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REGULATORY EFFECT OF HUMAN C-REACTIVE PROTEIN
ON HUMAN NEUTROPHIL ACTIVITIES: CHEMOTAXIS,
RESPIRATORY BURST, AND THEIR SIGNALING.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

by

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* * * * *

The Ohio State University
1998

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ABSTRACT

C-reactive protein (CRP) is the prototypic acute phase reactant of humans since its blood level can increase by over a thousand-fold within 48 hours of the onset of acute inflammation. CRP is a pentameric protein made of five identical protomers arranged in cyclic symmetry with non-covalent bonds between the subunits, and thus belongs to the "pentraxin" protein family. The structure and binding specificities of CRP have been conserved through several hundreds of million years of evolution, strongly suggesting an critical biological role for CRP.

CRP binds many substrates or ligands through its PC binding site, including microorganism, damaged cell membranes and chromatin. CRP is selectively deposited at the site of inflammation, and can be degraded into fragments by neutrophil cell surface proteinase at the same inflammatory site. There are a few reports that CRP and its digest product regulate leukocytes functions. The interaction between CRP and leukocytes are documented as receptor mediated, and involve intracellular Ca" mobilization. Since the effect of CRP on
leukocyte functions needs to be clarified, a series of experiments were initiated to focus on the signaling initiated by CRP with granulocytes.

Our research results showed that human CRP inhibited neutrophil chemotaxis induced by either the human chemokine IL-8 or the bacterial formyl peptide fMLPP in a dose-dependent manner at CRP concentration from 5 to 50 μg/ml. Two human CRP synthetic peptides 174-185 and 191-205 also significantly inhibited the chemotaxis induced by either IL-8 or fMLPP, thus confirming that CRP peptides have biological activities. This inhibition is MAPK related, since the MEK inhibitor PD 98059 could promote chemotaxis, while human CRP enhanced ERK2 activity induced by either fMLPP or IL-8 in HL-60 (G) cells. Human CRP by itself also activated PI-3K activity in these cells.

Human CRP also inhibits neutrophil superoxide production induced by fMLPP or PMA at CRP concentration ≥ 50 μg/ml, with the inhibitory effect more pronounced when PMA was the trigger. In cells stimulated with PMA, CRP inhibited the activation of PKC in terms of translocation to the membrane and serine autophosphorylation. This resulted in a decrease in the phosphorylation of serine residues on the p47-phox cytosolic component of NADPH oxidase and its translocation to the membrane. Therefore, human CRP blocked the assembly of
the functional NADPH oxidase and thus inhibited the respiratory burst of neutrophils.

The minimal sequence requirement and critical residues for the CRP cell binding peptide (Pep27-38) were demonstrated. Inhibition of $O_2^-$ production required residues 31-36 KAFTVC, with the Tyr33 and Ile37 being critical for its biological activity.

Neutrophil activation (chemotaxis, degranulation and respiratory burst) is critical for defending the host against bacterial infection. However, overactivation of neutrophils at the inflammatory site is responsible for the host tissue damage. Thus, regulating neutrophil activation is potentially significant for the therapy of inflammation in the practice of medicine. Human CRP is a naturally occurring plasma protein and likely to serve an important physiological and pathophysiological role in the development and regulation of inflammatory diseases.
To my parents
ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Richard F. Mortensen for guiding me through the research field with enthusiasm, understanding and insistency. Without his dedication to my project, I would not reach the point where I am now. Great thanks go to Dr. K. Mark Coggeshall, for his helpful discussion of my research as well as for his generosity letting me working in his laboratory and using everything I needed. Gratitude is expressed to Drs. Darrell R. Galloway, John M. Robinson and William R. Strohl for their interests in my research project and many suggestions. Thanks also go to my family for their patience and support.
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ABBREVIATION

APR  Acute phase reactant(s)
     Acute phase response
BALF Bronchoalveolar lavage fluid
BCS  Bovine calf serum
BSA  Bovine serum albumin
C5a  Complement 5 fragment a
C/EBP CAT box enhancer binding protein
CPS  C-polysaccharide
CRP  C-reactive protein
CRP-R CRP-receptor
DAG  Diacylglycerol
DMSO Dimethyl sulfoxide
D-PBS Dulbecco's PBS
EBSS Earle's balanced salt solution
ECL Electrochemiluminescence
E-CPS Erythrocytes-CPS
EGF  Epithelial growth factor
ELIZA Enzyme linked immunosorbent assay
ERK Extracellular signal-regulated kinase
FBS  Fetal bovine serum
FcR  Receptor for Ig Fc portion
fMLP Formyl-Met-Leu-Phe
fMLPP FMLP-Phe
G   Granulocytic
GBSS Gey's balanced salt solution
HNF Hepatic nuclear factor
HRP Horseradish peroxidase
[I²¹⁵] Iodine-125
Ig  Immunoglobulin
IL-1 Interleukin 1
IL-2 Interleukin 2
IL-6 Interleukin 6
IL-8 Interleukin 8
IL-8Ra IL-8 receptor alpha
IL-10 Interleukin 10
JNK  c-JUN N-terminal kinase
LPG Lipophosphoglycon
LPS  Lipopolysaccharide
Mφ  Macrophage
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated extracellular signal-regulated kinase kinase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide-adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>NFIL-6</td>
<td>Nuclear factor activated by IL-6</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>p38</td>
<td>A member of MAPK family</td>
</tr>
<tr>
<td>PAO</td>
<td>Phenyl arsine oxide</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphocholine</td>
</tr>
<tr>
<td>Phox</td>
<td>Phagocyte oxidase</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil(s)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl-methyl-sulfonyl-fluoride</td>
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<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTX-3</td>
<td>Pentraxin 3</td>
</tr>
<tr>
<td>RID</td>
<td>Radial immunodiffusion</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum amyloid P component</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
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<tr>
<td>SH3</td>
<td>Src homology domain 3</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain containing inositol phosphatase</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>snRNP</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<td>TSG-14</td>
<td>TNF-stimulated gene 14</td>
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INTRODUCTION

During acute inflammation caused by either microbial infection or tissue destruction in animals, the host system will initiate a cascade of nonspecific events termed "acute phase response" which is characterized by the hepatocytic production of a large group of blood proteins called "acute phase reactants" (APR). The APRs include coagulation proteins (fibrinogen), complement proteins (C2 through C9), protease inhibitors (α1-antitrypsin, α2-antiplasmin), metal-binding proteins (ceruloplasmin) and other proteins (lipopolysaccharide-binding protein, the LBP). C-reactive protein (CRP) is the prototypical APR in humans since its blood level can increase a thousand-fold within 24-48 hours of the onset of inflammation from a base level of 100 ng/ml. Thus CRP levels serve as an indication of acute inflammation in patients. The increased serum CRP level during the acute phase response is mainly the result of accelerated rates of transcription of the gene in liver hepatocytes, but the secretion rate is also increased due to the down regulation of a specific binding site by which CRP is retained within the endoplasmic reticulum (ER). CRP belongs to a unique protein
family termed "pentraxin" because it is composed of five identical, non-covalently associated protomers. Human CRP is coded by a single copy gene on chromosome 1 with a polypeptide of 206 amino acids without modification such as glycosylation and phosphorylation. The three dimensional structure of CRP had been recently solved by a group using X-ray chrystography with a resolution at 3 Å, showing a flat, pentameric disk with cyclic symmetry. The overall structural fold of CRP is similar to that of serum amyloid P component (SAP), another member of the pentraxin family. The basic structure of the CRP protomer is a jellyroll, with 14 antiparallel β-strands arranged into two β-sheets and a single α-helix. The two Ca\(^{2+}\) binding sites are coordinated by residues Asp60, Asn61, Glu138, Gln139, Asp140, Glu147, Gln150, and a phosphocholine (PC) binding site.

CRP and the pentraxin family are ancient proteins which are conserved for both structure and binding activity throughout evolution. CRP and SAP have been found in a wide array of animal species as primitive as the invertebrate *Limulus polyphemus* (horseshoe crab), through the lower level vertebrate *Mustelus canis* (dogfish), and all mammals examined to date. The amino acid sequence alignment shows significant homology among the CRPs from different species, as distant in evolution as limulus and man. Rabbit and human CRP disclose
a 75% residue identity. The remarkable conservation of structure and binding specificity of CRP through > 500 million years of evolution strongly suggests a critical biological role for CRP.

CRP binds to many targets through its PC binding site, including microorganism, damaged cell membrane and chromatin. Many studies have described biological activities of CRP, including complement activation, opsonization, cytokine induction, and a general protective effect against microbial infection and tumorigenesis. However, all these activities of CRP are nonspecific and elusive. A unique biological function of CRP needs to be defined if it exists. CRP selectively deposits at the site of inflammation, binds and opsonizes infecting microbial organisms and damaged host cells for phagocytosis through specific membrane receptor on leukocytes. CRP also has been degraded into fragments by neutrophil cell surface protease at the inflammatory sites. Heat modified CRP and CRP-derived peptide have been demonstrated modulating biological activities of certain leukocytes. CRP enhances O$_2^-$ production in macrophages but inhibits it in neutrophils. CRP peptide enhances monocyte/macrophage tumoricidal activity. The interaction between CRP and leukocytes is documented as receptor mediated, and involves intracellular Ca$^{2+}$ mobilization. But questions concerning what kinds of
functions of leukocytes, especially neutrophils (because they are the first group of leukocytes migrated into the acute inflammatory site where CRP is also deposited) are regulated by CRP and how CRP regulates these functions need to be clarified. So a series of studies was initiated to investigate the role of human CRP in the regulation of neutrophil activities.
A. History of C-reactive protein (CRP)

CRP was discovered in 1930 by two physicians Tillett and Francis (1) serum component from patients with acute pneumococcal pneumonia and was able to bind and precipitate the C-polysaccharide (CPS) from the acid extracts of \textit{Streptococcus pneumoniae} and thus named C-precipitin. The C-precipitin was only found in the blood of patients with acute bacterial infections by the CPS precipitation method, which is Ca$^{2+}$-ion dependent because this activity could be blocked by the anticoagulant citrate (2). The C-precipitin was classified as an "acute phase reactant" (APR) and later also as "acute phase protein" in 1941 when the protein nature of this C-precipitin was determined and thus renamed C-reactive protein (2). Since then the serum level of CRP is regarded as an indicator or measure of the severeness of infectious diseases (3). In a more sensitive study, a rabbit antibody against the purified CRP reacted with acute phase serum strongly, but not the normal sera (2). By tracing the $^{14}$C-labeled amino acids incorporated into the newly synthesized
proteins in animal model in the 60's, Hurlimann et al. demonstrated that the liver is the major organ synthesizing CRP (4).

B. Biosyntheses of CRP in the liver

In an animal model study, Kushner et al. demonstrated that as early as 8 hours after initiation of inflammation the production of CRP could be detected in the periportal area in the liver and then spread to the whole liver by 16 hours with the maximum appearing at 38 hours (5). In a mouse model, the mRNA of serum amyloid P component (SAP), the counterpart of human CRP in mice, was massively induced in liver within 4 hours of inflammatory stimulus and reached a peak by 8-12 hours (6). Electron microscopic study showed that CRP accumulated in certain areas within the hepatocytes, especially the rough (RER) and smooth endoplasmic reticulum (SER), the Golgi apparatus and secretory vesicles (7). It was later found that CRP was retained in the ER binding to a 60 kDa receptor protein, and during acute phase this receptor could lower its affinity for CRP and increase the secretion rate of CRP (8). The 60 KDa receptor protein turned out to be a carboxylesterase containing carboxyl terminal ER retention signals (9). But the overall increase of serum CRP is mainly the result of increased de novo synthesis of the
protein itself by liver hepatocytes (10, 11).

C. Gene structure of CRP and regulation of CRP synthesis

Human CRP gene is a single copy gene mapped to chromosome 1 using cDNA hybridization (12). Within 16 kb pairs upstream and downstream of the locus for the human CRP gene, only one pseudogene with 50-80 % homology could be found (13). The human CRP gene has two exons separated by a 278 bp intron. The first exon encodes a leader peptide and the first two amino acids of the mature protein, while the second exon encodes the remaining 204 amino acids (14). There is a typical promoter region containing the sequences TATAAAAT (TATA box) and CAAT at -81 and -29 base pairs upstream of the mature mRNA cap site (14). Two IL-6 responsive elements conferring inducible expression of the human CRP gene in hepatoma cell lines were located proximal to the site of initiation of transcription (15). Two acute phase response elements (APREs) at position -94 to -50 and -137 to -106 of CRP promoter are responsive to IL-6 and have the C/EBP consensus sequences TG(G/A)AA (16). The first APRE contains a binding site for the liver-specific transcription factor HNF-1, and the second APRE contains both a HNF-1 and a NF-IL-6α binding site (17). NF-IL-6α is a transcription factor of the C/EBP family induced by IL-6 (18), and
activated when phosphorylated on Ser105 by protein kinase C (PKC) (19). There is also an IL-1-responsive sequence between -42 and +15 in the human CRP gene (16). The 300 bp 5'-flanking region contains a total of 7 specific binding sites for five different positive/negative nuclear factors, from the families such as C/EBP, HNF-1α, HNF-3/Octamer-like factors (20). The human CRP gene promoter has STAT3 (signal transduction and activator of transcription) and C/EBP sites (165). Indeed, STAT 3 is necessary for IL-6-induced CRP synthesis in Hep 3B cells (165). Very recent data from our lab shows that the promoter region of the mouse CRP gene also contains consensus STAT3 binding sequences which are recognized by IL-6-induced STAT3 (Ochrietor). STAT 3 is a newly found transcription factor, which is recruited and activated through tyrosine phosphorylation by an IL-6 receptor-associated JAK kinase upon IL-6 stimulation (21). It was demonstrated by transient transfection in the human hepatoma PLC/PRF/5 cell line with site-specific mutations that the binding of NF-IL-6α to the two inducible IL-6 responsive elements resulted in synergistic induction of the human CRP gene (22). In cancer patients, the increase of IL-6 level is correlated with an increase in CRP serum levels (23,24). A more recent in vivo study using human CRP transgenic mice showed that IL-6 is necessary, but not
sufficient to induce human CRP gene transcription (25). IL-6 was demonstrated as the major cytokine to induce CRP production in a rat model and in the human cell line Hep3B using transgenic technology (26,27). However, maximal gene expression occurs only when both IL-1 and IL-6 are present (16,28). The IL-1 receptor antagonist protein was shown to selectively block the in vivo synthesis of human CRP (29). Other cytokines and hormones may also be involved in the regulation of CRP synthesis. TGF-β was reported to increases CRP production induced by IL-1 and IL-6 (30), but TNF-α inhibits the production (31). Interestingly, testosterone also regulates constitutive human CRP production, but not the lipopolysaccharide (LPS)-induced production (32,46).

Serum CRP is synthesized and secreted almost exclusively by hepatocytes (5). Sequences 540 bp upstream and 1.2 kb downstream of the start site were sufficient for liver-specific, constitutive expression (33). The increase of CRP during acute phase is the result of accelerated transcription of the gene in the liver (11), and also a result of an increase in the secretion rate due to the down regulation of a specific binding site by which CRP is retained within the endoplasmic reticulum (ER) (8). However, CRP is also synthesized extrahepatically by NK cells (34,35), Kupffer cells (36), alveolar macrophages (37) and monocytes (38) as
a membrane bound neo CRP (single CRP protomer) which was proposed to be a galactose-specific opsonin(34,35,39).

D. STRUCTURE OF CRP

Human CRP is composed of five identical subunits (protomers), each of which a non-glycosylated polypeptide chain of 206 amino acids with a calculated m.w. of 23,017 (40). Two cysteine residues at positions 36 and 96 that are conserved in the family are involved in an intrachain disulfide bond (41). Human CRP shares 51% amino acid identity and 59% nucleotide sequence identity with human SAP (42).

Using electron microscopic images in 1977, a group revealed that CRP subunits are arranged in a planar pentameric disc with cyclic symmetry (43). A recent infrared spectroscopic analysis suggested that each of the CRP protomer contains substantial β-sheets (~50%), 12% α-helix, and 24% β turn (44). Using X ray diffraction at a resolution at 3 Å, a recent study completely revealed the three dimensional structure of human CRP (45). The overall structure is similar to that of human SAP, but with obvious difference at every structural level (46). The CRP protomer consists of 14 antiparallel β-stands arranged in two β-sheets in a jellyroll structure; the core containing a hydrophobic region inside composed of mostly aromatic side chains of the
primary structure (45). This tertiary fold is similar to that of legume lectins, such as concanavalin A. The domain of residues 168-176 forms a long α-helix lying against one of the β-sheets, and together with the loop 177-182 forms part of a wall of an extended V-shaped hydrophobic pocket, while parts of the N- and C-termini form the other wall of this pocket (45). This pocket does not seem to be influenced by the presence or absence of bound calcium, and thus is speculated to be involved in non-phosphocholine, calcium-independent binding. On the opposite side of this hydrophobic pocket are two calcium binding sites, coordinated mainly by loop 140-150 with the help from residues Asp60 and Asn61. A hydrophobic spot beside the two calcium binding sites, together with the two Ca²⁺ ions, coordinates the phosphocholine (PC) binding site (46).

E. CRP ligand binding sites

CRP was first discovered when it precipitated the pneumococcal C-polysaccharide (CPS) (1) and was later found to be calcium dependent (2), and inhibitable by various phosphate monoesters (47). In the early 70's, it was found that the phosphocholine (PC) group on the C-polysaccharide was the actual ligand for CRP (48). There is a valence of one PC for each of the CRP protomer in the pentamer with an
association constant of $1.6 \times 10^5 \text{ M}^{-1}$ (49).

CRP has also been reported to bind to other PC-containing compounds, including phospholipids (phosphatidylcholine and sphingomyelin in the lipid bilayer (50,51) and various ligands that do not contain PC, such as chromatin (52), Histones H1, H2A and H2B (53), small nuclear ribonucleo-protein particles (snRNPs) (54,55), galactose-containing polysaccharides (56), polycationic substances (57), the repeating phosphorylated disaccharide units of the lipophosphoglycon (LPG) on the surface of parasite Leishmania (58), nuclear envelope protein, fibronectin (59-61), and ribonuclease-resistant autoimmune antigens, and the Sm-D and Sm-D' snRNP proteins as well.

1. *Binding site for phosphocholine.*

Binding of most of these ligands is inhibitable by PC (11), indicating that a single ligand binding site in CRP can accommodate somewhat diverse structural groups. The binding site on CRP for PC was first proposed as the tetrapeptide sequence of residues 39-Phe-Tyr-Thr-Glu-42 based on the conserved sequences of other known PC-binding proteins, such as myeloma Ig proteins (62). Later, a site-directed mutagenesis study of this region by Volanakis and colleagues demonstrated that this tetrapeptide does not play a major
role in the formation of the PC binding site of CRP because the mutated CRP binds CPS as well as the wide type CRP (63). In 1990, Mortensen and colleagues reported that a CRP derived synthetic peptide corresponding to CRP amino acid sequences 47-63 could bind to PC Ca"-independently, and competed with pentameric CRP for PC binding (64). Mutagenesis studies (65,66) of CRP disclosed that three conserved residues in CRP, the Trp67, Lys57, and Arg58 are important for keeping a proper conformation of the PC binding site. The Thr76 actually is a determinant in forming a hydrophobic pocket which accommodates the choline group. The structure derived from that of human SAP supports these results.

However, the latest human CRP x-ray crystallography structure (45) indicates that the phosphate group of PC binds the two Ca" ions, and the three choline methyl groups fit in the nearby large hydrophobic pocket formed by Phe66, Leu64 and Thr76 with a direct hydrophobic interaction between the methyl groups and the exposed face of the Phe66, which is conserved in human, mouse, rabbit, rat and frog CRP. The negatively charged Glu81 has an interaction with the positive choline nitrogen. The formerly indicated Trp67 does not have direct interaction with the methyl groups of choline, but rather interferes with the positioning of the Phe66.
2. Ca\(^{2+}\) binding site

A very early study (2) showed that binding of CRP to the CPS is calcium dependent, with two Ca\(^{2+}\) binding sites per subunit of equal affinity $K_d = 6 \times 10^{-5}$ (40, 67). Based on the comparison with Ca\(^{2+}\) binding sequences in other proteins, the highly conserved region between residues 134 to 148 in both CRP and SAP was predicted as the Ca\(^{2+}\) binding site (68), and proteolytic cleavage between residue 145 and 146 decreased Ca\(^{2+}\) binding, and calcium binding inhibits proteolytic cleavage (67). Mullenix and Mortensen reported that two synthetic CRP peptides (residues 134-148 and 152-176) bind calcium with dissociation constants of $5.2 \times 10^{-4}$ and $1.7 \times 10^{-4}$ M respectively (69). The recently published X-ray crystallography structure of CRP showed that the first Ca\(^{2+}\) is coordinated by Asp60, Asn61, Glu138, Gln139, Asp140 and the second Ca\(^{2+}\) is coordinated by Glu138, Asp140, Glu147 and Gln150 (45).

3. Binding to polycations

CRP also binds polycations, such as poly-L-lysine, poly-L-arginine, protamine and the lysine-rich and arginine-rich histones, myelin basic protein and leukocyte cationic protein (70-73), which is inhibitable by Ca\(^{2+}\) in a dose-dependent manner. However, PC-binding enhances the precipitation of
CRP with poly-L-lysine, suggesting the polycation-binding site is modulated by both the calcium- and the PC-binding site, but is a separate site (71).

4. Binding to chromatin

The affinity of CRP for chromatin was found to be greater than for PC (52), but the evidence showed that both ligands bound to the same site and was Ca"-dependent. The ratio of CRP vs. chromatin is 160 bp DNA per CRP, so it was suggested that CRP binds to chromatin at the DNA-histone region, where a basic group of lysine or arginine from the histone is adjacent to a negatively charged phosphate group of the DNA backbone (52).

5. Binding to galactosyl group

CRP also has a binding specificity for galactosyl derivatives as documented by Higginbotham since CRP precipitated the de-pyruvylated pneumococcal IV capsular polysaccharide(74). Results from several other laboratories supported this conclusion by showing that CRP bound to a number of snail galactans (75), agarose (76), leishmania galactan (77) and lipophosphoglycan (58). It had been found that immobilization of CRP onto polystyrene surfaces or latex beads was required for its lectin-like binding to galactosyl
groups on macromolecules such as plasma glycoproteins (Igs), synthetic glycoproteins (79), and polysaccharides (74, 80-83). This immobilization might cause a conformational change which expose a new binding site for the galactosyl groups.

6. Binding site for SAP

Human SAP could also recognize immobilized CRP (84, 85), but not vise versa (85). This binding is Ca""-dependent and not lectin-like. Using both monoclonal antibodies and synthetic CRP peptides, Christner demonstrated that the CRP residues 134-148 and 191-206 were recognized by SAP. Thus, binding site on SAP is located on the C-terminal 60 a.a. region (84). The biological meaning of CRP to SAP binding is not clear.

F. Biological Activities of CRP

The conservation of CRP throughout evolution from the horseshoe crab (Limulus polyphemus) to all the mammals studied to date suggests an essential biological role for CRP. CRP has a very broad spectrum of biological activities, ranging from complement activation, opsonization, regulation of leukocyte activity, affecting both the cellular and humoral immuneresponses. Over the years, the consensus viewpoint of CRP is that it is a nonspecific mediator of the
host defense system against microbial infection.

1. Complement Activation

Complement activation by CRP containing complexes probably is the first biological activity attributed to CRP that links it to the host humoral immuneresponses. It was first found in 1974 by Kaplan and Volanakis that CRP complexed with C-polysaccharide (CPS) or certain phospholipids could activate the complement (C) classical pathway (50, 86). It was subsequently shown that complexes of CRP with a variety of other ligands such as polycation (73), PC-containing phosphatidylcholine liposomes (76, 87) and nuclear DNA (88) also activated the classical C pathway. There was evidence that C4b α-chain could form a covalent bond with CRP (63).

The biological role of the CRP-activated complement classical pathway was later demonstrated by the hemolysis of CPS-coated sheep erythrocytes (E-CPS) which was dependent on the CRP concentration. The deposition of the larger b fragments of C3 and C4 on the erythrocyte enhanced their phagocytosis by monocytes (89, 90).

Aggregated CRP itself could cause complement activation only under mild acidic conditions with an optimum pH at 6.3, a pH often seen at an inflammatory site (91). Under these
conditions, CRP undergoes a pH-dependent conformational change which enhances its reactivity with the Cl component of the C system. These results indicate that the binding site for complement on CRP is probably not accessible in the native form of CRP.

a. Complement binding site

The interaction between CRP and complement has been investigated extensively. Based on the knowledge of the Clq-binding region of immunoglobulin (Ig), where a positively charged core region contains Glu318, Lys320, Lys322 (92), a similar region on CRP corresponding to residues Asp112, Lys114 and Arg116 was identified as a potential binding site. Using oligonucleotide-directed site-directed mutagenesis, the negatively charged Asp112 was shown to play a major role in forming the binding site as well as keeping the complement-activation efficient (93). The nearby positively charged Lys114 and Arg116 playing an important but indirect role, although the depletion of the two positively charged residues increased the binding affinity and C activating ability (93).

The newly revealed three dimensional structure of CRP indicates that these residues are involved in protomer-protomer interaction (45). Thus a conformation change is necessary to expose this site for complement activation and
may explain why CRP-ligand complexes could activate the complement pathway, but not the native CRP pentamer.

In contrast to immunoglobin (Ig) molecule, CRP binds Clq and activates C1 via a distinct site on the amino-terminal collagen-like stalk region of the ClqA polypeptide instead of the carboxyl-terminal globular-like region (94). Using synthetic peptides derived from ClqA, two cationic regions of residues 14-26 (which contain four arginines and one lysine) and 76-92 were identified as the binding site for CRP (95). Indeed the peptide 14-26 was able to inhibit the complement pathway activated by the CRP complex (95).

2. **Opsonic Activity of CRP**

CRP plays an important role in host defense against bacterial infection and this protection is mainly through the clearance of bacteria. The first evidence that CRP is an opsonin was the facilitated ingestion of certain bacterial species by neutrophil after incubation of bacteria with CRP-containing serum (96). It was later independently confirmed by Ganrot (97) and Kindmark (98) that CRP was opsonic for a variety of bacterial species, including both the Gram (+) and Gram (-) bacteria.

In additional studies using a chemiluminescence assay, Edwards, et al. (99) and Mold, et al. (100) showed that the
ingestion of CRP-coated *Streptococcus pneumoniae* by human neutrophils was dependent on the complement activation by CRP and the presence of PC on the bacterial capsule. Volanakis's lab reported that phagocytosis of CRP-opsonized sheep erythrocytes by human neutrophil is inducible by PMA treatment of the PMNs, but independent of the FcyRIII on the neutrophil (101). Mortensen's lab demonstrated that the phagocytosis of CRP-coated sheep erythrocytes by monocytes was dependent on the presence of both CRP and the complement cleavage products, C3b and C4b, on the surface of erythrocytes (90); the phagocytosis appeared to be FcγR-dependent since aggregated IgG inhibited this process (102).

Animal studies showed that human CRP as well as rabbit CRP could passively protect mice against fatal infection by *S. pneumoniae* (103,104) by mediating the clearance of pneumococci from blood in mice (105). Using human CRP transgenic mice, Volanakis's group showed that CRP could protect mice against *S. pneumoniae* infection, extending the median survival time and increasing the survival by two-fold for the transgenic mice compared to control mice. This protection is mainly through the clearance of bacteria since there were four-hundred-fold fewer pneumococci in the spleens of transgenic mice than in the control mice (106,107).
3. Anti-tumor Activities of CRP and CRP Peptide

Several reports have described the biological activities of CRP and its peptides in leukocyte activation (108-116). Barna, et al. reported in 1984 that liposome-associated CRP could initiate macrophage tumoricidal activity against mouse tumor cells in vitro (117). Zahedi and Mortensen in 1986 confirmed that human CRP enhanced mouse macrophage tumoricidal activity (118). A synthetic peptide corresponding to human CRP residues 174 through 185 increased macrophage tumoricidal activity and cytokine secretion from both normal human monocytes and alveolar macrophage in vitro (119,120). Another study showed that administration of this peptide to mice decreased the lung metastases of a transplantable mouse fibrosarcoma and this response was associated with increased macrophage tumoricidal activity (119). This anti-tumor activity can be enhanced by co-administration of IL-2 (121).

4. Protective Effect of CRP in Inflammation Models and Septic Shock

Experimental pulmonary alveolitis in animal models showed that neutrophil infiltration and protein leakage into alveolar airspace induced by the chemotactic agent C5a des arg were significantly reduced by increased levels of CRP,
accomplished by either induction of endogenous CRP synthesis (123), or by intravenous infusion of purified rabbit CRP (122). Similar results were achieved by the same group using other chemoattractant reagents (fMLPP, LTB and IL-8) in rabbit CRP transgenic mice where expression of rabbit CRP could be induced to elevated levels (139). In a mouse study, infusion of two CRP derived peptides 77-82 or 201-206 could significantly reduce neutrophil infiltration induced by fMLP des arg into the bronchoalveolar lavage fluid by up to 90%, as well as inhibit protein leak by 55%; both effects reached comparable levels that were observed with native CRP (167). Samols's group demonstrated that high levels of induced rabbit CRP could protect transgenic mice from LPS-induced, PAF-induced, and TNF-α- and IL-1β-induced septic shock (124).

5. CRP and Autoimmune Disease (Systemic lupus erythematosus or SLE)

It was found that CRP selectively deposited at an inflamed site, closely associates with the damaged cell membrane, but not intact, healthy cells of normal animal tissues (7). This can probably be explained simply since the damaged cell membrane would have its phospholipids exposed to CRP. It has long been thought that the binding
of CRP to damaged cell components, such as chromatin, histones, Sm-D antigen and snRNPs were associated with their facilitated removal from the host system, thus altering the course of autoimmune diseases and protecting host. In animal studies of SLE, the administration of human CRP could increase survival time in mice injected with chromatin; in addition, a transient decrease of autoantibodies to histones and DNA was observed (125).

6. Effect of CRP on Cytokine Synthesis

It was first noted that digested products of CRP generated by neutrophil surface proteinase could stimulate IL-1 production by human monocytes (126). Studies in vitro showed that human CRP (1-10 μg/ml) could stimulate synthesis of IL-1α, IL-1β, TNF-α by human macrophages (127). The synthetic CRP peptide 174-185, a peptide initiating tumoricidal activity by both human and mouse monocyte/macrophage, could also stimulate production of cytokines by human monocyte/macrophage (119). This peptide was also able to induce the production of monocyte chemoattractant protein 1 (MCP-1) (128). This peptide by itself was not chemoattractant to macrophages, while CRP did modulate monocyte chemotaxis response (129). Pue, et al. reported that although human CRP enhanced IL-1β production
by human peripheral blood monocytes, the production of IL-1β and IL-1ra by human alveolar macrophages was inhibited (130). Very recently, Samol's group found that high levels of circulating transgenic CRP in mice suppressed both the resting and LPS-induced mRNA levels of the inflammatory cytokines TNF-α, IL-1β and IL-6 (164). These findings suggest that CRP has anti-inflammatory effects.

Recently, it was also found that CRP increased the ability of human monocytes to produce tissue factor (TF), which contributes to disseminated intravascular coagulation (DIC) (131), and this effect could be inhibited by IL-10 stimulation (132). By contrast, CRP digestive products (peptides < 14 kDa) have been demonstrated as inhibitors of platelet aggregation due to the decrease secretion of ADP and serotonin by platelets, but the intact CRP itself did not inhibit platelet aggregation (135).

7. CRP and Other Disease

Rat CRP enhances the uptake and degradation of low density lipoproteins (LDL) by macrophage scavenger receptors (136). CRP may play some role in atherosclerosis disease, but the physiological and pathophysiological meaning remains uncertain.
G. CRP-Leukocyte Interaction

Over the decades, there have been several reports that CRP and CRP peptides bind and activate monocytes/macrophages and neutrophils, promoting phagocytosis, modulating neutrophil responsiveness, monocyte activity (137), and NK cell function (138). Human CRP was shown to bind to human neutrophils with high affinity (116) and then degraded into peptides by neutrophil surface serine proteases (110-112). Similarly, rat CRP binds specifically to rat peritoneal macrophages, and then is internalized and degraded into small peptides (140). CRP-binding cells have also been found to be subsets of the T cell, B cell and NK cell categories.

CRP has been reported to be synthesized and expressed on the cell surface by NK cells (34,35,39) and by rat liver macrophages (36,141) associated with a specific 60 KDa binding protein on the cell membrane. Later, two forms of this 60 kDa membrane protein were identified as two carboxyl-esterases, which regulated the secretion of CRP (9). An anti-CRP antibody inhibited the Antigen-Dependent Cell Cytotoxicity (ADCC) activity of NK cells (142).

1. CRP receptor

CRP promotes the phagocytosis of microorganisms as an opsonin, by binding to receptors on phagocytes, including
monocyte/MΦ (102) and neutrophil (114). There are many reports on the binding of CRP peptides and CRP complexes or CRP aggregates (114) to the phagocytes, modifying their responsiveness. An earlier study showed that phagocytosis of CPS-coated sheep erythrocytes by mouse MΦ was Fc-R mediated and thus it had been thought that CRP bound directly to FcRs, or to very similar receptors, on phagocytes (102). Binding studies using fluorescein-labeled heat modified CRP also showed that 36% of neutrophils, 70% of the monocytes and 8% of lymphocytes could bind to CRP, but CRP only binds to a small portion (12%) of FcγR bearing cells (109). Kilpatrick, et al (144) demonstrated that neither the mAb 3G8 (anti-FcγRIII), nor a human myeloma IgGl protein could inhibit binding of CRP to neutrophils. Zeller, et al. reported that aggregated CRP enhanced the respiratory burst of human phagocytes (both neutrophils and macrophages) initiated by aggregated IgG (109,137). However this priming effect could be reduced by the monoclonal antibody IV.3, specific for the IgG-binding site on FcγRII, but did not block CRP binding to the cells (137). This indicated that the CRP-R is different from FcγRII. Neither mAbs to monocyte FcγRI, nor to the neutrophil FcγRIII reduced the enhancing effect of CRP on cell activation (137,145,146), although they reduced the activity induced by aggregated IgG. Mortensen's lab reported
that CRP did not inhibit the binding of $^{125}$I-IgG to U937 cells (147). Recent research result from the DuClos's lab showed that the binding of CRP to the FcγR only accounted for about 10% of the total specifically bound CRP on cell surface (148). All of the above reports suggest that the CRP-R is distinct from FcγRs.

Results of formal ligand-binding studies using $^{125}$I-CRP and human neutrophils fit into a two-site or two-population model (116,149), with a high affinity (Kd = 3.7 x $10^{-10}$ M) receptor population with about 1.4 x $10^4$ sites per cell and a low affinity (Kd = 4.2 x $10^{-8}$ M) receptor population (116). These results are consistent with Tebo's measurements of CRP-R binding to U937 cells (147).

Efforts to determine the primary structure of the CRP-R have been made. Mortensen's lab reported that human monocyte cell line U937 and the mouse macrophage cell line PU51.8 had CRP receptors by ligand-affinity chromatography and obtaining partially isolated purified membrane proteins of about 40 kDa and 57-60 kDa, and antibodies against them were generated (147,150). Qin reported that an 80 kDa cell membrane protein could be purified from the same cell lines using a CRP-coated agarose beads (151). This 80 kDa protein was rapidly degraded into more stable 40 kDa and 55 kDa proteins. All three of these membrane proteins could be recognized by the
antibodies generated against the CRP-R. The amino acid sequence from the 55 kDa protein is unique. Using degenerative oligonucleotide probes, the Mortensen group obtained an open reading frame (ORF) from a HL-60 cell cDNA library. This ORF encodes a polypeptide sequence of more than 200 a.a. on the amino-terminus that contains four hydrophobic regions and several potential transmembrane domains of 19 to 21 residues (unpublished data).

Using a cell binding study, Qin demonstrated that the binding of human $^{125}$I-CRP to the U937 cells was inhibitable by a 100-fold excess of either human CRP or a synthetic CRP peptide (152) corresponding to residues 27-38 of the CRP subunit, which was designated as "cell binding domain" (CB peptide), a domain mediating the binding of human CRP to cells (153,154).

H. Other Members of the Pentraxin Family

Serum amyloid P component (SAP) was first identified in 1965 as a pentagonal protein associated with amyloid fibrils and later found to be identical to a constitutive protein. SAP identical subunits have 204 amino acids and is exclusively N-glycosylated on Asn27 to approximately 11% of its total molecular weight of 25,500 (155). The crystal structure of SAP shows that SAP is composed of ten protomers
in the form of two symmetric, flat pentamaric rings bound face-to-face (42,156). SAP is not an acute phase reactant in most mammals and vertebrates, with only a two-fold increase in humans during acute inflammation. The exception is the mouse, where SAP is a major APR and increases significantly during acute inflammation to levels obtained by human CRP (158,159). Human SAP shares 50 % amino acid sequences identity with human CRP and has the same overall crystal structure (42,45,157). One recent study found that SAP also bind to PC in a Ca**-dependent manner, but with a much lower affinity when compared with that of CRP (160).

It was long thought that CRP and SAP are the only two members of the pentraxin family until the early 1990's when two groups independently identified and cloned new members from human endothelial cells and fibroblasts stimulated by TNF-α or IL-1 (161,162). This gene was named TSG-14 ( TNF-α stimulated gene number 14 ) and PTX3 (Pentraxin 3), respectively, but was shown to be identical (163). The TSG14/PTX3 gene encodes for 381 amino acids and is organized into 3 exons with the third one encoding the C-terminal pentraxin motif, which is up to 27% homologous to human CRP and SAP sequences. The N-terminal 181 residues do not show significant homology with the pentraxins, or any other proteins (163). Immunohistochemistry analysis indicate that
the majority of the PTX3 protein was produced extrahepatically (163). Both lipopolysaccharide (LPS) and IL-1β significantly enhance the transcription of the TSG14/PTX3 gene in human peripheral blood mononuclear cells as well as in human myelomonocytic cell lines HL-60, U937 and THP-1 (166). However, transcription of the PTX3 gene cannot be detected in neutrophils, T and B lymphocytes, and NK cells under the same conditions. The biological function of the PTX3 protein has yet to be determined.
A. INTRODUCTION

During bacterial infection or body injury, the host cells, especially monocytes/macrophages, will synthesize inflammatory cytokines to initiate a host defense response. The synergistic action of the inflammatory cytokines IL-1β, TNF-α, and IL-6 reorchestrates the pattern of gene expression in hepatocytes and greatly enhances the synthesis of a group of acute phase blood proteins, more broadly termed acute phase reactants (APR) (11, 168, 169). These inflammatory cytokines also rapidly elicit the production of the chemokine IL-8 by monocytes and endothelial cells (170, 171).

CRP is the prototype of the APR in humans and increases over 1000-fold during the first 24–48 hours of acute inflammation (11, 172); it is widely used as a gauge for the
presence and extent of systemic inflammation (168). Circulating CRP is a pentamer of noncovalently associated identical subunits, each of which displays Ca\(^{2+}\)-dependent binding to phosphocholine (PC) and other monophosphate esters (173). CRP selectively accumulates at the site of tissue damage (7, 174) and is capable of being digested by membrane proteinases on activated PMNs into peptides that influence the activities of both monocytes and neutrophils (111, 126, 175). In fact, data from transgenic mice expressing the human CRP gene showed decreased PMNS infiltration in the lung during experimental streptococcal pneumonia (106). Rabbit CRP transgenic mice also showed a decreased response to LPS induced septic shock (124), a severe systemic inflammatory situation. Infusion of two CRP derived peptides 77-82 or 201-206 significantly decreased neutrophil infiltration and protein leakage in mice with alveolitis induced by fMLP or C5a des arg (167). Most in vitro studies showed that CRP down-regulated PMNS activated in response to other activators.

Interleukin-8 (IL-8) is a chemokine of the CXC subfamily that specifically activates neutrophils. IL-8 levels at inflammatory sites and in blood dramatically increase and correlate with the extent of neutrophil infiltration and host tissue damage at inflammatory
sites (170, 171). In fact, neutralizing antibodies to IL-8 protect against PMNS-mediated tissue damage. Recruitment of granulocytes to inflamed sites is a crucial event for both host resistance and eventual tissue repair; it is dependent on both chemokines as well as a group of poorly defined extrinsic factors that control the cell's response to chemokines (170,171,183,185). IL-8 also augments the respiratory burst and triggers degranulation in neutrophils (176-178). IL-8 gene expression can be induced in many different cell types, mainly endothelial cells and tissue macrophages responding to pro-inflammatory stimuli such as IL-1β, TNF-α, and LPS (170,171). Two receptors for IL-8 exist on neutrophils: IL-8R-α (type I) with a high affinity (Kd = 1 nM) only for IL-8 and IL-8R-β (type II), which has a similar affinity for both IL-8 and several other CXC chemokines (179). The two IL-8 receptors share 77% amino acid sequence identity and belong to the serpentine superfamily of seven transmembrane, trimeric G-protein-coupled receptors (179-181). The most closely related leukocyte receptors, based on both amino acid sequence and receptor function, are those for C5a and the bacterial peptide N-formyl-Met-Leu-Phe (fMLP) (179,181, 182), both of which are chemoattractant for neutrophils. The molecular mechanism(s) regulating the activities of these receptors is
is also produced. Despite extensive studies of CRP activities in vitro, a unique role for CRP has yet to be determined. Since PMNs possess specific receptor for CRP, I examined the influence of CRP on the response of neutrophils to both IL-8 and fMLP, as well as the signaling pathway behind the cellular phenomena. The studies described here show that CRP inhibits the IL-8 and fMLPP-induced neutrophil chemotactic response, but augments phosphatidylinositol-3 kinase (PI-3K) signaling and the mitogen activated protein kinase (MAPK) pathway, suggesting that CRP influences PMNS responses via a distinct signaling pathway.
B. MATERIALS AND METHODS

1. Reagents. The fMLP, fMLPP, PMA, DMSO, DTT, Wortmannin, ferricytochrome C (from horse heart), phosphatidylinositol (PI) were obtained from Sigma (St.Louis, MO). The PMA and fMLPP were stored concentrated in DMSO at -20°C and diluted with buffer just prior to use. The p-aminophenyl phosphocholine (PC)-Sepharose was purchased from Pierce Chemical (Rockford, IL). Recombinant human IL-8 isoforms of both 72 and 77 amino acids were purchased from Sigma and Harlan Bioproducts (Madison, WI), respectively. 125I-labeled IL-8 was obtained from Amersham Life Sciences (Arlington Heights, IL). The γ-32P-ATP was purchased from DuPont NEN (Boston, MA). The ERK kinase inhibitor PD098059 was obtained from Calbiochem (La Jolla, CA). The chemiluminescent substrate kit was from KPL (Gaithersburg, MA). The X-ray films were purchased from Wolf Scientific (W. Hempstead, NY) and Kodak (BIOMAX-ML).

2. Cells. The promyelocytic cell line HL-60 was obtained from the ATCC (Rockville, MD) and grown in RPMI-1640 plus 4% defined FBS (HyClone, Logan, Utah), 6% defined bovine calf serum (HyClone), 1 X glutamine and 1 X NEAA (non-essential
amino acids) (Gibco BRL) with 20 mM HEPES (pH 7.4). HL-60 cells were differentiated into granulocytic (G) cells by incubating 2x10^5 cells/ml with 1.2% DMSO for 6 or 7 days, after which 85-95% of the cells rapidly reduced NBT dye and stained positively for cytoplasmic esterase (193). The HL-60(G) cells were washed twice into Earle's balanced salt solution (EBSS) containing 10 mM HEPES (pH 7.4) and adjusted to 10^7 cells/ml and kept on ice for functional or binding assays.

3. Synthetic peptides. Synthetic peptides numbered according to the derived sequence for the 206 amino acids present in each of the human CRP subunits (49) were synthesized by the Ohio State University Biochemistry Instrument Center using t-boc synthesis on a Model 9500 Peptide Synthesizer (Milligen/Biosearch, Millipore, Burlinton MA). CRP peptide 174-185 is a gift from Dr. Barbara Barna (Cleveland Clinic). Peptide sequences are listed below:


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4. Purification of CRP. CRP was purified as described elsewhere (147,154). Briefly, serum amyloid P-component was removed from CRP-containing pleural or ascitic fluids by passage through a column of agarose beads. The eluted protein was then passed through a 10 ml (20 mm diameter) column of \( p \)-aminophenyl-phosphocholine (PC)-sepharose, washed extensively with TBS + 2mM \( \text{Ca}^{2+} \) and the bound protein eluted in TBS + 10 mM EDTA. A second round of affinity purification on the PC-bearing matrix was used to remove trace amounts of other proteins. The concentration of CRP was determined by ELISA or by RID with sheep anti-human CRP. The protein was > 99% CRP based on reactivity in the competitive ELISA and by SDS-PAGE. The concentration of endotoxin in the purified CRP was 0.1-0.2 endotoxin units/mg protein (Chromogenic Limulus assay, M. A. Bioprod., Walkersville, MD), corresponding to an LPS concentration of <0.05 ng/mg of CRP.

5. 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 2-3% contaminating lymphocytes as judged by morphology. The neutrophils were washed twice and brought up to 5x10^6/ml in cold Gey's balanced salt solution (GBSS)
containing 0.1% BSA and 20 mM HEPES buffer at pH 7.2. Chemotaxis was assessed using modified-Boyden chambers with PVP-free polycarbonate Nucleopore® membranes with 5 μm pores. The chemotactic substance was added to the lower chamber and 2.5x10^5 PMNs added to the upper chamber. The chambers were incubated at 37°C for 90 min., the filters removed, fixed, and stained with Wright's Giemsa stain. A single-blind assessment of chemotaxis was conducted by coding the slides before the number of cells/oil-immersion field (450X) were counted. The data from at least 20 fields/filter were obtained from two independent examinations of the slides and the mean value of cells/high power field (hpf) calculated. The mAb HD2-4 was used to neutralize CRP and is described elsewhere (194).

6. IL-8 Binding Assay. ^125I-IL-8 was added to HL-60(G) cells (10^6/0.1 ml) at 1 nM in D-PBS with 1% BSA. The competing proteins, CRP and unlabeled IL-8 were added at the same time as ^125I-IL-8 and the binding allowed to continue for 60 min. on ice. The cells were centrifuged at 14,000 x g for 1 min. at 4°C through a phthalate oil mixture (0.5 ml) and the cell pellet counted immediately (151) in a Beckman Gamma 4000 counter. Results were expressed as percentage of control binding (5 - 20,000 cpm/10^6 cells) and the experiment repeated three times with triplicate samples.
7. MAPK activity. HL-60(G) cells at 5 x 10^6 per sample were lysed by TN-1 lysis buffer (1% NP40; 20 mM Tris, pH8.0; 150 mM NaCl; 10 mM each of EDTA, NaF, Na_3VO_4, and NaP~P; 10 μg/ml aprotinin and 10 μg/ml leupeptin) after stimulation with IL-8 or fMLPP. ERK2 was immunoprecipitated at 4 °C for 3 h by 20 μl protein A/G-sepharose beads precoated with 1 μg of IgG rabbit anti-ERK-2 (Santa Cruz Biochemicals, CA). The beads were washed 4x in TN-1 and then twice in the kinase assay buffer (MgCl_2, 20 mM; sodium ortho-Vanadate 0.1 mM; β-Glycerophosphate 20 mM; HEPES 30 mM, pH7.6). Myelin basic protein (MBP) at 6 μg was used as the substrate and 5 μCi γ-32P-ATP added per sample. The γ-32P phosphorylated MBP was detected by autoradiography after separation by SDS-PAGE and transfer to a nitrocellulose membrane. MAPK protein was detected by immunoblotting the same membrane.

8. JNK activity assay. Cell lysates were prepared the same way as above and immunoprecipitated with 30 μl protein A-sepharose beads (Zymed, CA) coated with 1 μg rabbit anti-JNK1 IgG (Santa Cruz Biochemicals) for 3 hours at 4 °C. Beads were washed 4 times in TN-1 buffer and twice in the kinase assay buffer. GST-c-JUN fusion protein 3 μg, cold ATP at 2 mM and 5 μCi γ-32P-ATP in kinase buffer were used as substrates for JNK1. Reaction was run for 30 min at 30 °C before being stopped by boiling in 5X SDS sample buffer. the γ-32P
phosphorylated GST-c-JUN was detected by autoradiography after SDS-PAGE and transferring to nitrocellulose membrane. JNK1 was probed with the same anti-JNK1 antibody (1:1000).

9. PI-3 Kinase activity. Either IL-8 or fMLPP-stimulated HL-60(G) cells (5x10^6 in 0.1 ml of RPMI-1640) were lysed with TN-1 lysis buffer 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 anti-serum to the p85 subunit of PI-3K. The antibody was a gift from Dr. K.M. Coggeshall and raised by injecting a purified GST-fusion protein containing the N-terminal SH2 domain of p85 (obtained from B. Schaffhausen, Department of Biochemistry, Tufts University School of Medicine, Boston, MA). The Ab did not cross-react with other SH2 domain-containing proteins (data not shown). The beads were washed three times in TN-1 lysis buffer. Half of the beads were boiled in 2X DTT-SDS sample buffer for 5 min before running the supernatant on SDS-PAGE. Proteins were transferred to a PVDF membrane (MSI, Westborough, MA) and blotted with the same rabbit Ab at 1:1000. Horseredish peroxidase (HRP) labeled goat-anti-rabbit-IgG (KPL, Gaithersburg, MD) was used as a secondary Ab (1:2000) and a chemiluminescence solution (KPL) used as the substrate. The remaining half of the beads were used for a
PI-3K activity assay. The beads were suspended in an assay buffer (30 mM HEPES, pH7.4; 30 mM MgCl$_2$; 1mM EDTA and 50 μM ATP), using 100 μg of exogenous phosphatidylinositol (PI) and γ-$^{32}$P-ATP (2 μCi) as substrates. The reaction was run for 15 minutes at room temperature and stopped with 0.1 M HCl. Lipids were extracted with a mixture of methanol : chloroform at 1:1 ratio. Detection of γ-$^{32}$P labeled PIP was done by running thin layer chromatography (TLC) on Silica G-60 plastic backed plate in solvent followed by autoradiography. Silica G-60 plate was prepared by immersing in CDTA buffer for 1 min, air dried, and then activated by baking in 80-100 °C for 10 min. CDTA buffer: CDTA.H$_2$O 4.55g; H$_2$O 165 ml; ethanol(95%) 330 ml; 10 M NaOH 3 ml. Solvent mixture: CH$_3$OH 75ml; CHCl$_3$ 60 ml; pyridine 45 ml; boric acid 12 g; H$_2$O 7.5 ml; acetic(99%) 3.0 ml; BHT 0.375 g and ethoxyquin 75 μl.

10. IL-8 receptor serine phosphorylation. HL-60 (G) cells were incubated with CRP (300 μg/ml) at 37 °C for 30 min. before being stimulated by either IL-8 or PMA at various concentrations. Cells were lysed in TN-1 lysis buffer and the nuclear pellets discarded. The IL-8 receptor A was immunoprecipitated using a monoclonal mouse-anti-IL-8RA antibody (9H1, a gift from Phamgen, south San Francisco, CA) (185) and protein G-sepharose beads overnight at 4 °C. The
immunoprecipitates were used for SDS-PAGE and immunoblotting on a nitrocellulose membrane (S & S, NH). A biotin-conjugated monoclonal mouse anti-phosphoserine IgG (PSR-45, Sigma) was used as primary antibody (1:1,000) and a streptavidin-conjugated horseradish peroxidase (StrepA-HRP)(KPL) was used as secondary reagent (1:5,000). Membrane was soaked in chemiluminescent reagent and exposed to X-ray film.

11. Study of protein tyrosine phosphorylation level. HL-60 (G) cells were stimulated by fMLPP with CRP preincubation and lysed in TN-1 buffer. The SHIP were immunoprecipitated from the whole cell lysate by rabbit anti-SHIP serum (a gift from Dr. K.M. Coggeshall). The tyrosine phosphorylation of SHIP was detected by an immunoblotting assay using an anti-phosphotyrosine antibody cocktail (PY72 + 4G10, 1:5000). HRP-anti-mo-IgG (1:5000) and chemiluminescent reagents were used to detect the protein bands.

The detection of tyrosine phosphorylation pattern of whole cell lysate proteins was similar, except that HL-60 (G) cells 5 x 10^6 were lysed in a 2X lysis buffer (0.1M Tris, pH8.5; 0.1M NaCl; 10mM NaF, Na,VO_, EDTA and Na,P-P; 2% NP40; 10µg/ml aprotinin and leupeptin), and the supernatant was used.
C. RESULTS

1. 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 was examined. Exposure of isolated neutrophils to CRP under ligand binding conditions (on ice) and subsequent examination of their chemotactic responsiveness to recombinant human IL-8 (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 monoclonal anti-human-CRP antibody, HD2-4, to CRP before incubation with cells neutralized CRP's 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
Figure 1. Effect of human CRP on neutrophil chemotaxis in response to IL-8 (A) and fMLPP (B). Isolated human peripheral blood PMNs were allowed to bind purified human CRP for 30 min on ice prior to examining their chemotactic response to either $10^{-9}$ M rhIL-8 or to $10^{-8}$ M of the bacterial oligopeptide fMLPP. Anti-CRP mAb HD2-4 at 10 µg/ml was added to 20 µg/ml of CRP (Δ). Data from one representative experiment of 5.
response to the more potent chemoattractant.

HL-60 (G) cells did not have chemotactic response neither to fMLPP not to IL-8 stimulation (data not shown).

2. Effect of CRP-Fragments on Chemotaxis. Since human CRP is digested by activated PMNs into biologically active peptides (110,126), 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 pmole/ml (20 µg/ml of pentamer) inhibited chemotaxis in a manner similar to intact CRP (Table I). Approximately 60-80% of the CRP was cleaved into polypeptide fragments of < 15 KDa by the PMA-activated PMNs as judged by SDS-PAGE under reduced conditions (110). The recovered CRP polypeptide fragments by themselves exerted only very weak chemotactic activity (Table II). Therefore, the net effect of the digested CRP on chemotaxis is one of inhibition.

3. 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 (153,154), was tested along with additional synthetic CRP peptides for chemotactic activity at 800 pmole/ml, the equivalent of 20 µg/ml of the intact CRP pentamer. Of the peptides listed in
Table I. Chemotactic activity of human CRP digest product.

<table>
<thead>
<tr>
<th>Chemotactic Factor</th>
<th>Concentration</th>
<th>% of Control chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP 5 pg/ml</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>CRP 20 pg/ml</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>CRP 50 pg/ml</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>IL-8 1 nM</td>
<td>100 (Contr.)</td>
<td></td>
</tr>
<tr>
<td>GBSS</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

Purified CRP at 10, 40 and 100 µg/ml were digested by fresh human PMNs (5x10⁴/ml) at 37 °C for 2 hours and digest products were put into the lower chamber to test for chemotactic activity.
Table II. Chemotactic activity of human CRP digest product.

<table>
<thead>
<tr>
<th>Chemotactic factor</th>
<th>Concentration</th>
<th>% of Control chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>5 µg/ml</td>
<td>85</td>
</tr>
<tr>
<td>CRP</td>
<td>20 µg/ml</td>
<td>144</td>
</tr>
<tr>
<td>CRP</td>
<td>50 µg/ml</td>
<td>114</td>
</tr>
<tr>
<td>IL-8</td>
<td>1 nM</td>
<td>100 (Contr.)</td>
</tr>
<tr>
<td>GBSS</td>
<td></td>
<td>74</td>
</tr>
</tbody>
</table>

Purified CRP at 10, 40 and 100 µg/ml were digested by fresh human PMNs (5 x 10⁶/ml) at 37 °C for 2 hours and digest products were put into the lower chamber to test for chemotactic activity.
Table III, only the CB-Pep displayed weak chemotactic activity when compared to fMLPP. When the concentration of the peptides was increased to 2 nmole/ml (equivalent to 50 µg/ml pentameric CRP), similar results were obtained (Table IV). Several of the CRP-synthetic peptides were also tested for their effects on neutrophil chemotaxis mediated by IL-8 at 1 nM. Of the peptides tested, only two of them at 800 pmole/ml concentration inhibited the chemotactic response: the tumoricidal peptide of residues 174-185 (119), and the C-terminal peptide of amino acids (191-205) (Table V). Additional experiments at peptide concentrations up to 5-fold higher did not alter the results (data not shown). When fMLPP (10 nM) was used as the chemoattractant, synthetic peptides corresponding to residues 174-185 and the C-terminal residues 191-205 of CRP, significantly inhibited the induced PMN chemotactic response (Table V). 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.

4. Effect of antibodies to the CRP-R on Chemotaxis. A series of mouse mAb was generated against isolated membrane proteins from U937 monocytic cells which were obtained by affinity chromatography on CRP-sepharose. These mAbs were screened for
Table III. Chemotactic activity of human CRP synthetic peptides at low concentration.

<table>
<thead>
<tr>
<th>Chemotactic factor</th>
<th>Concentration nmole/ml</th>
<th>% of Control chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep 1-15</td>
<td>0.8</td>
<td>21 ± 11.3</td>
</tr>
<tr>
<td>Pep 27-38</td>
<td>0.8</td>
<td>35 ± 17.4</td>
</tr>
<tr>
<td>Pep 47-63</td>
<td>0.8</td>
<td>5.5 ± 3.1</td>
</tr>
<tr>
<td>Pep 134-148</td>
<td>0.8</td>
<td>3.7 ± 2.7</td>
</tr>
<tr>
<td>Pep 152-176</td>
<td>0.8</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Pep 174-185</td>
<td>2</td>
<td>5.5 ± 3.1</td>
</tr>
<tr>
<td>Pep 191-205</td>
<td>2</td>
<td>14 ± 2.7</td>
</tr>
<tr>
<td>fMLPP</td>
<td>10 nM</td>
<td>100 (Contr.)</td>
</tr>
<tr>
<td>GBSS</td>
<td>--</td>
<td>20 ± 5.7</td>
</tr>
<tr>
<td>CRP</td>
<td>50 µg/ml</td>
<td>15 ± 3.1</td>
</tr>
</tbody>
</table>

Peptide solutions at 0.8 nmole/ml (equivalent to 20 µg/ml of pentameric CRP) in GBSS buffer are put into the lower chamber instead of fMLPP. GBSS buffer is the negative control, whereas fMLPP is the positive control. Data are obtained from two experiments.
Table IV. Chemotactic activity of human CRP synthetic peptides.

<table>
<thead>
<tr>
<th>Chemotactic factor</th>
<th>Concentration nmole/ml</th>
<th>% of Control chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep 1-15</td>
<td>2</td>
<td>13 ± 3.2</td>
</tr>
<tr>
<td>Pep 27-38</td>
<td>2</td>
<td>56 ± 8.5**</td>
</tr>
<tr>
<td>Pep 174-185</td>
<td>2</td>
<td>9 ± 2.1</td>
</tr>
<tr>
<td>Pep 191-205</td>
<td>2</td>
<td>14 ± 2.7</td>
</tr>
<tr>
<td>fMLPP</td>
<td>10 nM</td>
<td>100 (Contr.)</td>
</tr>
<tr>
<td>GBSS</td>
<td>--</td>
<td>16 ± 4.1</td>
</tr>
<tr>
<td>CRP</td>
<td>50 µg/ml</td>
<td>15 ± 3.1</td>
</tr>
</tbody>
</table>

Peptide solutions at 2 nmole/ml (equivalent to 50 µg/ml of pentameric CRP) in GBSS buffer are put into the lower chamber instead of fMLPP. GBSS buffer is the negative control, whereas fMLPP is the positive control. Data are obtained from two experiments.
Table V
Effect of Human CRP Peptides on Neutrophil Chemotaxis in Response to IL-8 and fMLPP

<table>
<thead>
<tr>
<th>CRP Peptide</th>
<th>% of Control CTX vs. Chemotaxin[^2]</th>
<th>IL-8 (1 nM)</th>
<th>fMLPP (10 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep 1-15</td>
<td>N.D.</td>
<td>79 ± 12.1</td>
<td></td>
</tr>
<tr>
<td>Pep 27-38</td>
<td>83.5 ± 19.1</td>
<td>106 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>Pep 47-63</td>
<td>N.D.</td>
<td>126 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Pep 134-148</td>
<td>N.D.</td>
<td>115 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Pep 152-176</td>
<td>N.D.</td>
<td>116 ± 11.2</td>
<td></td>
</tr>
<tr>
<td>Pep 174-185</td>
<td>60.5 ± 14.8*</td>
<td>53 ± 5.7*</td>
<td></td>
</tr>
<tr>
<td>Pep 191-205</td>
<td>56.5 ± 6.4*</td>
<td>33.8 ± 2.8*</td>
<td></td>
</tr>
</tbody>
</table>

[^1]: Peptides synthesized on the basis of the consensus sequence.
[^2]: PMNs exposed to the peptides (800 pmoles/ml) for 30 min before testing their chemotactic response to 10^-9 M rhIL-8 or 10^-8 M fMLPP. Mean values (±SEM) for n=4. * p ≤ 0.05.
specificity and their ability to inhibit specific ligand (CRP) binding as described elsewhere (147). 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 PMNs (Fig. 2). This concentration of the mAb is sufficient to occupy > 70% of the CRP-R calculated at 10⁴ receptor sites/PMN (147). Two rabbit polyclonal antibodies against the 40 KDa and 55 KDa CRP receptor proteins also inhibited neutrophil chemotaxis induced by either fMLPP or IL-8 (table VI). Thus, both the mAb and the polyclonal antibodies to the putative CRP-R mimicked the action of CRP itself on neutrophil chemotaxis.

5. 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 of pentamer) failed to significantly inhibit binding of 1 nM ¹²⁵I-rhIL-8 to HL-60(G) cells; however at 200 µg/ml, CRP inhibited only ~25% of the IL-8 binding at a 1,600-fold molar excess (Fig.3). In the same experiments, rhIL-8 itself inhibited ¹²⁵I-rhIL-8 binding by approximately 70% at a 100-fold molar excess of the
Figure 2. Effect of an anti-CRP-R mAb on neutrophil chemotaxis. Neutrophils (2.5 x 10⁶/0.1 ml) were allowed to bind purified IgM from the anti-CRP-R mAb RC10 (O) or the mouse IgM myeloma MOPC 104e (●) for 60 min. prior to exposure to 10 nM fMLPP. Data are mean values (±SEM) from two experiments.
Figure 2
Figure 3. Effect of human CRP on binding of $^{125}$I-IL-8 to HL-60(G) cells. Concentrations of rhIL-8 (O) at 1 to 10 nM and purified CRP (●) at 8 to 1600 nM (1 to 200 μg/ml) were allowed to compete for binding sites at 4 °C. The cpm for 100% of control binding was ~24 x 10^3 cpm/10^6 cells. Mean values from 4 separate cell-binding assays.
Figure 3
unlabeled ligand (Fig. 3). Thus, it seems unlikely that CRP inhibits IL-8 mediated chemotaxis by inhibiting ligand-receptor binding.

6. Effect of CRP on IL-8 receptor desensitization. The IL-8 receptors can be desensitized by phosphorylation on one or more Ser residues within their cytoplasmic C-terminus and lose activities (178,185). Because CRP inhibited IL-8-induced neutrophil chemotaxis, it might be possible that CRP desensitized IL-8 receptors. Thus the IL-8Rα isoform within neutrophil was immunoprecipitated and probed with PSR45, a mouse monoclonal anti-phosphoserine antibody (Sigma). The outcome of such assays was that human CRP at 200 µg/ml failed to alter the extent of Ser-phosphorylation of IL-8Rα in the presence or absence of IL-8 (Fig 4).

7. Effect of CRP on MAPK. In preliminary experiments, when the pattern of all 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 (Fig 5). Since MAPK is ~38-44 Kda and its activity was reported to be inducible by IL-8 over a 30 min interval reaching maximum levels at 3 min (186), CRP was tested for its influence on
Figure 4. Effect of CRP on the phosphorylation level of IL-8 receptor A. HL-60 (G) cells 10 x 10^6 were stimulated with IL-8 and lysed. The IL-8RA was immunoprecipitated with the 9H1 antibody. The membrane was first blotted with the anti-phosphoserine antibody and the image detected by ECL. The same membrane was then stripped, blocked and blotted with the 9H1 antibody. ECL was used. Cells in lane 7 was incubated with CRP first for 30 min.
71 KD

IB: anti-phosphoserine

71

IB: anti-IL-8RA

CRP, 300 ug/ml
IL-8, 50 nM, min

0 1 2 3 5 10 5

figure 4
Figure 5. Effect of CRP on protein tyrosine phosphorylation in HL-60 (G) cells stimulated by IL-8. The whole lysates were run on SDS-PAGE, transferred to nitrocellulose membrane, and then blotted with anti-phosphotyrosine antibody. Two protein bands around 33 KDa were increased by CRP.
**Immunoblotting: anti-phosphotyrosine**

( * is positive control, Na$_3$VO$_4$ stimulated )
MAPK activity measured as ERK-2. ERK-2 was increased by 2-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. 6A). A plot of the cpm of ³²P-labeled MBP present in each lane is shown for comparison (Fig. 6B). ERK-2 activity induced by fMLPP was also enhanced by preincubation with CRP (Fig. 7A). The same blotted membrane was probed with anti-ERK-2 antibody to demonstrate that approximately the same amount of ERK-2 protein was present in each of the lanes (Fig. 7B). 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 MAPKK) present in neutrophils (187,188), I 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 VI). Since PI-3K is upstream of MAPKK (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 VI). Therefore, PI-3K appeared to be a more logical target for the signaling triggered by CRP.
Figure 6. Effect of CRP on MAPK activity induced by IL-8.
(A) HL-60 (G) cells (5 x 10^6) were incubated with (lanes 3, 5, 7) or without (lanes 2, 4, 6) CRP at 200 µg/ml before stimulating with 50 nM IL-8 for 1 min (lanes 2, 3), 3 min (lanes 4, 5), and 10 min (lanes 6, 7). (B) Plot of γ-^32P~ counts from the membrane in A. Cell lysates were immunoprecipitated with rabbit anti-ERK-2, separated by SDS-PAGE, and the MBP was added as the substrate for in situ phosphorylation. The labeled MBP was detected by both autoradiography and by direct gel counting. Results are representative of 4 experiments.
Figure 6
Figure 7. Effect of CRP on MAPK activity induced by fMLPP. HL-60(G) cells were incubated either with CRP at 50 μg/ml (lanes 3, 5, 7) or without (lanes 2, 4, 6) before stimulating with 50 nM fMLPP for 1, 3, or 5 min. Cell lysates were immunoprecipitated with IgG anti-ERK-2, separated by SDS-PAGE, and MBP added for in situ phosphorylation (A). The immunoblot of the same membrane probed with anti-ERK-2 is shown in (B).
Figure 7
Table VI

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>Chemotactic Response to 10 nM fMLPP</th>
<th>Conc.</th>
<th>mean cells/hpf</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD98059</td>
<td></td>
<td>0 μM</td>
<td>52.6 ± 5.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 μM</td>
<td>86.4 ± 3.1</td>
<td>-64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 μM</td>
<td>71.0 ± 2.5</td>
<td>-35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 μM</td>
<td>66.0 ±10.0</td>
<td>-25</td>
</tr>
<tr>
<td>Wortmannin</td>
<td></td>
<td>0 nM</td>
<td>29.5 ± 3.3</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 nM</td>
<td>25.0 ± 2.8</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM</td>
<td>7.3 ± 4.0</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 nM</td>
<td>4.0 ± 1.6</td>
<td>86</td>
</tr>
</tbody>
</table>

1 The ED$_{50}$ for PD98059 is 5 μM and 10 nM for Wortmannin.

2 Mean number of PMNs/hpf (±SEM) for n=4.
8. Effect of CRP on JNK activities. Both the c-Jun N-terminal Kinase (JNK), or stress activated protein kinase (SAPK) and p38 are stress activatable and members of the MAPK family. In preliminary experiments, I found that IL-8 activated JNK, but not p38. JNK could also be activated by fMLPP. Therefore, I examined effect of CRP on JNK activity in activated HL-60 (G) cells. I found that CRP at 200 µg/ml (5 min) enhanced JNK activity induced by fMLPP stimulation but not by IL-8, as indicated by the increased phosphorylation of the JNK substrate GST-c-Jun fusion protein (Fig. 8).

9. Effect of CRP on PI-3K. PI-3K plays a pivotal role for both IL-8 signaling (234), as well as signaling by fMLP (235) 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 (234, 236). Preliminary experiments with fMLPP at 0.1 to 100 nM revealed that the maximum PI-3K activity was induced at 25 nM. Optimal PI-3K activity could be reached by 100 nM IL-8 stimulation (Fig. 9). Therefore, the effect of CRP on fMLPP-induced PI-3K activity was initially evaluated and PI-3K activity was found to be increased at CRP concentrations > 5 µg/ml (Fig 10). Therefore, the effect of treating the PMNs to different
concentrations of CRP alone was tested. PI-3K activity was significantly increased at CRP levels ≥5 μg/ml (Fig. 11A). When an immunoblot of the precipitated PI-3K was probed with an anti-p85 Ab specific for the regulatory subunit of PI-3K, the relative amount of p85 subunit per lane was the same (Fig. 11B). 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. 12A). The optimal PI-3K response takes 5 min with fMLPP. Thus, the kinetics of PI-3K activation by CRP are rapid. The immunoblot of the samples in Figure 11A & 12A indicated that similar amounts of pl10 catalytic activity of PI-3K were examined based on the presence of the p85 subunit (Fig. 11B & 12B). Thus, CRP may attenuate neutrophil responses via PI-3K-dependent reaction(s) in a time and dose-dependent fashion.

10. Study of tyrosine phosphorylation of SHIP. Since CRP itself increased PI-3K activities in both a time-dependent and a dose-dependent manner, and the PI-3K products 3-phosphotidylinositols are well documented signaling mediators (189,190), it is possible that these lipid products may also act as signal mediators in HL-60 (G) cells stimulated by CRP. These phospholipids are deactivated (or metabolized) quickly in cells, namely by lipid phosphatases (191). Plus, it has
Fig. 8. Effect of CRP on JNK activity induced by IL-8 or fMLPP. HL-60 (G) cells 5 x 10⁶ were incubated with (lanes 3, 5, 7, 9) or without (lanes 2, 4, 6, 8) human CRP before stimulated with IL-8 (lanes 2, 3 for 15 min; lanes 4, 5 for 30 min; and lanes 6, 7 for 60 min) or fMLPP 30 min (lanes 8, 9). JNK activities were detected by γ-³²P phosphorylation of a GST-c-Jun fusion protein.
Figure 8
Figure 9. Dose-response of PI-3K activity induced by IL-8. HL-60 (G) cells $5 \times 10^6$ were stimulated with IL-8 at 0 to 150 nM for 3 min. PI-3K was immunoprecipitated and in vitro kinase assay was carried out. Lane 7 is same as lane 5 except that 100 nM Wortmannin was added before adding substrate.
Figure 9

Immunoprecipitation: Rb-anti-p85 serum
Figure 10. Effect of human CRP on PI-3K activity induced by fMLPP. A, HL-60 (G) cells $5 \times 10^6$ were stimulated with fMLPP after incubation with CRP at 0 to 100 µg/ml (lanes 2 to 6). Lane 8 is normal serum precipitation. B, Immunoblotting result shows each sample had equal amount of PI-3K p85 regulatory subunit, indicating equal activity.
Figure 10
Figure 11. Effect of human CRP on PI-3K activity.

A, HL-60 (G) cells were allowed to bind different concentrations of CRP (lanes 3 through 7) for 30 min, or 25 nM fMLP for 3 min (lanes 2, 8, 9). The PI-3K was immunoprecipitated from cell lysates with anti-p85 before assaying PI-3K activity. The cell lysate in lane 8 was treated with 100 nM Wortmannin. B, The precipitated samples were probed with the anti-p85 Ab after SDS-PAGE.
Figure 11

**A**

![Diagram A](image)

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IP: Rb-anti-p85 serum

(* normal Rb serum IP *)

**B**

![Diagram B](image)

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IP: Rb-anti-p85 serum

(* normal Rb serum IP *)

Immunoblotting: Rb-anti-p85 serum
Figure 12. Time course of the PI-3K activity stimulated by CRP. CRP at 20 µg/ml was added to 5 x 10⁶ HL-60(G) cells for different intervals before the cells were examined for PI-3K activity as described in Figure 6. Stimulation with fMLPP at 25 nM for 3 min. in the presence and absence of Wortmannin (100 nM) served as the control. Representative results from three experiments.
CRP, min: 
FMLPP, min: 
Wortmannin: 

IP: Rb-anti-p85 serum

(* normal Rb serum IP)

Figure 12
been shown that in B cell negative signaling, SHIP (SH2 domain containing inositol phosphatase) was tyrosine phosphorylated upon stimulation and competes with Grb2 for the binding site on cell receptors to down regulate the Ras signaling pathway (192). An earlier study of mine suggested that CRP may be involved in the Ras signaling pathway by enhancing ERK2 activity in HL-60 cells stimulated by both IL-8 and fMLP. Therefore, the tyrosine phosphorylation level of SHIP in HL-60 (G) cells stimulated with fMLP and CRP was examined.

The SHIP tyrosine phosphorylation level was weak, but constant in all the samples over a 10 min period despite a moderate, but constant amount of SHIP on the same nitrocellulose membrane as detected by immunoblotting using anti-SHIP antibody. It seems that CRP did not alter the SHIP tyrosine phosphorylation level in both resting cells, as well as cells stimulated by fMLP (Fig.13).
Figure 13. Effect of CRP on SHIP tyrosine phosphorylation. HL-60 (G) cells 10 x 10⁶ were incubated with CRP before fMLPP (50 nM) stimulation. Cells were lysed in TN-1 buffer and SHIP was immunoprecipitated with 2 μl rabbit anti-SHIP serum. A, The membrane was first blotted with anti-phosphotyrosine antibody cocktail (PY20 + 4G10) and detected with ECL. B, The same membrane was stripped, blocked and blotted with the same anti-SHIP serum.
CRP, 300 ug/ml: - - - - - + + +
fMLPP, min: - .5 1 2 5 10 - 1 -
IP: \( \text{Rb-anti-SHIP} \) NS
D. DISCUSSION

The results described here demonstrate that purified human CRP inhibits agonist-induced neutrophil chemotaxis in vitro by altering chemokine-induced signaling. Furthermore CRP itself initiated signaling via the specific CRP-R and did not interfere with chemokine (IL-8) binding. CRP peptide fragments generated by neutrophil-mediated proteolysis, as well as certain synthetic CRP peptides, also inhibited PMN chemotactic responses. The significance of these findings is that CRP may function as a physiological regulator of leukocyte infiltration within the context of an acute inflammatory insult. CRP as an inducible APR, is an ideal candidate regulator of leukocyte activities since CRP blood levels in humans become elevated as early as 6 h after tissue injury and reach a maximum by 24 to 36 h, with a t½ of only 19 h (169,173,195). During inflammation the recruitment of particular leukocyte populations to a local site by chemoattractants is a critical event not only for host defense, but also governs the severity of any untoward tissue damage (171,183,196). Since leukocyte infiltration is a complex, multi-stage series of events, the mechanisms regulating the early events of inflammation, when there is greatly enhanced expression of proteins such as CRP is of
considerable interest because of the potential for the development of therapeutics.

Reports of the influence of human CRP on a variety of leukocyte activities in vitro are consistent with our findings of inhibition of chemotaxis. The earliest reports emphasized the ability of CRP to function as an opsonin (90, 97, 197, 198); subsequent studies with neutrophils reported that aggregated CRP potentiated the generation of intracellular reactive $O_2^-$ intermediates triggered via IgG-FcRs (146). By contrast, native pentameric CRP inhibited both degranulation and the respiratory burst of PMN, as detected by the extracellular release of reactive $O_2^-$ intermediates in response to a variety of agonists, including fMLP, PAF, C5a, as well as the PKC-activator PMA (199-201). The attenuation of the respiratory burst was proposed as a means to minimize tissue damage where CRP was invested (97). These pronounced effects of CRP on neutrophil functions have recently been extended to in vivo experiments using mice expressing either the human or rabbit CRP transgene that confers C-dependent resistance to pneumococci (105, 106), or protects against both neutrophil alveolitis initiated by C5a (167) and lethal endotoxemia (124), respectively. These findings emphasize a multifunctional role for CRP during inflammation.

The effects of CRP-derived peptides on leukocyte
functions as first described by Robey, et al. (126) clearly demonstrated that peptides generated by PMNs, but not intact CRP, were chemotactic for monocytes. Subsequent work by Shephard, et al. (110,111,175) 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 (111), contained within the synthetic peptide of residues 191-205 that I show here inhibits IL-8- and fMLPP-induced chemotaxis. Another peptide that inhibited chemotaxis was composed of residues 174 to 185, which was previously characterized as a macrophage-activating agent for tumoricidal activity in mice (119). Indeed, CRP peptides have recently been shown to inhibit neutrophil alveolitis (167). We did not observe chemotaxis with the intact CRP pentraxin, nor with most of the CRP peptides. 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 (202).

The signaling mechanism whereby CRP alters the NADPH oxidase complex activity in PMNs has not yet been determined; however, one study (200) did show an alteration in the pattern of protein phosphorylation in activated neutrophil 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 (203) and inhibition of intracellular Ca\(^{2+}\) mobilization (204). 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 chemoattractant receptors all belong to the seven-transmembrane-receptor superfamily coupled to heterotrimeric G-proteins (170,171). 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 that is PKC or PKA dependent (205-207). It seems unlikely that CRP stimulates 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 extend of IL-8-induced Ser-phosphorylation of the IL-8R A isoform in neutrophils failed to detect a significant difference. Rather, the experiments described herein with IL-8 and fMLPP suggests that the CRP-R mediates inhibition of chemotaxis by a process dependent on PI-3K. Indeed, our results show that Wortmannin blocks ligand-induced chemotaxis suggesting that
PI-3K is essential, yet CRP induces PI-3-kinase activity. This apparent contradiction may have one or more of the following explanations. CRP may block chemotaxis via a different mechanism 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 (210). CRP may activate a distinct isoform of PI-3K that is not sensitive to Wortmannin. CRP may also activate an inositol phosphatase that consumes any nascent 3-phosphoinositides (233). These alternative mechanisms in neutrophils exposed to CRP under various conditions are being examined. The CRP-triggered PI-3K-dependent pathway should also propagate reactions that inhibit the phosphorylation and assembly of the components of the NADPH oxidase (209).
CHAPTER 3

EFFECT OF HUMAN CRP ON RESPIRATORY BURST OF HL-60 GRANULOCYTE AND THE ASSEMBLY OF THE NADPH OXIDASE

A. INTRODUCTION

The respiratory burst, one of the most important neutrophil activation processes, has been credited not only for protecting host by killing invading microorganisms, but also for damaging host tissue due to its overreaction (226). During the acute phase of inflammation, CRP selectively accumulates at the site of inflammation (7, 174) and is capable of being digested by membrane proteinases into peptides that influence the activities of both monocytes and neutrophils (111, 126, 175). It was reported that heat modified CRP bound to human monocytes and neutrophils enhancing intracellular production of $O_2^-$ anion mediated by aggregated IgG (108, 109). However, the modified CRP alone did not elicit a respiratory burst, nor did it potentiate the response induced by PMA or serum opsonized zymosan (108, 109). By contrast, one study showed that CRP at concentrations of
50-100 μg/ml inhibited about 40-50% of O$_2^-$ release by PMNs induced by ligand stimulation (58). Indeed, transgenic mice carrying a diet-inducible rabbit CRP gene displayed a decreased response to LPS-induced septic shock (124), a severe systemic inflammatory crisis with broad tissue damage and multi-organ failure caused by excess inflammatory cytokines and extensive production of active O$_2^-$ anions. Digested CRP peptides significantly inhibited neutrophil superoxide production and chemotaxis in response to several stimuli (110,111). The synthetic CRP peptides of residues 77-82 and 201-206 were found to inhibit zymosan stimulated superoxide production in neutrophils (112) and protein leakage in mice with alveolitis induced by fMLP or C5a des arg (167). By contrast, degraded CRP enhanced the human monocytes superoxide burst response (113,114). CRP decreased significantly the extent of phosphorylation of several unidentified cell proteins in human neutrophils stimulated by PMA and fMLPP, correlating directly with superoxide production (227).

The NADPH oxidase is responsible for all of the superoxide production in the phagocytic respiratory burst. The complete NADPH oxidase complex is composed of the flavocytochrome b558 (heterodimer of gp91-phox and p22-phox) (215,216), the p40-phox, p47-phox and p67-phox (209,217,218).
Several missense mutations of the p47-, p67-, and gp91- phox proteins cause a hereditary disease termed Chronic Granulomatous Disease (CGD) (228-230). In unstimulated cells, only the flavocytochrome b558 is membrane bound, all of the other components are cytosolic proteins (209). The p47-, p67- and p40- phox proteins also exist in a complex form in the cytoplasm(209). Upon activation, the p47-phox and p67-phox are phosphorylated on serine residues by PKC and/or PKA (213,219,220), and then translocated to the inner membrane to assemble the intact, active NADPH oxidase (209,221). Among these cytosolic -phox proteins, the p47-phox acts critically as a bridge, binding to the docking sites ( SH3-domains ) on the COOH-terminus of both the gp91- and p22- phox components(222), and itself providing binding sites for the p40- and p67- phox proteins (223-225).

Protein kinase C (PKC), a large family of serine/threonine kinases, is composed of 3 subclasses and 12 members (214). PKC translocates from the cytosol to the cytoplasmic membrane during neutrophil activation (213). PKC phosphorylates many different protein substrates including itself (autophosphorylation) as well as the NADPH oxidase components p47-phox, p67-phox (213,214). The predominant isoform of PKC in the neutrophil is the classical member PKCβ2 (212). PKC was shown to be involved in the assembly of the
NADPH oxidase on the cell membrane (209,232).

Therefore, the purposes of the research described in this chapter were to clarify the effect of human CRP on the superoxide production in neutrophils, to reveal whereby CRP exerts its effects.
B. MATERIALS AND METHODS

1. Reagents. N-formyl-methionyl-leucyl-phenylalanine (fMLP), fMLP-phenylalanine (fMLPP), PMA, DMSO, and ferricytochrome 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 prior to use. The specific MEK kinase inhibitor PD098059 was obtained from Calbiochem (La Jolla, CA). Chymostatin, leupeptin, pepstatin A, antipain, sodium orthovanadate were from Sigma. Herbimycin A, chelerythrine chloride, KT5720 and phenyl arsine oxide (PAO) were from BioMol (Plymouth Meeting, PA). X-ray film was Kodak BIOMAX (ML).

The biotin-conjugated monoclonal anti-phosphoserine antibody (PSR45) and the polyclonal rabbit-anti-human PKCβ2 IgG (P3203) were purchased from Sigma. The polyclonal rabbit-anti-human PKCβ2 (PK20) IgG was from Oxford BioMed Inc. (Oxford, MI). The polyclonal rabbit-anti-human PKCβ antibody was from Gibco BRL. The goat-anti-p47-phox and goat-anti-p67-phox antibodies were generous gift from Dr. T. Leto (NIH). The mouse monoclonal anti-human-Rac IgG2b (23A8) was from UBI (Lake Placid, NY). The rabbit anti-human-Rac2 polyclonal antibody (C-11) was from Santa Cruz Biotech. (CA). The HRP-conjugated goat-anti-rabbit-IgG, the HRP-conjugated
strepavidin (StrepA-HRP), the HRP-conjugated goat-anti-mouse-IgG and the chemiluminescent reagents were purchased from KPL (MD). The protein-G-sepharose beads, the protein-G-conjugated-HRP (proteinG-HRP) were purchased from Zymed (CA).

2. Cells. The promyelocytic cell line HL-60 was obtained from the ATCC (Rockville, MD) and grown in RPMI-1640 plus 4% defined FBS (Sigma) and 6% defined bovine calf serum (HyClone, Logan, Utah). HL-60 cells were differentiated into granulocytic (G) cells by incubating 2x10⁵ cells/ml with 1.2% DMSO for 6 or 7 days. The HL-60(G) cells were washed twice into Earle's balanced salt solution (EBSS) containing 10 mM HEPES (pH 7.4) and adjusted to 10⁷ cells/ml and kept on ice for functional or binding assays.

3. Synthetic peptides. Synthetic peptides numbered according to the derived sequence for the 206 amino acids present in each of the subunits of human CRP were used. The truncated and mutated Pep 27-38 were also synthesized by the Ohio State University Biochemistry Instrument Center.

4. Superoxide production. Superoxide production by HL-60(G) was measured by cytochrome c reduction in a cuvette assay (51 of 98R). Briefly, HL-60(G) cells at 5x10^6 cells/ml in EBSS with 20 mM HEPES buffer (pH 7.4) were incubated with CRP, synthetic CRP peptide or kinase inhibitor at 4°C for 30 min. The cuvettes were placed in a heated (37 °C) cuvette tray at 2 x 10^6/ml in a total volume of 500 μl and a triggering stimulus of fMLPP or PMA was added. Mineral oil (40 μl) was layered onto the surface to prevent oxidation. The change in absorbance at 550 nm vs. the control was recorded over a 30-60 min period in a Beckman DU recording spectrophotometer.

5. Translocation of PKC. HL-60 (G) cells 10 x 10^6 in 200 ul EBSS buffer (pH 7.4) were preincubated ± CRP at 200 μg/ml for 30 min at 37 °C before stimulation with PMA (either 5 ng/ml or 40 ng/ml) for 3 min at 37 °C after. Reactions were stopped by sonicating the cells on ice for 1 min before centrifuged at 800xg, 4 °C for 10 min to remove nuclei and unbroken cells. The supernatants were then mixed with a modified extraction buffer (Tris 50 mM, pH 7.5, EGTA 2 mM, sodium orthovanadate 10 mM, leupeptin 1 μg/ml, apritinine 0.2 μg/ml, PMSF 2 mM) before centrifuged at 60,000 rpm, 4 °C (Beckman, TLA-100 rotor) for 30 min. The membrane pellet was washed once by resonicating in the extraction buffer and centrifugation under the same
conditions. The final membrane preparations were stored in the extraction buffer at -70 °C. The protein concentration of each membrane sample was measured by the BCA method (Pierce Chemical, IL) using BSA as the standard protein. An equal amount of protein from each sample was used for the immunoblotting assay with separation by 10% SDS-PAGE and transferred to a nitrocellulose membrane. A polyclonal rabbit anti-human PKC-β2 IgG (P-3203, Sigma) was used as the primary antibody (1:1,000) and HRP-labeled anti-rabbit-IgG (KPL, MD) as the secondary antibody (1:2,500). Chemiluminescent reagent was used to expose the film.

6. Translocation of the p47-phox and p67-phox proteins to the membrane. The same method described for PKC translocation was used. The separated proteins on membrane were probed using the goat-anti-p47-phox or anti-p67-phox antibody (1:2,000) and proteinG-HRP (1:4,000, Zymed, CA) was used as the secondary reagent.

7. Serine phosphorylation of the p47-phox and p67-phox. Immunoprecipitation of the p47-phox from lysate of HL-60(G) cells under different stimulation conditions were done using the goat anti-p47-phox serum (2 μl) or anti-p67-phox serum (3 μl) and proteinG-sepharose beads (10 μl) at 4 °C, overnight.
A goat anti-rabbit serum was used as an antibody control for immunoprecipitation. The beads were then washed 3 times in 1x D-PBS before denaturing, running on SDS-PAGE and immunoblotting. The phosphorylation of serine residues was detected using the PSR-45 monoclonal antibody and the StrepA-HRP. The relative amount of the p47-phox or p67-phox was detected by reprobing the membrane with the same goat antibodies (1:2,000) and proteinG-HRP (1:4,000, Zymed, CA).

8. Translocation of the Rac2 proteins to the membrane. The similar methods described above for 6 and 7 were used with modification. The membrane proteins isolated from 30 x 10⁶ HL-60 (G) cells were dissolved in TN-1 lysis buffer followed by immunoprecipitation with 8 µl rabbit polyclonal anti-Rac2 antibody (Santa Cruz Bio. CA) and protein-G-sepharose beads (20 µl) at 4 °C, overnight. A normal rabbit serum was used as an antibody control. The denatured immunoprecipitates were used for immunoblotting assay. The separated proteins on membrane were probed using the same anti-Rac2 antibody (1:500) and HRP-conjugated Go-anti-Rb-IgG (1:2,000, KPL, MD) was used as the secondary reagent.
C. RESULTS.

1. Effect of CRP on superoxide production.

Neutrophil activation during inflammation includes chemotaxis, phagocytosis, degranulation and respiratory burst. Since previous studies showed that purified human CRP at elevated concentrations of > 50 μg/ml inhibited the respiratory burst of neutrophils triggered by a variety of stimuli (175, 203), we examined the effect of the CRP on the $O_2^-$ production of HL-60(G) cells, which bind CRP with the same affinity, specificity, and number of binding sites per cell as U937 cells (147). HL-60 is a premyelocytic leukemia cell line which can be differentiated into neutrophil-like granulocyte.

Incubation of heat modified human CRP with HL-60 (G) cells inhibited $O_2^-$ production by the cells stimulated with both higher and lower levels of either PMA (5 and 40 ng/ml) or fMLPP (50 nM and 1 μM). CRP at acute phase concentration ≥ 50 μg/ml significantly inhibited the $O_2^-$ production. The inhibitory effect of CRP was more pronounced in the cells with PMA stimulation and was in a dose-dependent manner at CRP concentrations ranging from 20 to 200 μg/ml, with maximal inhibition of 75% (Fig.14). CRP had relatively weaker inhibitory effect when fMLPP was used as stimulator. CRP did not change the overall kinetics of $O_2^-$ production in cells stimulated by either PMA or fMLPP, although CRP inhibited the
Figure 14. Effect of human CRP on superoxide (O$_2^-$) production by HL-60 (G) cells. Cells (1 x 10$^6$) were preincubated with CRP at different concentration for 30 minutes before stimulation with either PMA (5 ng/ml, ●) or fMLPP (50 nM, ○). The absorbance of cytochrome C at 550 nm was recorded over a 30 minute interval. Data were representative of 3 experiments.
Fig. 14

Percent of Control vs. CRP ug/ml

- ○ IMLPP
- ● PMA

100
cell response from its early stage. PMA, a direct PKC activator, induced a very strong but somehow delayed $O_2^-$ production which began after 5 min and reach peak at ≥ 30 min with a ΔA ≥ 1.0 (Fig.15a). On the other hand, fMLPP, the ligand of the trimeric G-protein associated serpentine receptor, activated $O_2^-$ production on a much smaller scale but reached saturation as early as at 5 min and lasted more than 20 min (Fig.15b). Human CRP itself never initiate $O_2^-$ production in HL-60 (G) cells (data not shown).

2. Effect of the CRP-Peptides on Superoxide Production.

Since CRP is accumulated and degraded into biologically active peptides by leukocytes at the inflammatory site, we examined the effect of several CRP synthetic peptides on the $O_2^-$ response of HL-60(G) cells. Of the peptides examined, only Pep27-38, the cell binding peptide (153,154), inhibited $O_2^-$ production when HL-60(G) cells were exposed to the peptide prior to triggering the respiratory burst with 1 μM fMLPP. A dose-dependent inhibition of the fMLPP-driven response was observed over a range of Pep27-38 concentrations of 3.0 to 16,000 pmole/ml, with the threshold for inhibition occurring at 400 pmole/ml, or the equivalent of ~10 μg/ml of the intact CRP pentraxin, a modest acute phase level of CRP in the blood (Fig.16). HL-60(G) cells stimulated by 1.0 μM FMLPP generated
Figure 15. Effect of human CRP on the kinetics of superoxide production by HL-60 (G) cells. Cells (1 x 10^6) were incubated with CRP 200 μg/ml at different concentration for 30 minutes before stimulation with either PMA (5 ng/ml, a) or fMLPP (50 nM, b). Data were representative.
Figure 16. Effect of CRP peptides on fMLPP-induced respiratory burst of HL-60 (G) cells. The cells were exposed to different concentration of various peptides for 30 min prior to triggering $O_2^-$ production with 1.0 μM of fMLPP. The data represent the mean percentage inhibition from five experiments. The SD for each value was ≤ 6.0%.
% of Control O2- Response to fMLPP

1 10 100 1000 10000

pmoles/ml CRP Peptide

Figure 16
7.5 (± 1.7) nmoles O$_2^-$ /10$^6$ cells/15 min. Pep27-38 inhibited both the rate and extent of O$_2^-$ produced. None of the peptides tested by themselves was capable of inducing a significant respiratory burst. Thus, the CRP peptide that selectively binds to the CRP-R also altered a basic functional response of granulocytes associated with inflammation.

3. Effect of Modifications of the CB-Pep on Superoxide Production.

Since the Pep27-38 mediates the binding of CRP-R and carries biological activity—inhibiting O$_2^-$ production, I used truncated versions of this peptide and the Pep27-38 with substitutions of a single residue to test their ability on inhibiting O$_2^-$ production and better understand the requirement of receptor binding of CRP through this domain and its bioactivity.

Each of the truncated and substituted forms of Pep27-38 were evaluated for their ability to inhibit O$_2^-$ production in response to fMLPP. HL-60(G) cells were exposed to the various synthetic peptides just to triggering the respiratory burst. A reproducible, significant decrease in the response was observed with some of the truncated peptides and an assessment of their relative inhibitory activity revealed that the minimum length required for significant inhibition required
residues 31 through 37 or: **KAFTVC** (Table VII).

Synthetic peptides that correspond to the 12-residue CB-Pep that contain a single, conservative substituted amino acid were also compared to Pep27-38 for their ability to inhibit the fMLPP-driven respiratory burst. Peptides with substitutions at residues 33 and 37 lost their ability to significantly inhibit the $O_2^-$ response (Table VIII). Therefore, only the Phe-33 and Leu-37 in the CB-Pep appear to be critical for leukocyte CRP-R signaling.

4. Effect of protein kinase inhibitors on $O_2^-$ production.

To single out possible signal transduction pathway initiated by CRP stimulation, I examined effect of several protein kinase inhibitors on the $O_2^-$ production in HL-60 (G) cells.

**a. Effect of PTK inhibitor Herbimycin A.**

Since fMLPP stimulation causes changes of tyrosine phosphorylation of several proteins in HL-60 cells while CRP modified some of this effect as I showed in chapter 2, I investigated the effect of Herbimycin A, a protein tyrosine kinase (PTK) inhibitor, on $O_2^-$ production. Herbimycin A inhibited $O_2^-$ production induced by both PMA and fMLPP, in a dose-dependent manner at concentrations from 0.1 – 100 μM,
Table VII. Effect of Truncated CRP Cell-Binding Peptides on FMLPP-Induced Superoxide Production by HL-60(G) Cells

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<th>Peptide</th>
<th>Sequence</th>
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<tr>
<td>CB27-38</td>
<td>TKPLKAFTVCLH</td>
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<tr>
<td>CB28-38</td>
<td>KPLKAFTVCLH</td>
<td>74.0 ± 6.9</td>
</tr>
<tr>
<td>CB29-38</td>
<td>PLKAFTVCLH</td>
<td>83.1 ± 7.1</td>
</tr>
<tr>
<td>CB30-38</td>
<td>LKAFTVCLH</td>
<td>86.0 ± 7.7</td>
</tr>
<tr>
<td>CB31-38</td>
<td>KAFTVCLH</td>
<td>81.0 ± 7.2</td>
</tr>
<tr>
<td>CB32-38</td>
<td>AFTVCLH</td>
<td>108.0 ± 9.0</td>
</tr>
<tr>
<td>CB33-38</td>
<td>FTVCLH</td>
<td>110.1 ± 8.8</td>
</tr>
<tr>
<td>CB27-37</td>
<td>TKPLKAFTVCL</td>
<td>62.3 ± 6.4</td>
</tr>
<tr>
<td>CB27-36</td>
<td>TKPLKAFTVC</td>
<td>63.5 ± 7.1</td>
</tr>
<tr>
<td>CB27-35</td>
<td>TKPLKAFTV</td>
<td>95.8 ± 8.5</td>
</tr>
</tbody>
</table>

*Each peptide was tested at 400 and 2000 pmoles/ml with 10⁶ granulocytes triggered with 1.0 μM of FMLPP. Data shown obtained from 4 experiments with the peptide at 2000 pmoles/ml.
Table VIII. Effect of Single Amino Acid Substitutions within the CRP CB-Pep on FMLPP-Induced Superoxide Response of HL-60(G) Cells

<table>
<thead>
<tr>
<th>Peptide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence</th>
<th>% of Control(±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB27-38</td>
<td>TKPLKAFTVCLH</td>
<td>77.2 ± 6.9</td>
</tr>
<tr>
<td>CB30I</td>
<td>TKPIKAFTVCLH</td>
<td>89.0 ± 7.1</td>
</tr>
<tr>
<td>CB31R</td>
<td>TKPLRAFTVCLH</td>
<td>83.4 ± 7.4</td>
</tr>
<tr>
<td>CB32L</td>
<td>TKPLKLFTVCLH</td>
<td>80.0 ± 2.3</td>
</tr>
<tr>
<td>CB33Y</td>
<td>TKPLKAYTVCLH</td>
<td>117.0 ± 13.4</td>
</tr>
<tr>
<td>CB34S</td>
<td>TKPLKAFSVCLH</td>
<td>86.8 ± 11.1</td>
</tr>
<tr>
<td>CB35L</td>
<td>TKPLKAFTLCLH</td>
<td>87.5 ± 12.8</td>
</tr>
<tr>
<td>CB37I</td>
<td>TKPLKAFTVCIH</td>
<td>106.0 ± 10.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peptide is designated by the residue number that is substituted and the substituted amino acid is underlined.
with a similar ID₅₀ at about 4 - 7 µM (Fig.17a). This indicates that PTK(s) is important for the signaling pathway in O₂⁻ production initiated by fMLPP and PMA, fitting with my earlier results in chapter 2.

b. Effect of PKC inhibitor cherelythrine chloride.
Cherelythrine chloride is a potent and specific PKC inhibitor. At 10 µM, cherelythrine chloride inhibited O₂⁻ production by 80% with 1 min preincubation and a 90% inhibition was reached by 10 minutes preincubation in HL-60 (G) cells stimulated by either PMA and fMLPP (Fig.17b). So PKC seems playing a crucial role in O₂⁻ production.

c. Effect of PI3-K inhibitor wortmannin.
Wortmannin, a fungal metabolite, is a potent and selective PI-3K inhibitor. Superoxide production in HL-60 (G) cells induced by fMLPP was inhibited by wortmannin in a dose-dependent manner at concentrations from 0.1 nM to 200 nM (Fig.17c), reaching a maximal ~70% inhibition. But wortmannin only had weak inhibitory effect on PMA-induced O₂⁻ production, ~20% inhibition at 200 nM. Results here indicate that fMLPP receptor signaling pathway is more dependent on the activity of PI-3K activity.
d. **Effect of protein tyrosine phosphatase inhibitors.**

Two inhibitors of protein tyrosine phosphatase, sodium orthovanadate (Na$_3$VO$_4$) and phenyl arsine oxide (PAO), inhibited $O_2^-$ production induced by fMLPP and PMA in a similar way, both in a dose-dependent manner (Fig.17d & 17e). This implies protein tyrosine phosphatase(s) is important for generation of respiratory burst.

e. **Effect of protein kinase A inhibitor KT5720.**

KT5720 is a specific PKA inhibitor with a reported IC$_{50} =$ 0.056 µM. At the range from 0.01 to 1 µM, KT5720 had no obvious inhibitory effect on $O_2^-$ production induced by either PMA or fMLPP (Fig.17f).

5. **Effect of CRP on translocation of PKC.**

One early event in the assembly of the NADPH oxidase is the phosphorylation of multiple serine residues on the cytosolic p47-phox component of the oxidase by PKC (209,213). Activation of PKC itself is related to its translocation from cytosol to membrane, and autophosphorylation (212). Therefore, we examined the translocation of PKCβ2 (the dominant isoform in PMNs) to the membrane following cell's exposure to CRP. CRP at 200 µg/ml inhibited PKCβ2 translocation to cell membrane in cells stimulated with PMA.
Figure 17. Effect of protein kinase and phosphatase inhibitors on superoxide production. HL-60 (G) cells $1 \times 10^6$ in 0.5 ml EBSS (pH 7.4) were preincubated with different protein kinase inhibitors, (a) herbimycin A, 37 °C for 30 min, (b) chelerythrine chloride (10 μM), (c) wortmannin, 37 °C for 30 min, (d) sodium orthovanadate (Na$_3$VO$_4$), 37 °C for 30 min, (e) PAO, 37 °C for 10 min, (f) KT5720, 37 °C for 30 min. Cells were then stimulated with either PMA (5 ng/ml) or fMLPP (50 nM) and absorbance changes were measured. Data for each curve are from at least 2 experiments and expressed in mean ± SD.
Effect of Herbimycin A on O2- production

![Graph showing the effect of Herbimycin A on O2- production.](image)

Effect of C.C. on O2- Production

Chelerythrine Chloride

![Graph showing the effect of C.C. on O2- production.](image)

Figure 17 (to be continued on p.114)
Effect of Wortmannin on O· production

![Graph C](image)

Effect of Vanadate on O· production

![Graph D](image)

(to be continued on p. 115)
Effect of PAO on O2- Production

![Graph showing the effect of PAO on O2- production. The y-axis represents the percent of control (no PAO) and the x-axis represents PAO concentration in uM. The graph includes lines for IMLPP 50 nM and PMA 5 ng/ml.]

Effect of KT5720 on O- production

![Graph showing the effect of KT5720 on O- production. The y-axis represents the percent of control (no KT5720) and the x-axis represents KT5720 concentration in uM. The graph includes lines for IMLPP 50 nM and PMA 5 ng/ml.]

(end of figure 17)

115
ng/ml) for 5 min, revealed here by immunoblotting results probed with an anti-PKCβ2 polyclonal antibody (Fig.18) The inhibition in cells stimulated with 40 ng/ml PMA was not significant. The CRP concentration 200 µg/ml chosen is because of its maximal inhibition of $O_2^-$ production. The efforts to detect a dose-response effect of CRP over the range of 20 to 200 µg/ml on PKC translocation failed, but a threshold concentration of CRP at ≥ 100 µg/ml was indicated (Fig.19). CRP by itself did not induce PKCβ2 translocation.

Since CRP (at ≥ 200 µg/ml) also inhibits $O_2^-$ production in HL-60 (G) cells stimulated by fMLPP (Fig.14), I investigated the effect of CRP on PKCβ2 translocation induced by fMLPP. The signal of PKCβ2 detected with immunoblotting was very weak and no distinguishable differences was noted. The weak activity of fMLPP compared to that of PMA in inducing $O_2^-$ production may explain this.

6. Phosphorylation of PKC isoform.

PKC autophosphorylates itself when activated. So the serine phosphorylation level of PKCβ2 in cells following stimulation with CRP and PMA was examined. Acute phase concentrations of human CRP as low as 20 µg/ml significantly inhibited the serine phosphorylation level of PKCβ2 in HL-60(G) cells stimulated by PMA (Fig.20), but dose response is not clear as comparing lane 5 to 6. CRP itself did not
change the serine phosphorylation level of PKCβ2.

7. Effect of CRP on translocation and phosphorylation of the p47-phox protein.

Upon cell activation, the p47-phox and p67-phox are phosphorylated on serine residues by PKC and translocate to the membrane-bound Cyt.b558 assembling the intact, active NADPH oxidase. Among these cytosolic proteins, the p47-phox acts critically as a bridge. Thus the effect of human CRP on serine phosphorylation and translocation of p47-phox was first examined.

Human CRP at 200 μg/ml inhibited significantly the translocation of the p47-phox to cell membrane in HL-60 (G) cells stimulated by 5 ng/ml but not 40 ng/ml PMA (Fig.21). CRP itself did not alter the p47-phox translocation.

The serine phosphorylation of the p47-phox immunoprecipitated from whole cell lysates was decreased by about 50% although the phosphorylation extent of the p47-phox in the positive control was low (Fig.22). There was no obvious dose-response of CRP, however, a threshold of 50 μg/ml was noted.

In this immunoprecipitation assay, the p47-phox and the heavy chain of the antibody (IgH, ~55 KDa) separated on either a 7.5% or a 10% SDS-PAGE minigel were very close to each other as shown by both of the Ponceau S staining and immunoblotting.
Figure 18. Effect of human CRP on the translocation of cytosolic PKCβ2 to the cell membrane. HL-60 (G) cells (10 x 10⁶) were preincubated with CRP for 30 min before PMA stimulation (5 min). Cell membranes were prepared and an equal amount of protein was added to each lane for SDS-PAGE. (a) Immunoblotting was done on nitrocellulose membranes and detected by chemiluminescent reagents. (b) The intensity of the bands in (a) was measured by luminescense.
Figure 18

a

CRP, ug/ml
- 200  - 200  - 200
PMA, ng/ml
- 5  5  40  40

IB : Rb-anti-PKC β2 Ab
Figure 19. Effect of CRP on PKC β2 translocation to the membrane. HL-60 (G) cells (10 x 10⁶) were incubated with CRP for 30 min (lane 2, 4, and 5) or PKC inhibitor chelerythrine chloride (10 mM, lane 6) for 10 min before stimulation by PMA for 5 min. An equal amount of protein (40 µg per lane) from the membrane preparation was used for SDS-PAGE. Lane 7 is whole cell lysate. (a) Immunoblot. (b) Relative intensity of the immunoblotted bands.
Figure 19

IB: Rb-anti-PKC β2
Figure 20. Effect of human CRP on serine phosphorylation of PKCβ2. HL-60 (G) cells (10 x 10⁶) were treated with CRP for 30 min and then stimulated with PMA. The whole cell lysates were immunoprecipitated with 4 µl of polyclonal rabbit anti-PKCβ2 and 10 µl protein G beads. Lane 1 is the rabbit antibody control (pre-immune). Membrane was immunoblotted with mouse monoclonal anti-phosphoserine. (a) Result of immunoblot. (b) Relative intensity of the serine phosphorylated PKC β2 bands.
Figure 20
Figure 21. Effect of human CRP on the translocation of the p47-phox component to the cell membrane (dose response). HL-60 (G) cells (10 x 10^6) were incubated with CRP at different concentration for 30 min before stimulation with PMA for 5 min. An equal amount of protein (40 μg per lane) from the membrane preparation was used for SDS-PAGE. (a) Immunoblot. (b) Relative intensity of the immunoblotted bands.
Figure 21

IB: Goat-anti-P47-phox serum

PMNA, n/g/ml: 5 5 40 40
CRP, 200 n/g/ml:

P47-phox

44 KDa

71 KDa
Figure 22. Effect of CRP on serine phosphorylation of the p47-phox. HL-60 (G) cells (10 x 10^6) were treated with CRP for 30 min and stimulated with PMA. The whole cell lysates were used for immunoprecipitation with 2 μl antiserum and 10 μl protein G beads. Lane 6 is the goat antibody control (pre-immune). Samples were assayed by immunoblotting. (a) Results of immunoblots. (b) Normalized data for the relative intensity of the serine phosphorylated p47-phox bands.
Figure 22

A

\[ \text{IgH} \]
\[ p47-P_s \]

(CB: Rb-anti-phosphoserine)

\[ p47 \]

CRP, ug/ml:
- - 50 100 200 -
PMA, 5 ng/ml:
- + + + + +

(CB: Gt-anti-p47-phox)

IP: goat-anti-p47-phox serum

B

Relative Intensity

0 1 2

1 2 3 4 5 6

Figure 22

127
(in this case the binding of the proteinG-HRP to the denatured IgH was weaker than the binding to the anti-p47 IgG molecule and thus washed away by longer washing; the difference was also indicated by the lack of the p47 band in the immunoprecipitate from a control antibody), thus the serine phosphorylation signal was interfered by the stronger signal from the IgH due to the large amount of this protein and its strong unspecific binding to either the StrepA-HRP or the proteinG-HRP.


The translocation of the p67-phox component to the membrane was not significantly altered by preincubation cells with human CRP (Fig.23). The phosphorylation extent of the p67-phox immunoprecipitated from whole cell lysates was very weak and did not indicate altered by CRP incubation.


Human CRP at 200 μg/ml significantly inhibited the translocation of the Rac2 to cell membrane in HL-60 (G) cells stimulated by 40 ng/ml PMA (Fig.24). CRP itself did not alter the Rac2 translocation. Large amount of cells were used because there was not enough Rac2 from 10 million cells. The antibody control did not precipitated the Rac2.
Figure 23. Effect of CRP on the p67-phox translocation to the membrane. HL-60 (G) cells (10 x 10⁶) were incubated with CRP for 30 min before stimulation by PMA for 5 min. An equal amount of protein (80 µg per lane) from the membrane preparation was used for SDS-PAGE. (a) Immunoblot. (b) Relative intensity of the immunoblotted bands.
Figure 23
Figure 24. Effect of human CRP on the translocation of cytosolic Rac2 to the cell membrane. HL-60 (G) cells (30 x 10⁶) were incubated with CRP for 30 min before PMA stimulation (15 min). Cell membranes were prepared and an equal amount of protein (300 μg) was used for immunoprecipitation. The immunoprecipitates were used for SDS-PAGE. (a) Immunoblotting was done on nitrocellulose membranes and detected by chemiluminescent reagents. (b) The intensity of the bands in (a) was measured by luminescence.
CRP, 200 µg/ml: - - + - + - -
PMA, 40 ng/ml: - + - + + -
IP: * + + + + - WCL

IP & IB: rabbit-anti-human Rac2

(* preimmune rabbit serum)
D. DISCUSSION

The receptor for fMLPP is a trimeric G-protein associated receptor. Upon ligand binding to the fMLP-R, the G protein will dissociate into Gα and βγ dimer, which are both able to activate downstream enzymes. The proximal enzyme downstream is phospholipase C β2 (PLCβ2) which digests phosphotidyl-inositol into the secondary messengers diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate (IP3) (237). The IP3 stimulates intracellular calcium mobilization. The DAG will, together with the released intracellular calcium ions, activates PKC to propagate a series of reactions (237). Other signaling molecules are also involved in the activation process since fMLP stimulates the Ras signaling pathway (238), and protein tyrosine kinases likely branches at the level just after trimeric G-protein activation (239). On the other hand, PMA is a direct, powerful activator of PKC and therefore, its signaling pathway for activating the NADPH oxidase complex, would be more direct.

Since PMA and fMLPP use somewhat different pathways to activate the NADPH oxidase to produce the O2⁻ anion, and human CRP more efficiently inhibited the O2⁻ production induced by PMA (stronger stimulus) than fMLPP, then it is reasonable to propose that CRP acts at different points to exert its modification effect on O2⁻ production. One such important
target among these is the protein kinase C (PKC), since direct activation of PKC by PMA can be blocked by CRP incubation (fig.14), which is similar to the effect of the specific PKC inhibitor cherelythrine chloride (fig.17b). The other site is the PI3-K. Wortmannin inhibited $O_2^-$ production induced by fMLPP more efficiently with an $ID_{50} = 40$ nM, but it only had minor effect on PMA-induced $O_2^-$ production. This may be explained by the following reasoning: If PKC is downstream of PI3-K, and PMA is a direct PKC activator, then PMA activation of cells bypasses any inhibitory effect of wortmannin. By contrast, fMLPP-induced $O_2^-$ production may be largely PI-3K-dependent, and thus more readily inhibited by wortmannin. As I had shown that CRP could increase PI3-K activity, and fMLPP may have PKC-independent mechanism of activating $O_2^-$ production, thus although CRP may inhibit PKC activity, the overall inhibitory effect of human CRP on $O_2^-$ production in cells stimulated by fMLPP was less efficient than that in cells stimulated by PMA. Some protein phosphatases may also contribute to the production of $O_2^-$ as shown by fig.17d and 17e, but their relationship with CRP signaling needs to be delineated.

As I showed, CRP incubation inhibited both the translocation and phosphorylation of PKCβ2, thus its overall activation induced by PMA. So the phosphorylation of its
substrates will be lower in the inhibited cells, as I showed here in figure 22 that the serine phosphorylation of the p47-phox protein was noticeably decreased. Because the assembly of the intact, functional NADPH is dependent on the translocation of the cytosolic proteins, the p47-phox, p67-phox and p40-phox to the membrane-bound cyt. b558 (gp91-phox and p22-phox heterodimer), during which the p47-phox bridges the assembly (209). Since the translocation of p47-phox is dependent on its serine phosphorylation, so we can expect that human CRP will block or inhibit the assembly of the functional NADPH oxidase complex and thus decrease the $O_2^-$ production. Because I did not detect significant block of p67-phox translocation, there may be some other mechanisms that human CRP regulates the respiratory burst, such as downregulating the activity of the Cyt.b558.

The activity of NADPH oxidase is also regulated by a small GTPase, Rac (209). Rac (21 kDa) is a member of the low molecular weight GTP-binding protein (LMWG) Ras superfamily. Rac2 is the predominant (96%) isoform in neutrophils (240,241). Rac is a cytosolic protein associated with Rho GDP-dissociation inhibitor (RhoGDI) in resting cells (242). Upon cell activation, Rac dissociates and translocates independently of the p47- and p67-phox proteins to the inner membrane and then associated with the Cyt. b558 and the p67-
phox (240,241). The Rac activity is also closely related to the rearrangement the cytoskeleton (243). Human CRP inhibited the translocation of the Rac2 to the membrane in activated cells. The implication of this inhibition is that it may represent a direct mechanism for reducing $O_2^-$ production by an otherwise assembled NADPH oxidase complex. This effect of CRP on Rac is entirely consistent with the observation that CRP inhibits PMN chemotaxis since Rac is required for polymerization of actin filaments for neutrophil membrane ruffling (243).

Figure 25 is a working model to explain the mechanism by which CRP inhibits $O_2^-$ production by blocking the assembly of the NADPH oxidase.
Figure 25. Proposed mechanism for C-reactive protein down-regulation of NADPH oxidase activity. Part of the diagram adopted from F.R. Deleo's paper in J. Leuk. Biol., vol 60, 677, 1996.
SUMMARY

C-reactive protein (CRP) is the prototype of all the acute phase reactants in humans since its blood levels increase dramatically by 48 hours after the onset of inflammation. The increase in serum CRP concentration during acute phase response is primarily the result of increased rates of transcription of the gene in the liver.

The remarkable conservation of structure and binding specificity of CRP through both vertebrate and invertebrate evolution strongly suggests a critical biological role for CRP. CRP binds many targets through its PC-binding site, including microorganisms, damaged cell membranes and chromatin to fulfill many biological activities, including complement activation, opsonization, cytokine induction, tumor destruction, and innate immunity against microbial infection. These activities attributed to CRP are still poorly defined. CRP becomes selectively deposited at sites of tissue damages, and can be degraded into fragments by neutrophil cell surface protease at these inflammatory sites. A few reports suggested that CRP regulates leukocyte functions. Heat modified CRP and
CRP-derived peptide were reported to modulate biological activities of certain leukocytes, such as enhancing $O_2^-$ production of macrophages but inhibiting it in neutrophils. CRP peptides also enhance monocyte/macrophage tumoricidal activity. The interaction between CRP and leukocytes was documented as receptor mediated, and involves intracellular Ca$^{2+}$ mobilization. But several questions concerning the kind of leukocyte functions regulated by CRP and the mechanism by which CRP regulates these functions need to be elucidated.

My research results show that human CRP inhibits both neutrophil chemotaxis and respiratory burst. Human CRP inhibits human neutrophil chemotaxis induced by either the human chemokine IL-8, or the bacterial-derived chemoattractant formyl peptide fMLPP in a dose-dependent manner at CRP concentration from 5 to 50 μg/ml. Two human CRP synthetic peptides 174-185 and 191-205 also significantly inhibited chemotaxis induced by either IL-8 or fMLPP, confirming that CRP peptides do have significant biological activities. This inhibition is MAPK related, since the MEK (MAPKK) inhibitor PD 98059 could promote chemotaxis, while human CRP enhanced ERK2 activity in HL-60 (G) cells induced by either fMLPP or IL-8. Human CRP by itself also activated PI-3K activity in granulocytes.

Human CRP also inhibited neutrophil superoxide production
induced by either fMLPP or PMA at CRP concentration ≥ 50 μg/ml, with the inhibitory effect more potent for PMA. In cells stimulated with PMA, CRP inhibited the translocation to the inner membrane of PKCβ2 and its serine phosphorylation. This in turn decreased the phosphorylation of serine residues on the p47-phox cytosolic component of NADPH oxidase and its translocation to the membrane. The translocation of the Rac2 protein which is directly related to NADPH oxidase activity and actin polymerization also was inhibited by human CRP. I also demonstrated that the minimal sequence required for the CRP cell binding peptide (Pep27-38) for its ability to inhibit $O_2^-$ production consists of residues 31-37 KAFTVC, with the Tyr33 and Ile37 being critical. Thus, human CRP and CRP-derived peptides block the assembly of a functional NADPH oxidase to inhibit the respiratory burst of neutrophils.
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