Sigma and Harlan Bioproducts (Madison, WI), respectively. 125I-labeled IL-8 (125I-IL-8) was obtained from Amersham Life Sciences (Arlington Heights, IL). The PI-3K inhibitor, wortmannin, was purchased from Sigma. The specific ERK kinase inhibitor PD098059 was obtained from Calbiochem (La Jolla, CA).

Cells

The promyelocytic cell line HL-60 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI 1640 plus 4% defatted FBS (HyClone, Logan, UT) and 6% defined bovine calf serum (HyClone). HL-60 cells were differentiated to granulocytic (G) cells by incubating 2 × 10^6 cells/ml with 1.2% DMSO for 6 or 7 days, after which 85 to 95% of the cells rapidly reduced nitroblue tetrazolium dye and were electrophoresed with running buffer containing 1 mM glutathione. A variant of the protein alone was given 30 days later. After 3 days, spleen cells were harvested and were fused with the P3X63.AG853 nonsecreting mouse myeloma variant (ATCC). Supernatants from Ig-secretion hybrids were screened for reactivity with the immune in a direct ELISA and for reactivity with HL-60 cells, which do not share HLA with U937 cells, but possess the CRP-R. Selected hybridomas were subcloned twice, rescreened, and expanded for the production of ascites. The clones were all found to be of the mouse IgM (e) isotype, which was purified from ascites using a mannann-binding IgM affinity purification kit (Pierce). The IgM concentration was determined by absorbance at 280 nm and by radial immunodiffusion versus the mouse IgM myeloma MOPC-104e. The RC10.2 clone was used for the anti-CRP mAb in these studies since it inhibits ligand (125I-labeled CRP) binding to HL-60 and U937 cells (27).

A mouse mAb to IL-8RA designated 9H1 was obtained from Genentech (So. San Francisco, CA) and has been shown to bind to the NH2 terminus of the IL-8R and neutralized IL-8 binding (29).

Neutrophil chemotaxis

Human peripheral blood PMNs were obtained from heparinized blood by centrifugation on a one-step polymorph purification solution (Accurate Chemical & Scientific, Westbury, NY). The purity of the PMNs was always >95% with 2 to 3% contaminating lymphocytes, as judged by morphological criteria. The neutrophils were washed twice and brought up to 5 × 10^9 cells/ml in cold Gey’s balanced salt solution (EBSS) containing 10 mM HEPES (pH 7.4), and adjusted to 10^6 cells/ml and kept on ice for functional or binding assays.

Synthetic peptides


Purification of CRP

CRP was purified as described elsewhere (27, 28). Briefly, serum amyloid A (SAA)-enriched PMNs were solubilized in 10 ml of a lysis buffer of 20 mM Tris, pH 7.5, 110 mM NaCl, 1% Nonidet P-40, 2 mM PMSF, 10 g/ml pepstatin A, and 10 g/ml aprotinin by tumbling 1 hour on ice. The cells were centrifuged at 14,000 × g for 1 min at 4°C through a phthalate oil mixture (0.5 ml), and the pellet was collected immediately (27, 28) in a Beckman Gamma 4000 counter. Results were expressed as percentage of control binding (5–20,000 cpm/10^6 cells), and the experiment was repeated three times with triplicate samples.

MAPK activity

HL-60(G) cells at 5 × 10^6/sample were lysed by TN-1 (1% Nonidet P-40; 20 mM Tris, pH 8; 150 mM NaCl; 10 mM each EDTA, NaF, Na3VO4, and NaP2; 10 µg/ml aprotinin; and 10 µg/ml leupeptin) solution after stimulation with IL-8 or FMLP. MAPK was immunoprecipitated at 4°C for 3 h by 1 µg of protein A/G-Sepharose beads precoated with 1 µg of IgG rabbit anti-ERK-2 (Santa Cruz Biochemicals, Santa Cruz, CA). The beads were washed four times in TN-1 and then twice in the kinase assay buffer (MgCl2 20 mM; sodium orthovanadate, 0.1 mM; β-glycerophosphate, 20 mM; and HEPES, 30 mM, pH 7.6). Myelin basic protein (MBP) at 6 µg was used as the substrate, and 5 µCi (γ-32P)ATP (DuPont, Wilmington, DE) was added per sample. MBP was incubated by autoradiography after separation by SDS-PAGE and transfer to a nitrocellulose membrane. MAPK protein was detected by immunoblotting the same membrane.

PI-3K activity

Either IL-8- or FMLP-stimulated HL-60(G) cells (5 × 10^6/ml of RPMI 1640) were lysed with TN-1 solution, and immunoprecipitation of the p85 regulatory subunit of PI-3K was done overnight at 4°C using protein A/G-Sepharose beads precoated with a rabbit polyclonal IgG Ab to the p85 subunit of PI-3K. The Ab was raised by injecting a purified glutathione-S-transferase fusion protein containing the N-terminal SH2 domain of

single-stained band of a glycosylated protein of ~40 kDa (27) was dis-
Results

Effect of human CRP on neutrophil chemotaxis

Since the chemotactic activity of blood PMNs is an indicator of the cellular inflammatory response, the effects of purified human CRP on the neutrophil chemotactic response to the chemokine IL-8 and the bacterial chemotactic peptide FMLPP were examined. Brief exposure of isolated neutrophils to CRP under ligand-binding conditions (on ice) and subsequent examination of their chemotactic responsiveness to rhIL-8 (1 to 10 nM) revealed that CRP significantly inhibited the response at a concentration of >10 μg/ml CRP, as shown in a representative experiment (Fig. 1A). Using the same experimental approach with FMLPP at 10 nM, CRP inhibited neutrophil chemotaxis at concentrations ≥20 μg/ml (Fig. 1B). In these experiments, addition of the CRP alone to the lower chamber had no effect on chemotaxis, nor did CRP alone, or in combination with IL-8 or FMLPP, have any effect on cell viability, as judged by dye exclusion. The addition of the high affinity mAb to human CRP, HD2-4, neutralized its inhibitory activity on chemotaxis (Fig. 1B). Thus, CRP affects neutrophil chemotaxis in response to both IL-8 and FMLPP, but required a greater concentration of CRP to inhibit the response to the more potent chemotaxin.

Effect of CRP fragments on chemotaxis

Since human CRP is digested by activated PMNs into biologically active peptides (15, 31), the activity of CRP-derived peptides generated by incubation with PMA-activated PMNs on neutrophil chemotaxis was examined. The products of CRP digestion at 800 pmol/ml (20 μg/ml) inhibited chemotaxis in a manner similar to intact CRP (Table I). Approximately 60 to 80% of the CRP was cleaved into polypeptide fragments of <25 kDa by the PMA-activated PMNs, as judged by SDS-PAGE under reduced conditions (31). The recovered CRP polypeptide fragments, from which intact CRP was removed, by themselves exerted only very weak chemotactic activity (data not shown). Therefore, the net effect of the digested CRP on chemotaxis is one of inhibition.

Effect of human CRP synthetic peptides on chemotaxis

The synthetic cell-binding peptide of residues 27–38 within each CRP subunit, which binds to the CRP-R on leukocytes (28, 32), was found to significantly inhibit the chemotactic response of neutrophils to IL-8 and FMLPP (Fig. 1B). Each of the six residues in the core domain of human CRP was digested by PMNs (5 × 10⁵/ml) to yield PMN-digested human CRP. PMNs digested by PMNs (5 × 10⁵/ml) were allowed to digest purified human CRP for 2 h at 37°C. The CRP peptide fragments were allowed to bind to freshly isolated PMNs for 30 min before testing their chemotactic response to 1 nM of rhIL-8.

Table II. Effect of human CRP peptides on neutrophil chemotaxis in response to IL-8 and FMLPP

<table>
<thead>
<tr>
<th>CRP Peptide Residues③</th>
<th>% Control Chemotaxis vs Chemotaxin④</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-8 (1 nM)</td>
</tr>
<tr>
<td>NH2–1–15</td>
<td>83.5 ± 19.1</td>
</tr>
<tr>
<td>27–38</td>
<td>126 ± 8.5</td>
</tr>
<tr>
<td>47–63</td>
<td>116 ± 11.2</td>
</tr>
<tr>
<td>134–148</td>
<td>60.5 ± 14.8*</td>
</tr>
<tr>
<td>152–176</td>
<td>126 ± 8.5</td>
</tr>
<tr>
<td>174–185</td>
<td>ND</td>
</tr>
<tr>
<td>191–205</td>
<td>56.5 ± 6.4*</td>
</tr>
</tbody>
</table>

③ Peptides synthesized on the basis of the consensus sequence.
④ PMNs exposed to the peptides (800 pmol/ml) for 30 min before testing their chemotactic response to 10⁻⁷ M rhIL-8 10⁻⁷ FMLPP. Mean values ± SEM for four experiments.

⁎ p ≤ 0.05.

Table I. Effect of intact and PMN-digested human CRP on the chemotactic response of neutrophils to IL-8

<table>
<thead>
<tr>
<th>CRP Treatment③</th>
<th>CRP Concentration (μg/ml)</th>
<th>% Inhibition of Chemotaxis④</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>43.5 ± 7.8</td>
</tr>
<tr>
<td>20</td>
<td>66.0 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>Digested by PMN</td>
<td>5</td>
<td>54.0 ± 12.3</td>
</tr>
<tr>
<td>20</td>
<td>69.4 ± 2.3</td>
<td></td>
</tr>
</tbody>
</table>

③ PMNs (5 × 10⁵/ml) were allowed to digest purified human CRP for 2 h at 37°C. The CRP peptide fragments were allowed to bind to freshly isolated PMNs for 30 min before testing their chemotactic response to 1 nM of rhIL-8.

④ Average percent inhibition from two experiments with triplicate samples.
was tested along with additional synthetic CRP peptides for chemotactic activity at 800 pmol/ml, the equivalent of 20 μg/ml of the intact CRP pentamer. Of the peptides listed in Table II, only the cell-binding peptide of residues 27 through 38 displayed weak chemotactic activity when compared with FMLPP. Several of the CRP-synthetic peptides were also tested for their effects on neutrophil chemotaxis mediated by IL-8. Of the peptides tested, only two of them inhibited the chemotactic response: the macrophage-activating peptide of residues 174–185 (33), and the C-terminal peptide of amino acids 191–205 of CRP significantly inhibited the induced PMN chemotactic response (Table II). Thus, two of the CRP peptides, Pep 174–185 and Pep 191–205, inhibited the chemotactic response triggered by concentration gradients of either FMLPP or IL-8.

**Effect of a mAb to the CRP-R on chemotaxis**

A series of mouse mAbs was generated against isolated membrane proteins from U937 monocytic cells that were obtained by affinity chromatography on CRP-Sepharose. These mAbs were screened for specificity and their ability to inhibit specific ligand (CRP) binding, as described elsewhere (27, 32). The IgM mAb RC10.2, which inhibited labeled CRP binding, was tested for its ability to alter the chemotactic response of PMNs to FMLPP. Chemotaxis was inhibited at concentrations >0.1 μg/ml of purified IgM mAb per 10⁶ PMN (Fig. 2). This concentration of the mAb is sufficient to occupy >70% of the CRP-R calculated at 10⁴ receptor sites/PMN (27). Thus, the mAb to the putative CRP-R mimicked the action of CRP itself on neutrophil chemotaxis.

**Effect of CRP on IL-8 binding**

Since one possibility for the inhibitory action of CRP on the chemotactic response is that CRP blocks the chemokine-receptor binding interaction, the effect of CRP on labeled IL-8 binding was examined. Human CRP at concentrations from 1 to 100 μg/ml (800 nM) failed to significantly inhibit binding of 1 nM ¹²⁵I-IL-8 to HL-60(G) cells; however, at 200 μg/ml, CRP inhibited only ~25% of the IL-8 binding at a 1600-fold molar excess (Fig. 3). In the same experiments, rhIL-8 itself inhibited ¹²⁵I-IL-8 binding by approximately 70% at a 100-fold molar excess of the unlabeled ligand (Fig. 3). Thus, it seems unlikely that CRP inhibits IL-8 mediated chemotaxis by inhibiting ligand-receptor binding.

**Effect of CRP on MAPK**

In preliminary experiments, when the pattern of all of the tyrosine-phosphorylated proteins from HL-60(G) cells stimulated with IL-8 or FMLPP after exposure to human CRP was examined, the intensity of two prominent phosphorylated proteins of 35 to 40 kDa was increased. Since MAPK is ~38 to 44 kDa and its activity was reported to be inducible by IL-8 over a 30-min interval, reaching maximum levels at 3 min (34), CRP was tested for its influence on MAPK activity measured as ERK-2. ERK-2 was increased by two-fold in response to CRP at concentrations of 200 μg/ml at 1, 3, and 10 min after stimulation with 50 nM of IL-8 (Fig. 4A). A plot of the cpm of ³²P-labeled MBP present in each lane is shown for comparison (Fig. 4B). ERK-2 activity induced by FMLPP was also enhanced by preincubation with CRP (Fig. 5A). The same blotted membrane was probed with anti-ERK-2 Ab to demonstrate that approximately the same amount of ERK-2 protein was present in each of the lanes (Fig. 5B). CRP appears to augment MAPK activity by increasing its phosphorylation.

Since bacterial chemotactic peptides have been shown to activate the two isoforms of mitogen-activated extracellular signal-regulated kinase kinases (MEK or MAPK kinase) present in neutrophils (35, 36), we tested the effect of the MEK-specific inhibitor, PD98059, on FMLPP-induced chemotaxis. This inhibitor at its ED₅₀ of 5 μM and higher concentrations potentiated the chemotactic response (Table III). Since PI-3K is upstream of MAPK kinase (MEK) and has also been implicated in the regulation of the neutrophil chemotactic response, we examined the effects of the PI-3K-specific inhibitor, Wortmannin, and found that it inhibited the chemotactic response at its ED₅₀ of 10 nM (Table III). Therefore, PI-3K appeared to be a more logical target for the signaling triggered by CRP.

**Effect of CRP on PI-3K**

PI-3K plays a pivotal role for both IL-8 signaling (37), as well as signaling by FMLP (38) in PMN; therefore, the effects of CRP on
PI-3K were tested. PI-3K activity is sensitive to the inhibitor wortmannin, which down-regulates the activity of Raf-1, B-Raf, and MAPK, but not Ras in PMNs stimulated by IL-8, C5a, and FMLP (37–39). Preliminary experiments with FMLPP at 0.1 to 100 nM revealed that maximum PI-3K activity was induced at 25 nM. Therefore, the effect of CRP on FMLPP-induced PI-3K activity was initially evaluated and found to be increased at CRP concentrations ≥5 mg/ml; however, the controls containing CRP in the absence of any FMLPP also displayed enhanced PI-3K activity. Therefore, the effect of treating the PMNs to different concentrations of CRP alone was tested. PI-3K activity was increased significantly at CRP levels ≥5 mg/ml (Fig. 6A). When an immunoblot of the precipitated PI-3K was probed with an anti-p85 Ab specific for the regulatory chain of PI-3K, the relative amount of p85 per lane was the same (Fig. 6B). Exposing the HL-60(G) cells to CRP alone for different intervals from 1 to 60 min indicated that an exposure time of only 1 min was sufficient to elevate PI-3K activity (Fig. 7A). The optimal PI-3K response takes 3 min with FMLPP. Thus, the kinetics of PI-3K activation by CRP are rapid. The immunoblot of the samples in Figure 7A indicated that similar amounts of p110 catalytic activity of PI-3K were examined based on the presence of the p85 subunit (Fig. 7B). Thus, CRP may attenuate neutrophil responses via PI-3K-dependent reaction(s) in a time- and dose-dependent fashion.

Table III. Effect of the PI-3K inhibitor Wortmannin and the MEK inhibitor PD98059 on the chemotactic response of PMNs to FMLPP

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentrationa</th>
<th>Mean cells/hpfb</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD98059</td>
<td>0 μM</td>
<td>52.6 ± 5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 μM</td>
<td>86.4 ± 3.1</td>
<td>−64</td>
</tr>
<tr>
<td></td>
<td>25 μM</td>
<td>71.0 ± 2.5</td>
<td>−55</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>66.0 ± 10.0</td>
<td>−25</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>0 nM</td>
<td>29.5 ± 3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 nM</td>
<td>25.0 ± 2.8</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>10 nM</td>
<td>7.3 ± 4.0</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>50 nM</td>
<td>4.0 ± 1.6</td>
<td>86</td>
</tr>
</tbody>
</table>

a The ED50 for PD98059 is 5 μM and 10 nM for Wortmannin.

b Data are the mean number of PMNs/hpf ± SEM for four experiments.

Therefore, the effect of treating the PMNs to different concentrations of CRP alone was tested. PI-3K activity was increased significantly at CRP levels ≥5 μg/ml (Fig. 6A). When an immunoblot of the precipitated PI-3K was probed with an anti-p85 Ab specific for the regulatory chain of PI-3K, the relative amount of p85 per lane was the same (Fig. 6B). Exposing the HL-60(G) cells to CRP alone for different intervals from 1 to 60 min indicated that an exposure time of only 1 min was sufficient to elevate PI-3K activity (Fig. 7A). The optimal PI-3K response takes 3 min with FMLPP. Thus, the kinetics of PI-3K activation by CRP are rapid. The immunoblot of the samples in Figure 7A indicated that similar amounts of p110 catalytic activity of PI-3K were examined based on the presence of the p85 subunit (Fig. 7B). Thus, CRP may attenuate neutrophil responses via PI-3K-dependent reaction(s) in a time- and dose-dependent fashion.
The effects of CRP-derived peptides on leukocyte functions, as first described by Robey et al. (15), clearly demonstrated that peptides generated by digestion of CRP by PMNs, but not intact CRP, were chemotactic for monocytes. Subsequent work by Shephard et al. (16, 17, 31) revealed that CRP proteolysis by PMNs generated peptides that inhibited both neutrophil chemotaxis and O$_2$ production. Indeed, one of the most active peptides consisted of residues 201 to 206 (16), contained within the synthetic peptide of residues 191–205 that we show, in this work, inhibits IL-8-induced chemotaxis. Another peptide that inhibited chemotaxis was composed of residues 174 to 185, which were characterized previously as a macrophage-activating agent for tumoricidal activity in mice (33). CRP peptides have also more recently been shown to inhibit neutrophil alveolitis (47). We did not observe chemotaxis with the intact CRP pentraxin, nor with most of the CRP peptides; however, the peptides including residues 174 to 185 and 191 to 205 displayed reproducible chemotactic activity. The mAb, RC10.2, which inhibits receptor binding of CRP, mimics the effects of CRP by inhibiting chemotaxis. The effects of CRP on the two phagocytic leukocyte populations are distinct with activation of the monocyte/macrophage population and inhibition of the granulocytic responses (7).

The signaling mechanism whereby CRP alters the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex activity in PMNs has not yet been determined; however, one study (19) does show an alteration in the pattern of protein phosphorylation in activated neutrophils that was very similar to our initial observations on changes in the pattern of protein tyrosine phosphorylation induced by CRP. The effects of CRP on PMN also correlated with a pronounced increase in cytosolic cAMP (45) and inhibition of intracellular Ca$^{2+}$ mobilization (48). Whether the direct effect of CRP on the respiratory burst of activated PMNs involves the same signaling pathway(s) regulating their chemotactic response remains to be determined.

The chemotaxtactant receptors all belong to the seven-transmembrane-receptor superfamily coupled to heterotrimeric G proteins (4, 5). Both forms of the IL-8R (CXCR1 and CXCR2), as well as the FMLP-R, are regulated by a desensitization process that requires phosphorylation of Ser and Thr residues in the C-terminal cytoplasmic region, a process that is protein kinase C or protein...
kinase A dependent (49–51). It seems unlikely that CRP stimu-
lates IL-8R degradation via homologous desensitization, since
CRP did not bind or compete for IL-8 binding sites. Attempts
to demonstrate that CRP altered the extent of IL-8-induced Ser phos-
phorylation of the IL-8RA isoform in neutrophils failed to detect a
significant difference. Rather, the experiments described here
with IL-8 and FMLPP suggest that the CRP-R mediates inhibition
of chemotaxis by a process dependent on PI-3K. Indeed, our re-
sults show that wortmannin blocks ligand-induced chemotaxis,
suggesting that PI-3K is essential, yet CRP induces PI-3K activity.
This apparent contradiction may have one or more of the following
explanations. CRP may block chemotaxis via a different mecha-
nism than through PI-3K, e.g., inhibition of the activities of the
small G proteins, such as Rac and/or Rho, that regulate neutrophil
cytoskeletal functions (52). CRP may activate a distinct isoform
of PI-3K that is not sensitive to Wortmannin and also fails to generate
the appropriate 3-phosphoinositide product (53). CRP may also
activate an inositol phosphatase that consumes any nascent 3-phos-
phoinositides (54). We are presently examining these alternative
mechanisms in neutrophils exposed to CRP under various condi-
tions. The CRP-triggered PI-3K-dependent pathway should also
propagate reactions that inhibit the phosphorylation and assembly
of the components of the nicotinamide adenine dinucleotide
phosphate (NADPH) oxidase (55).

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