Proteolysis of Human C-reactive Protein Produces Peptides with Potent Immunomodulating Activity*

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We have studied the ability of human C-reactive protein to modulate the immune response in vitro. Whereas native C-reactive protein did not induce phagocytic leukocytes to chemotax or to produce superoxide, treatment of purified C-reactive protein with human neutrophil-derived acid proteases produced substances with potent effects on leukocyte function. Close examination of the primary structure of human C-reactive protein revealed three regions evenly distributed throughout the protein each of which contain peptide sequences closely resembling the amino acid sequence of the immunomodulator peptide tuftsin, Thr-Lys-Pro-Arg. We have synthesized the three peptides which include Thr-Lys-Pro-Leu ([Leu]tuftsin), Gly-Lys-Pro-Arg ([Gly]tuftsin), and Thr-Lys-Pro-Gln ([Gln]tuftsin) and assayed them for biological activity. The three synthetic peptides were found to stimulate phagocytic leukocytes to chemotax, produce superoxide, and induce mononuclear cells to produce interleukin 1 in vitro at concentrations similar to those concentrations required for tuftsin to induce these phenomena. These results support a potentially important role for C-reactive protein as a possible immunomodulator during inflammation.

Within the first 24-48 h following the onset of several types of inflammation, there appears in the blood of man a protein not easily detected in normal serum. This protein, termed C-reactive protein (CRP), may increase by as much as 2000-fold during the inflammatory process (1, 2). This dramatic increase in CRP, first described in 1930 (3), suggests an important role for CRP in inflammation. Several of the biological properties of CRP resemble those immunoglobulins including the ability to promote reactions of precipitation (3, 4), phagocytosis (5, 6), and complement fixation (7, 8). Furthermore, it was recently shown that chromatin may be a physiological ligand for CRP (4) since CRP mediates the solubilization of chromatin by complement (8). A defect in the CRP complement-dependent mechanism to solubilize chromatin may occur in patients with systemic lupus erythematosus and may result in the formation of anti-DNA antibodies found in many of these patients due to the inability to clear the chromatin (8).

In pursuit of other functional contributions of CRP to the inflammatory response, we have undertaken the study of possible biological activity of CRP digests in modulating inflammatory cell activity. Furthermore, phagocytosis and degradation of CRP by phagocytic cells such as macrophages or PMNs may lead to the release of biologically active peptides.

As we show in this paper, treatment of CRP with PMNs or PMN lysates at pH 5.0 for 18 h results in the digestion of the CRP molecule and the formation of substance(s) with biological activity. In addition, peptides identified within the intact CRP molecule and resembling tuftsin, Thr-Lys-Pro-Arg, an immunomodulating peptide found in the CH2 domain of the Fc segment of the immunoglobulin heavy chain residues 289-292 (30), were synthesized and assayed for immunoregulation of leukocyte function. Digestion of CRP may result in the release of such molecules with potent immunoregulatory potential.

MATERIALS AND METHODS

fMLP was from Peninsula Laboratories; phorbol 12-myristate 13-acetate from Sigma; and crude C5a was prepared as described previously (9). Purified human CRP was isolated from the plasma of rheumatoid arthritis patients as described previously (8).

Biosynthesis and Isolation of S-Labeled CRP—The biosynthesis of [16S]methionine-labeled CRP was carried out by treating a heparin-treated human mononuclear cell cell line PLC/PRF/5 with supernatant from endotoxin-treated human mononuclear cells. The culture was labeled with [16S]methionine (New England Nuclear) for 24 h. Further details are given in Ref. 10 and purification was done as described for nonradiolabeled CRP (8).

Isolation of Phagocytic Leukocytes—Human monocytes were isolated from heparinized blood of normal volunteers by Ficoll-Hypaque density gradient sedimentation as previously described (11). PMNs were isolated by gently removing the buffy coat on top of the red cell pellet formed in the Ficoll-Hypaque density gradient sedimentation step. Contaminating red blood cells were lysed by addition of NH4Cl lysis buffer at 4 °C for 10 min (Media Unit, National Institutes of Health). Cells were quantitated using a ZBI Coulter Counter and suspended in Gey's balanced salt solution containing 2% bovine serum albumin (Media Unit, National Institutes of Health) for chemotaxis studies or in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin for cultures.

Assays for Biological Activity—Chemotaxis was carried out in 48-well microchamber plates (Neuroprobe) as described (12, 13). Chemotactic activity was determined by measuring the number of monocytes or PMNs that migrated through the 5-μm polycarbonate filters.
following 90-min incubation of the tested material with the cells at 37 °C. Positive control chemotactic stimuli included the synthetic peptide fMLP and human C5a des-Arg prepared from endotoxin-activated human serum in the absence of carboxypeptidase inhibitors (9). Buffer only served as a negative control.

Superoxide production was measured by the reduction of ferricytochrome c in microtiter plate wells according to the method of Pick and Mizel (14).

Supernatants were assayed for IL-1 activity using thymocytes from 6- to 8-week-old C57/HesJ mice as previously described (15).

**Digestion of Purified CRP by PMNs or PMN Extracts**—Purified CRP (500 ng/ml) was dialyzed into either a buffer containing 0.15 M NaCl, 10 mM CaCl2, 50 mM Tris, pH 7.5, or 0.15 M NaCl and 0.1 M sodium acetate at pH 5.0. The solutions containing CRP were added directly to pellets of PMNs and the cells were evenly suspended at concentrations of 5-8 × 10^6 cells/ml. The digestion of CRP by the whole cell suspensions or the cell lysates proceeded at 37 °C for 18 h. Cells were lysed by freeze-thaw methods using an ethanol/dry ice bath to freeze the cells and a 37 °C water bath for thawing. For biological activity assays, an aliquot of the digestion mixture or PMN solution without CRP for the control was withdrawn, an equal volume of cold 0.1 M HCl was added to the withdrawn aliquot and this solution was dried in a Savant vacuum centrifuge equipped with a cold trap. To the dried residue was added either 20% trichloroacetic acid which denatured and precipitated soluble proteins such as undigested CRP or 1 ml of Gey's balanced salt solution for biological testing of the digest. Prior to using the digests in biological assays, the solutions were centrifuged and sterilized on a 0.22-μM Millipore membrane.

**Synthesis and Purification of Peptide Analogs to Tuftsin**—The three tetrapeptides, [Gly]tuftsin, [Gln]tuftsin, and [Leu]tuftsin, were synthesized by solution methods. Peptide [Gly]tuftsin was synthesized by condensing H-Arg-(Mts)-OH with Z(0Me)-Lys-(Z)-Pro-OH (16,17) using N-hydroxy-5-norbornene-2,3-dicarboximide (18) to give Z(OMe)-Lys(Z)-Pro-Arg(Mts)-OH which after trifluoroacetic acid treatment was condensed with Z(OMe)-Gly-OH by the p-nitrophenyl active ester procedure (19). All protecting groups were removed by 1 M fluoromethanesulfonic acid thioanisole (20). For the synthesis of [Glu]tuftsin, Z(OMe)-Lys(Z)-Pro-OH was condensed with H-Gln-OBzl using N-hydroxy-5-norbornene-2,3-dicarboximide (18) to give Z(OMe)-Lys(Z)-Pro-Gln-OBzl which after trifluoroacetic acid treatment was coupled with Z(OMe)-Thr-NHNH2 by the azide procedure (21) to give the protected tetrapeptide from which all protecting groups were removed by the same deprotection procedure as referenced above. For the synthesis of [Leu]tuftsin, Z(OMe)-Lys(Z)-Pro-OH was condensed with H-Leu-OH using N-hydroxy-5-norbornene-2,3-dicarboximide to give Z(OMe)-Lys(Z)-Pro-Leu-OH which after trifluoroacetic acid treatment was condensed with Z(OMe)-Thr-NHNH2 by the azide procedure to give the protected tetrapeptide from which all protecting groups were removed as described above. The crude products obtained were converted to the corresponding acid by Amberlite CG-4B (acetate form) treatment followed by gel filtration on a Sephadex G-10 column, and by ion exchange chromatography on carboxymethylcellulose. The purity of the final products was ascertained by thin layer chromatography and amino acid analysis. Tuftsin was synthesized as described previously (22).

**Tests for Endotoxin**—All substances used to assay biological activity were tested for the presence of endotoxin using the limulus amebocyte lysate test as described (23).

### RESULTS

**Proteolysis of CRP by PMNs**—Since native CRP has little or no effect on leukocyte migration or activation, experiments were conducted to study the proteolytic degradation of CRP and the potential activity of the digestion products. Proteolysis under a variety of conditions was evaluated by measuring the amounts of [35S]methionine-labeled CRP released and soluble in 10% trichloroacetic acid at measured time intervals. In the presence of whole PMNs or PMN lysates, the digestion of CRP was approximately 80% complete after 18 h regardless of whether the PMNs/CRP incubation was at pH 5.0 (Fig. 1) or at pH 7.4 (data not shown). Similar results were obtained using the PMNs lysates to digest CRP (data not shown).

**Chemotaxis of Monocytes and PMNs to CRP Digests**—CRP digestion mixtures were tested for their ability to induce chemotaxis of peripheral blood monocytes and PMNs as shown in Figs. 2 and 3, respectively. For both cell populations it is clear that only the solutions containing CRP which had been digested by the leukocytes stimulated cells to chemotax. Neither monocytes nor PMNs migrated to CRP which had been incubated in buffer at pH 5.0 for 18 h at 37 °C at any concentration tested above that observed for the buffer control (data not shown). Following neutralization, the supernatants from the leukocyte suspension at pH 5.0 or the leukocyte extract alone at pH 5.0 did not cause either cell type to...
migrate significantly above controls when added to the chemotaxis chambers.

A comparison of Figs. 2 with 3 shows that the PMNs were slightly more sensitive to the cleavage products than monocytes, since the molar concentration of digested CRP required for optimal chemotaxis of monocytes is $2.5 \times 10^{-7}$ M, whereas for PMNs the optimal concentration is $6 \times 10^{-7}$ M. The reason(s) for this is not clear.

Biologically Active Peptides in CRP—Because of the chemotactant activity revealed following digestion of CRP we next evaluated the native molecule for potential chemotactic peptides. Based on previous findings which implied a relationship between CRP and the $F_r$ portion of immunoglobulin (see "Discussion"), it became readily apparent that there are three peptides in the parent CRP molecule which closely resemble the potent immunomodulator peptide tuftsin, Thr-Lys-Pro-Arg, which is located in the $F_r$ fragment of human IgG (24). These peptides are evenly distributed throughout the CRP parent molecule (25, 26) and are shown enclosed in boxes in Fig. 4. By comparing the CRP peptides shown in Fig. 4 with tuftsin, Thr-Lys-Pro-Arg, it is obvious that the only difference is a single substituted amino acid at either the amino- or carboxyl-terminal amino acid residues of tuftsin. Thus, because of their similarities to tuftsin and because of the potent immunoregulatory importance of such CRP cleavage products in inflammation, a significant effort was committed to the synthesis of these peptides in parallel with tuftsin and the subsequent analysis of these peptides for biological activity.

Chemotaxis by Synthetic CRP Peptides—Both monocytes and PMNs were tested for their ability to chemotactically respond to the synthesized peptides over a broad dose-response curve and the synthetic peptides were found to have chemotactic activity comparable to that seen with tuftsin. Although the chemotactic activity of each peptide varied with different individuals' monocytes, the chemotactic response was dose-dependent and, maximal activity was frequently observed at approximately 10 ng/ml concentrations of the peptides (Fig. 5, A and B). PMN chemotactic responsiveness to the peptides was similar to that described for monocytes (data not shown).

Superoxide Production by Monocytes Treated with CRP Peptides—Although the digested CRP fragments could not be properly evaluated for their ability to induce superoxide production because the leukocyte extract alone also stimulated superoxide production by cultured monocytes, the synthetic peptides were evaluated and, as shown in Fig. 6, stimulated superoxide production by monocytes. Optimal activity was observed at 1 mg/ml of the peptides.

The kinetics of superoxide production by monocytes treated with the CRP-derived [Gly']tuftsin were compared to those induced by 1 $\mu$M fMLP and the data are shown in Fig. 7. The induction of superoxide by [Gly']tuftsin was found to parallel that of fMLP, being detectable within 60 min, plateauing between 2 and 4 h, and subsequently declining.

CRP Peptide-induced IL-1 Production by Monocytes Treated with CRP Peptides—In additional studies, the effect of CRP peptides on IL-1 production was determined. The results of IL-1 production induced by the CRP peptides and by tuftsin are given in Table I. A dose-dependent stimulation of monokine synthesis was evident for each of the peptides. Nonstimulated supernatants treated with the peptides just prior to assay in the IL-1 assay were inactive.

Tests for Endogenous Pyrogen—All samples were tested for the presence of endotoxin using the limulus amebocyte lysate...
test (23). Any samples showing values greater than 1.25 units by comparison to an IL-1 standard assigned 100 units/ml. Which CRP appears to be an acute phase reactant (27), the discovered in a wide variety of species such as the rabbit in 1930 (3). Based on a functional endotoxin units/ml, undiluted, were omitted from the study.

### DISCUSSION

The existence of CRP as an acute phase reactant in humans was first demonstrated in 1930 (3). Based on a functional definition, namely, the Ca$^{2+}$-dependent binding to phosphocholine, proteins closely related to human CRP have been discovered in a wide variety of species such as the rabbit in which CRP appears to be an acute phase reactant (27), the dogfish shark Mustelus canis (28), and the invertebrate Limulus polyphemus (29), in which high levels of CRP-like proteins appear to be constituents of normal blood.

Functionally, CRP appears to closely resemble the $F_c$ portion of immunoglobulin because, like the $F_c$ portion, CRP appears to mediate phagocytosis (5, 6) and complement fixation (7, 8). In Limulus, an invertebrate containing no immunoglobulin, and in the dogfish shark, a primitive animal containing very few immunoglobulins, CRP levels are quite high (28, 29). Interestingly, dogfish CRP is a disulfide-linked dimer with reduced and unreduced molecular weights quite similar to $F_c$ (28). Thus, CRP may be the progenitor of the immunoglobulin $F_c$.

Tuftsin, Thr-Lys-Pro-Arg, a peptide found in the CH2 domain of the $F_c$ segment of the immunoglobulin heavy chain, residues 289–292 (30), is well established to be a natural activator of phagocytic cells (24). Based on partial sequence data, Osmand et al. (34), described the presence of parts of the tuftsin molecule in human CRP as far back as 1977. As we have clearly demonstrated in this paper, human CRP contains three peptides closely resembling tuftsin and each of the CRP-derived synthetic peptides are quite similar to tuftsin in their ability to cause monocytes and PMNs to chemotax, produce superoxide, and induce monocytes to produce IL-1.

The possibility that some or all of the tuftsin-like peptides from CRP may be formed is substantiated by the demonstration (Fig. 1) that PMNs are capable of digesting human CRP and that the resulting components of the digestion mixture have chemotactic activity toward PMNs and monocytes. Why PMNs were slightly more responsive than monocytes to the CRP digestion products is not known. Perhaps future studies characterizing the CRP digestion products and comparing the receptors for the peptides on PMNs versus monocytes will be informative and may lead to an explanation as to why PMNs are first to arrive at sites of tissue damage in vivo.

Using the computer program described by Saroff and Pretorius (31) we have searched the available data bases for human proteins which may contain peptides resembling tuftsin or tuftsin-like CRP peptides. The only limitation placed on our search was that the searched for peptides must have three out of four of the amino acids found in tuftsin with allowable substitutions only at the one and four positions. In contrast to our expectations, very few human proteins with known sequences contain peptides meeting these requirements: human glutamate dehydrogenase (Thr-Lys-Pro-Gly), human mitochondrial hypothetical protein Z (Thr-Lys-Pro-Thr), human $\lambda$ light chain subgroup V (Thr-Lys-Pro-Ser), and human thyrotropin (Thr-Lys-Pro-Gln). Thr-Lys-Pro-Gln is also found in CRP and, as we have shown, has biological activity. We have also found Gly-Lys-Pro-Lys in human haptoglobin and this may be active like CRPs [Gly'ltuftsin. Like CRP, haptoglobin is considered to be an acute phase protein (32).

Thus, although Monte Carlo analyses of the peptides described in this paper may predict that these peptides should occur often in humans, based on the available sequences for human proteins, this does not seem to be true. In fact, that human CRP contains three unique peptides with biological activity suggests that the formation of the peptides in vivo would be a unique function involving CRP. This would support the hypothesis that “the specific biologic functions of some proteins may reside in their unique subsequences, with their functions being expressed upon the degradation of the parent protein” (31).

Physiologically, the question arises as to whether the concentrations of the CRP peptides for maximum biological activity in vitro may be found in vivo. As we have shown, the optimal activity for superoxide production by monocytes treated with the CRP peptides is 1 $\mu$g/ml. On a molar concentration basis, this is approximately 2 $\mu$M. For the parent CRP molecule, 2 $\mu$M is equal to 50 $\mu$g/ml, assuming a subunit molecular weight for CRP of approximately 25,000. During the acute phase response, CRP levels may reach concentrations in excess of 50 $\mu$g/ml providing adequate substrate for the formation of physiologically active levels of peptides in vivo.

Optimal activity for IL-1 production was found to be higher although monocytes were clearly stimulated to produce IL-1 by 100 ng/ml peptide. CRP may have to be concentrated at sites of inflammation beyond the serum levels such as at a site where exposed chromatin (4) could act as an affinity matrix to accumulate circulating CRP, favoring the regional formation of high levels of peptides following the solubilization (8) and possible digestion of chromatin, and possibly CRP, by digestive enzymes. This can be conceptualized by the hypothetical “CRP cycle” in which CRP bound to exposed

### TABLE I

| Peptide concentration | Interleukin 1 |
|-----------------------|--------------|
| $\mu$g/ml             |                |
| Tuftsin*   | [Gly]$^{a}$Tuftsin | [Lac]$^{b}$Tuftsin | [Glu]$^{c}$Tuftsin |
| 10        | 275           | 123            | 60              | 76           |
| 1.0       | 53            | 57             | 30              | 53           |
| 0.1       | 28            | 34             | 20              | 12           |

$^{a}1 \times 10^6$ adherent monocytes/ml were cultured with the indicated concentrations of peptides for 48 h and the supernatants harvested.

$^{b}$Three-fold dilutions of the supernatants were assayed for IL-1 activity in the thymocyte proliferation assay. Data transformed into units by comparison to an IL-1 standard assigned 100 units/ml.
chromatin acts as an initiator of a cascade of events resulting in the stimulation of hepatocytes to produce more CRP (35). The rapid return of CRP levels in the blood to normal following the acute phase response suggests that CRP may be consumed during the completion of its biological function.

In conclusion, based on the in vitro evidence presented here, CRP may function as an immunomodulator in addition to functioning as a scavenger for chromatin (4, 8) and/or platelet-activating factor (33). The “immunomodulator” role for CRP may present itself during or after the “scavenger” role as in the CRP cycle (35), or may be independent and act to give the immune system a boost during inflammation.

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