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Neutrophil lysosomal degradation of human CRP: CRP-derived peptides modulate neutrophil function

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SUMMARY

Hydrolysis of human C-reactive protein (CRP) at pH 4·5 and pH 7·4 with neutrophil-derived lysosomal enzymes yielded 10% trichloroacetic acid soluble peptides ($M_r < 14{,}000$). These peptides inhibited neutrophil superoxide production, chemotaxis, degranulation and phagocytosis at 2 μg/ml. This inhibition was not observed with native CRP or intermediate peptides ($M_r > 14{,}000$). CRP peptides ($M_r < 14{,}000$) also caused a dose-related inhibition of Quin-2 fluorescence indicating interference with intracellular calcium movements during cell activation. These results point to a potential regulatory role for CRP-derived degradation products on neutrophils during inflammation.

Keywords C-reactive protein neutrophil function lysosomal enzymes peptides

INTRODUCTION

C-reactive protein (CRP) is normally a trace plasma protein but during the acute phase response, macrophage-derived monokines effect its rapid synthesis and release with concentrations increasing to 100–500 μg/ml (Goldman & Liu, 1987). The precise biological role of CRP has not been fully elucidated but suggestions have been made that CRP aids the clearance of foreign pathogens and debris of necrotic tissue, in particular chromatin (Robey *et al.*, 1984). However, with respect to chromatin this seems less likely as serum amyloid P component has recently been shown to be the major chromatin binding protein in the plasma (Pepys & Butler, 1987). Inspection of the amino acid sequence of CRP reveals the existence of regions similar in structure to the tuftsin peptide (Thr-Lys-Pro-Arg) found in the CH2 domain of the Fc segment of IgG (Woo, Kronenberg & Whitehead, 1985). Tuftsin is produced *in vivo* by cleavage by IgG by a neutrophil membrane derived enzyme and has known pro-inflammatory potency (Najjar, 1980). It has been suggested that on degradation of CRP bioactive peptides might be released which could modulate the inflammatory process. This hypothesis has been investigated using various synthetic peptides corresponding to the regions in the CRP molecule containing the tuftsin related sequences (Robey *et al.*, 1987; Buchta *et al.*, 1987a). Conflicting results obtained with respect to biological effects of the synthesised peptides is probably due to the slight differences in amino acid sequence of the peptides used as it has been shown that even one amino acid alteration in a peptide sequence can totally reverse the bioactivity (Fridkin & Gottlieb, 1981). Neutrophil leukocytosis is often concomitant with the acute phase response. If CRP is indeed involved in ligand clearance by phagocytic cells then lysosomal enzyme degradation of CRP is likely during these events. We have studied the degradation of CRP by lysosomal enzymes at neutral and acid pH and the functional effects of the cleaved products generated during digestion on neutrophil function was investigated. Marked suppression of superoxide production, chemotaxis, phagocytosis and degranulation was found using CRP-peptides of $M_r < 14{,}000$. This functional inhibition was associated with altered Ca^{2+} influx.

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MATERIALS AND METHODS

Unless indicated otherwise all chemicals and reagents were obtained from the Sigma Chemical Co. (St Louis, Mo, USA).

CRP preparation and iodination

CRP was isolated as described by De Beer & Pepys (1982) and iodinated using the iodogen technique (Fraker & Speck, 1978). The specific activity of CRP was 0·4 μCi/μg protein ($2{\cdot}5 \times 10^5$ ct/min/μg protein). Purity was confirmed on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and autoradiography (Kodak XAR).

Preparation of neutrophils from human blood

Purified human blood neutrophils (Shephard *et al.*, 1986) were washed and resuspended in 0·15 M phosphate-buffered saline

(PBS, pH 7·4) for the preparation of lysosomal enzymes for CRP degradation and Hanks' balanced salt solution for assays of neutrophil functions.

Preparation of lysosomal enzymes for proteolysis of CRP
Lysosomal enzymes were obtained by the release of granule constituents (degranulation) from neutrophils (40×10^6/ml) activated (15 min, 37°C) with 1 μM of the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) and 5 μg/ml cytochalasin B in a final volume of 1 ml PBS (Bentwood & Henson, 1980). Cell lysates prepared by freeze-thawing neutrophils (40×10^6/ml) three times were also used as a source of lysosomal enzyme. The lysosomal enzyme-rich cell-free supernatant was incubated immediately after preparation with CRP. For standardization of the lysosomal enzyme batches, the supernatants were assayed for myeloperoxidase and lysozyme using colourimetric (Paul, Selvaraj & Sbarra, 1978) and turbidometric (Parry, Chandan & Shahani, 1965) methods respectively.

Proteolysis of CRP by lysosomal enzymes
To study CRP degradation reaction mixtures contained 200 μg ^{125}I-labelled CRP, 500 μl lysosomal enzyme prepared from 20×10^6 degranulated cells (200 μg protein) in either 0·1 M sodium acetate, pH 4·5 or 0·02 M Tris–HCl, pH 7·4 in a final volume of 1000 μl. Enzyme of ^{125}I-labelled CRP was omitted in control degradations. When required either pepstatin (100 μg/ml final) leupeptin (100 μg/ml final), phenylmethylsulphonic acid (PMSF, 2 mM final) or diisopropylfluorophosphate (DFP, 1 mM final) was added to the reaction. Reactions were done at 37°C and stopped by the addition of 50% (w/v) tricholoroacetic acid (TCA) to a final concentration of 10% (w/v) to duplicate samples of the total reaction mixture. For SDS-PAGE (Laemmli, 1970) a fraction of the total reaction mixture was added to an equal volume of 4% SDS sample buffer containing 10% mercaptoethanol and boiled for 3 min. Where necessary the remainder of the reaction mixture was microfuged (2 min 4°C) and SDS sample buffer was added to the supernatant and pellet. An amount corresponding to 2 μg starting CRP was layered in each track and resolved by 12% SDS-PAGE followed by autoradiography (Kodak XAR).

Preparation of CRP-derived peptides
In order to prepare CRP peptides of $M_r < 14{,}000$ for testing in assays of neutrophil function CRP was proteolysed for 18 h, 37°C. Reaction mixtures contained 3 mg CRP, enzyme from 150×10^6 lysed cells (10 mg protein) and either 0·1 M sodium acetate, pH 4·5 or 0·02 M Tris-HCl, pH 7·4 in a final volume of 10 ml. Enzyme controls omitted CRP. Reactions were terminated by the addition of 50% TCA (w/v) to a final dilution of 10% (w/v). the precipitated proteins were removed by centrifugation and the TCA in the supernatant was extracted four times with 4 volumes of diethyl ether to obtain the soluble peptides of $M_r < 14{,}000$ at neutral pH. The aqueous medium was freeze dried and peptides and enzyme residue in the control tube redissolved in sterile distilled water (10% of original volume). The ability of the CRP peptides of $M_r > 14{,}000$ to modulate neutrophil function was also tested. For this purpose unlabelled CRP was degraded with lysosomal enzyme and the insoluble peptides ($M_r > 14{,}000$) produced during proteolysis at pH 4·5 were collected by centrifugation, washed with sterile distilled water, dried and redissolved in dimethylsulphoxide (DMSO). The final concentration of the CRP-derived peptides was calculated from a control proteolysis reaction using ^{125}I-labelled CRP.

Assays of neutrophil functions
For these assays peptides derived from unlabelled CRP were tested for their ability to modulate neutrophil function.

Superoxide assay. This was measured using lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) as previously described (Dahlgren, Aniansson & Magnusson, 1985) using an LKB Wallac 125/luminometer (Turku, Finland) in the presence of the following activators, at the final optimal concentrations shown in parenthesis: (a) phorbol 12-myristate 13-acetate (PMA, 10 ng/ml); (b) opsonized zymosan (1 mg/ml); (c) FMLP (1 μM); (d) the calcium ionophore A23187 (1 μM); and (e) arachidonic acid (20 μM). LECL readings were integrated for 5 s intervals and recorded as millivolts (mV) s. Superoxide scavenging by the CRP-derived peptides ($M_r < 14{,}000$) was investigated using an xanthine/xanthine oxidase superoxide generating system. Ten micrograms of the peptides were incubated with 0·2 mM xanthine, 100 milliunits of xanthine oxidase and 0·2 mM lucigenin in a final volume of 1 ml PBS and LECL measured as above.

Assay of neutrophil migration. This was measured using modified Boyden Chambers (Wilkinson, 1982; Shephard *et al.*, 1986) with 5 μm pore size nitrocellulose filters (Sartorius membrane filter, Gottingen, Federal Republic of Germany). Neutrophils were pre-incubated for 15 min at 37°C with varying concentrations of the CRP-derived peptides, appropriate enzyme controls or a fixed concentration of 50 μg/ml of CRP. The neutropil suspensions (0·2 ml containing 10^6 neutrophils) were then introduced into the upper compartments of the Boyden chambers and 1 ml of leucoattractant was placed in the lower compartment together with CRP-derived peptides at concentrations equal to those present in the upper chamber. Two leuco-attractants were used, (i) FMLP at an optimal chemotactic concentration of 10 nM, and (ii) fresh autologous endotoxin activated serum. These results are expressed as neutrophils per microscope high-powered field.

Assay of the leucotactic potential of CRP-derived peptides To investigate possible leucoattractant inactivating activity, the CRP-peptides, at a fixed concentration of 10 μg/ml, were incubated with 10 μM FMLP for 60 min at 37°C and the FMLP diluted to 10 nM and tested for leucotactic activity for neutrophils. Neutrophil migration and orientation in positive gradients of the CRP-derived peptides (0·01–10 μg/ml) were measured using the Boyden chamber method and the orientation assay of Zigmond (1977). The orientation assay was performed as previously described (Zigmond, 1977; Shephard *et al.*, 1986). Negative and positive (0·1–10 nM FMLP gradient) control systems were included. The effects of the CRP-peptides on neutrophil orientation in the positive gradient of FMLP were also investigated. A minimum of 100 polarized neutrophils were scored. These results are expressed as the percentage of neutrophils oriented towards the CRP-peptides or strongest concentration (10 nM) of FMLP.

Phagocytosis assays. Neutrophils (5×10^6) were co-incubated for 15 min at 37°C with the CRP-peptides followed by the addition of pre-opsonized *Candida albicans* (2×10^7) (laboratory isolate) or *Staphylococcus aureus* (ATCC 25923, 5×10^7). The

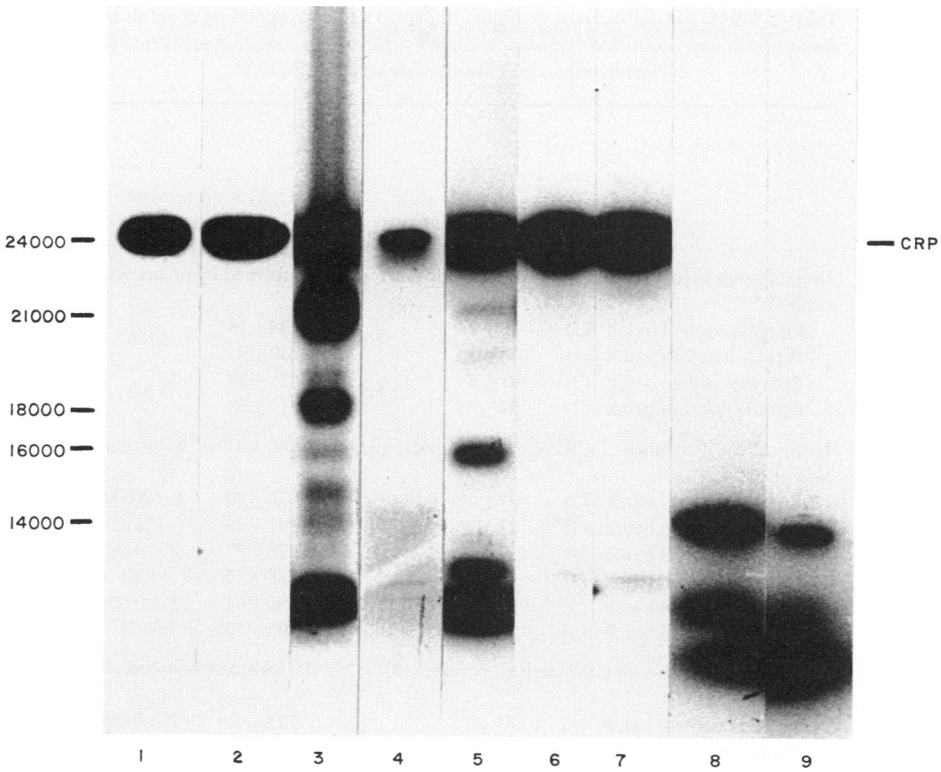

Fig. 1. Autoradiographic analysis of the ^{125}I-labelled CRP cleavage products produced at 18 h by lysosomal enzymes separated on 12% SDS-PAGE. Track 1, CRP standard; Track 2, CRP precipitate in the absence of enzyme at pH 4·5; Track 3 and 4, CRP cleavage products generated by acid hydrolysis in the total reaction suspension and supernatant respectively; Track 5, CRP cleavage at pH 7·4; Track 6 and 7, inhibition of proteolysis at pH 7·4 with PMSF and DFP respectively; Track 8 and 9, Nature of CRP-derived peptides in the 10% TCA supernatant concentrated 10-fold by lyophilization as produced by hydrolysis at pH 4·5 and 7·4 respectively.

tubes were rotated for 25 min at 37°C and complement-mediated phagocytosis of *Candida albicans* was measured by light microscopy, and antibody-mediated phagocytosis of *Staphylococcus aureus* by fluorescence microscopy (Pruzanski, Saita & Nitzan, 1983).

Degranulation assays. Neutrophils (5×10^6) were co-incubated with the CRP-peptides for 15 min at 37°C then activated with 1 μM FMLP and 5 μg/ml of cytochalasin B. The neutrophils were removed by centrifugation and the supernatants assayed for myeloperoxidase and lysozyme as described earlier. Total levels of both enzymes were determined using neutrophil sonicates.

Assay of intracellular calcium. This was measured by fluorescence spectrophotometry as described by Tsien, Pozzan & Rink (1982) using Quin 2 (Calbiochem, La Jolla, Calif. USA) FMLP (1 μM) -activated neutrophils.

RESULTS

Nature of ^{125}I-labelled CRP degradation products

Degradation of ^{125}I-labelled CRP was monitored by SDS-PAGE as well as by the production of 10% TCA soluble products which was linear up to 18 h. SDS-PAGE analysis of the reaction mixture indicated a time-dependent decrease in the concentration of native CRP accompanied by an increase in the production of cleavage products optimal at pH 7·4 and pH 4·5. At both pH values, all the Coomassie Blue stained protein bands proved to be radioactive. No Coomassie stained protein bands could be detected on SDS-PAGE in a control track loaded with enzyme alone (data not shown).

Incubation of ^{125}I-labelled CRP (18 h, pH 4·5 37°C) in the absence of enzyme resulted in the formation of a precipitate (containing approximately 90% of the total radioactivity) which on SDS-PAGE analysis revealed a single band at $M_r = 24{,}000$ (Fig. 1, track 2). The radioactivity remaining in the supernatant proved to be non-degraded CRP (data not shown). The nature of the ^{125}I-labelled CRP cleavage products in the reaction suspension at 18 h generated by lysosomal acid hydrolysis is shown in Fig. 1, track 3. Major degradation products were observed at $M_r = 21{,}000$ and $M_r = 18{,}000$ (Fig. 1, track 3). The precipitate formed during the reaction proved to contain uncleaved ^{125}I-labelled CRP and the hydrolysis products of $M_r > 14{,}000$ (data not shown) while the supernatant contained the products of $M_r < 14{,}000$, and some non-precipitated ^{125}I-labelled CRP (Fig. 1, track 4). Quantification of the radioactivity in the major bands of a representative degradation experiment showed that at 18 h approximately 10%, 30% and 10% of the loaded radioactivity could be accounted for in the CRP band at $M_r = 24{,}000$ and the degradation products at $M_r = 21{,}000$ and $M_r = 18{,}000$ respectively. Degradation for 18 h resulted in approximately 40% of the radioactivity being 10% TCA soluble. This degradation was not inhibitable by pepstatin or leupeptin.

Less than 10% of the radioactivity precipitated during lysosomal enzyme degradation of CRP for 18 h at pH 7·4 (18 h). The nature of the ^{125}I-labelled CRP cleavage products in the

Table 1. Effects of CRP-derived peptides (10 μg/ml) and corresponding controls on spontaneous and opsonized zymosan activated superoxide generation measured by lucigenin enhanced chemiluminescence (LECL).

	LECL			
	Spontaneous		Opsonized zymosan activated	
Two different batches of CRP-derived peptides ($M_r > 14{,}000$) formed by proteolysis at pH 4·5				
Enzyme control batch (1)	18±3	NS	708±18*	NS
Peptide fraction batch (1)	18±5		740±9	
Enzyme control batch (2)	91±3	NS	576±18	NS
Peptide fraction batch (2)	84±5		612±31	
Three different batches of CRP-derived peptides ($M_r < 14{,}000$) formed by proteolysis at pH 4·5				
Enzyme control batch (3)	37±18	NS	670±70	$P<0{\cdot}0005$
Peptide fraction batch (3)	22±7		191±26	(71)†
Enzyme control batch (4)	37±2	NS	522±27	$P<0{\cdot}005$
Peptide fraction batch (4)	30±2		385±15	(26)
Enzyme control batch (5)	30±3	$P<0{\cdot}005$	792±61	$P<0{\cdot}005$
Peptide fraction batch (5)	13±2		303±13	(62)
Two different batches of CRP-derived peptides ($M_r < 14{,}000$) formed by proteolysis at pH 7·4				
Enzyme control batch (6)	41±7	NS	775±5	$P<0{\cdot}005$
Peptide fraction batch (6)	26±5		437±4	(44)
Enzyme control batch (7)	31±3	NS	779±32	$P<0{\cdot}005$
Peptide fraction batch (7)	29±10		434±21	(44)

Mean values ± s.e.m. as mV. sec of four determinations.

* Peak LECL values observed approximately 10 min after addition of opsonized zymosan.

† ( ) = percentage inhibition.

reaction suspension at 18 h is shown in Fig. 1, track 5. Of the radioactivity loaded, approximately 40% could be accounted for in the CRP band at $M_r = 24{,}000$, while 12% was accounted for in the major degradation band at $M_r = 16{,}000$. Degradation for 18 h resulted in 40% of the radioactivity being 10% TCA-soluble. Four identical degradation experiments gave similar results. The cleavage product of $M_r = 16{,}000$ and the 10% TCA soluble products produced during degradation at pH 7·4 were inhibited by PMSF and DFP (Fig. 1, tracks 6 and 7). The presence of 1·5 mM $CaCl_2$ slowed the rate of degradation without altering the nature of the intermediates, to yield 50% less TCA soluble products at 18 h (data not shown).

Nature of CRP-derived peptides in the 10% TCA soluble supernatant

The 10% TCA soluble peptides obtained by precipitation of the proteins after degrading unlabelled CRP (18 h, pH 4·5 or pH 7·4) were used as the source of soluble peptides in assays of neutrophil function. Analysis of the 10% TCA supernatant of an identical reaction containing ^{125}I-labelled CRP indicated that the peptides were smaller than 14,000 molecular weight (Fig. 1, tracks 8 and 9. Further analysis on a 15–20% gradient SDS-PAGE gel using low molecular weight markers showed the presence of peptides at $M_r = 13{,}000$, $M_r = 11{,}000$, $M_r = 9{,}200$, $M_r = 6{,}000$, $M_r = 5{,}000$ and $M_r = 4{,}000$ in addition to peptides of $M_r < 2{,}500$ which could not be separated with this system (data not shown). Depending on the pH of the peptide preparation the intensities of these bands were different.

Effect of CRP-derived peptides on neutrophil function

The results are expressed as the mean values with standard errors (s.e.m.).

Effect of native CRP (50 μg/ml) and CRP-derived peptides (10 μg/ml) on the spontaneous and opsonized-zymosan-activated neutrophil LECL responses. CRP did not influence superoxide generation by unstimulated and zymosan-activated neutrophils. The values for unstimulated neutrophils were 19±1 mV.s and 36±5 mV.s for control and CRP-treated cells respectively. The corresponding values for opsonized zymosan-activated neutrophils were 526±22 mV.s and 600±15 mV.s respectively. Likewise the spontaneous and zymosan-activated LECL responses of neutrophils treated with 10 μg/ml of DMSO solubilized CRP-derived peptides of $M_r > 14{,}000$ produced by degradation at pH 4·5 did not differ from control values (Table 1). However CRP-peptides of $M_r < 14{,}000$ produced by proteolysis of CRP at pH 4·5 or pH 7·4 caused marked inhibition of opsonized-zymosan-activated LECL responses (Table 1). This was consistently observed with different preparations of these CRP-derived peptides (Table 1). The effects on peak LECL, responses, measured 10 min after stimulus addition, are shown in Table 1. The kinetics of opsonized zymosan-activated LECL

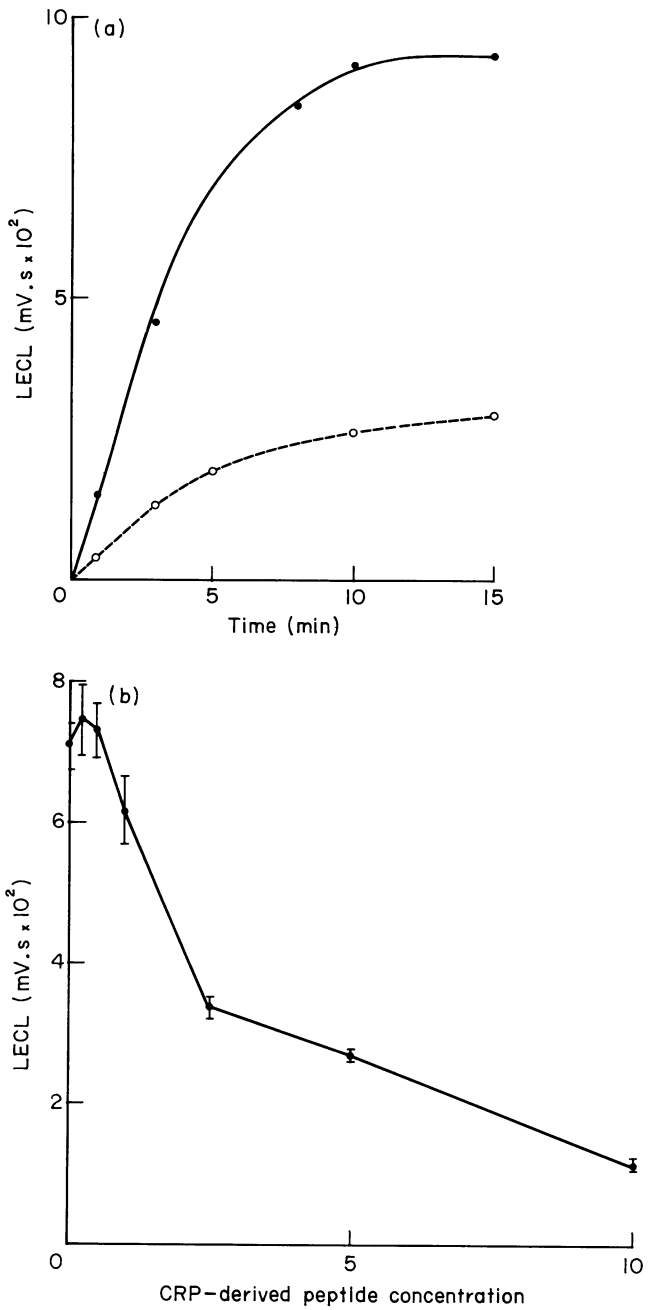

Fig. 2. Opsonized zymosan-activated LECL responses of neutrophils co-incubated with CRP derived peptides ($M_r < 14,000$). (a) Effects of 10 μg/ml CRP-derived peptides on the LECL response of neutrophil activated with opsonized zymosan (●). Enzyme control; (○) CRP-derived peptides. Background unstimulated value = 44 mV.s. (b) Effects of varying concentrations of CRP-derived peptides (0·25–10 μg/ml) on peak LECL responses of neutrophils activated with opsonized zymosan. Each point is the mean s.e.m. peak values 10 min after stimulus addition. Background unstimulated value = 62 mV.s.

responses of neutrophils coincubated with 10 μg/ml of CRP-derived peptides are shown in Fig. 2a. The results of a dose-response experiment with CRP-derived peptides (0·25–10 μg/ml) are shown in Fig. 2b. Inhibition of LECL responses was observed with concentrations of 1 μg/ml and greater of the CRP-derived peptides. For all further studies CRP-derived peptides ($M_r < 14,000$) were generated by acid hydrolysis.

Effects of CRP-derived peptides ($M_r < 14,000$, 10 μg/ml) and enzyme controls on the peak LECL responses of neutrophils activated with PMA (10 ng/ml), calcium ionophore, (1 μM), FMLP (1 μM) and arachidonic acid (20 μM).

These results are shown in Table 2. The peak LECL responses in neutrophils activated with PMA, calcium ionophore, FMLP and arachidonic acid were observed at approximately 15 min, 7 min, 1 min and 5 min respectively after addition of the stimulus. In a single dose response experiment the concentrations of the CRP-derived peptides which caused 50% inhibition of the LECL responses in neutrophils activated with PMA, calcium ionophore, FMLP, arachidonate and opsonized zymosan were 8·6 μg/ml, 2 μg/ml, 2·1 μg/ml, 2·1 μg/ml and 2·5 μg/ml respectively.

Superoxide scavenging activity of CRP-derived peptides. Relative to the enzyme control, the CRP-peptides (10 μg/ml) did not interfere with superoxide generation by a cell-free xanthine/xanthine oxidase system. The peak LECL values (reached after 6 min) were 271 ± 7 mV.s and 294 ± 7 mV.s for the control and CRP peptide systems respectively.

Neutrophil orientation and migration. No chemotactic activity (using the orientation and microphore filter assays) nor affect on orientation of neutrophils in an FMLP gradient was found using native CRP or CRP-derived peptides of $M_r > 14,000$ (data not shown). However the low molecular weight peptide fraction ($M_r < 14,000$) consistently inhibited neutrophil random and leucoattractant-induced migration. These results are shown in Table 3. The CRP-peptides (10 μg/ml) did not inactivate FMLP or influence the cells morphologically (data not shown).

Phagocytosis. The mean percentages (± s.e.m.) of *Candida albicans* phagocytosed by control neutrophils and neutrophils treated with 5 and 10 μg/ml CRP-peptides ($M_r < 14,000$) were 99 ± 1, 95 ± 1 ($P < 0·025$) and 71 ± 1 ($P < 0·005$) respectively. With the fluorescence assay and *Staphylococcus aureus* as the test microorganism the CRP-peptides at 10 μg/ml inhibited phagocytosis by 22% ($P < 0·025$; 10·4 ± 0·8 bacteria versus 8·1 ± 0·6 bacteria per neutrophil).

Neutrophil degranulation. CRP peptides ($M_r < 14,000$ at concentrations of 1 μg/ml and 10 μg/ml caused 15% ($P < 0·005$) and 16% ($P < 0·005$) inhibition respectively of myeloperoxidase release by FMLP/cytochalasin B-activated neutrophils. The corresponding values for inhibition of lysozyme release were 14% ($P < 0·025$) and 25% ($P < 0·005$).

Quin-2 fluorescence. Neither the CRP-peptides of $M_r > 14,000$, (10 μg/ml) nor native CRP affected Quin-2 fluorescence. However CRP-peptides of $M_r < 14,000$ caused a dose-related inhibition of Quin-2 fluorescence. The mean percentage inhibition for concentrations of 1 μg/ml and 10 μg/ml CRP-derived peptides were 38% ($P < 0·025$) and 89% ($P < 0·005$) respectively.

DISCUSSION

The CRP molecule contains three tuftsin-like peptides, i.e. Thr-Lys-Pro-Leu (residues 27–30), Gly-Lys-Pro-Arg (residues 113–116), and Thr-Lys-Pro-Gln (residues 200–203) (Woo *et al.*, 1985). This has lead to speculation that upon degradation bioactive peptides are liberated capable of influencing phagocy-

Table 2. Effects of CRP-derived peptides ($M_r < 14{,}000$) (10 μg/ml) on superoxide generation by neutrophils activated with PMA, calcium ionophore, FMLP and arachidonic acid

Stimulus	Peak LECL responses with Enzyme control	10 μg/ml CRP-peptides	
PMA (10 ng/ml)	1126±49	830±51 ($P<0.025$)	(26)
Calcium ionophore (1 μM)	179±45	99±29 ($P<0.005$)	(45)
FMLP (1 μM)	83±17	20±4 ($P<0.025$)	(76)
Arachidonate (20 μM)	1189±328	99±29 ($P<0.025$)	(92)

( ) = Percentage inhibition.

Results as mean values (mV. s) ± s.e.m. of four determinations.

The unstimulated background values for control and CRP-peptide treated neutrophils were 15±2 and 11±1 mV. s respectively.

Table 3. Effects of varying concentrations of CRP-derived peptides ($M_r < 14{,}000$) on neutrophil random movement and on migration to the leuco-attractants FMLP and EAS

PMNL co-incubated with	Random migration	Migration to EAS (10%)	Migration to FMLP 10 nM
Medium only	26±8	141±5	121±4
50 μg/ml native CRP	32±5	159±2	127±8
10 μg/ml enzyme control	24±2	146±3	124±7
0·15 μg/ml CRP-derived peptides	30±2	130±5	117±7
0·3 μg/ml CRP-derived peptides	24±3	146±3	117±7
0·6 μg/ml CRP-derived peptides	14±1(46)	113±4(20)†	73±6(40)*
2·5 μg/ml CRP-derived peptides	6±1(77)	46±2(67)*	46±2(62)*
5 μg/ml CRP-derived peptides	1±0·4(96)	6±1(96)*	16±5(87)*
10 μg/ml CRP-derived peptides	0	0	0

† $P<0.005$; † $P<0.01$; ‡ $P<0.05$.

Results as mean value ± s.e.m. of four determinations as neutrophils per microscope high-powered field.

( ) = Mean percentage inhibition.

tic function. In our experiments, uncleaved CRP as previously reported (Shephard *et al.*, 1986; Zeller *et al.*, 1986) did not affect neutrophil function. This is in contrast to other published data where CRP *per se* has been found to inhibit the function of activated neutrophils (Buchta *et al.*, 1987b). These differences in the ability of native CRP to modulate the biological activity of neutrophils could be a function of the concentration of CRP cleavage products inherently present in the preparation of CRP and only visible on silver staining following SDS-PAGE (Fiedel & Gewurz, 1986). This proposal arises from the finding that CRP preparations containing low molecular weight CRP subunit fragments inhibit platelet activity (Fiedel *et al.*, 1982). Subsequently pronase digestion of CRP confirmed that only the CRP-derived fragments of $M_r = 14{,}000$–$8{,}000$ were responsible for the inhibition of platelet aggregation (Fiedel & Gewurz, 1986). These studies suggest that an investigation into the affect of biologically derived CRP-peptides on the classical function of neutrophils is warranted. We have confirmed that CRP can be proteolytically cleaved by neutrophil lysosomal enzymes at pH 4·5 or pH 7·4 (Robey *et al.*, 1987). The fact that neither leupeptin nor pepstatin inhibited degradation at pH 4·5 indicates that cathepsin B and cathepsin D are not involved. The marked inhibition of degradation of PMSF and DFP suggests that the enzymes involved at pH 7·4 are serine proteases. Only the CRP-derived peptides of $M_r < 14{,}000$ inhibited the classical biological function of stimulated neutrophils; inhibition of superoxide production, chemotaxis, degranulation and phagocytosis. These peptides appear to act by suppressing increases in intracellular calcium levels which occurs during activation, rather than by the scavenging of radicals. The relative resistance

of superoxide production by PMA-stimulated neutrophils to inhibition by CRP-derived peptides supports this conclusion. Peptides of various lengths containing the tuftsin-related sequences from the primary sequence of human CRP have been synthesized and both abrogation and enhancement of neutrophil function have been shown depending on the variation in the sequences synthesized (Robey *et al.*, 1987; Buchta *et al.*, 1987a). Tetrapeptides corresponding to the tuftsin regions enhanced chemotaxis, phagocytosis and superoxide production by phagocytic cells (Robey *et al.*, 1987) Peptides corresponding to residues 181–187 (Thr-Lys-Pro at the N-terminus), residues 51–58 and residues 37–58 (Thr-Lys-Arg at the C-terminus) inhibited neutrophil superoxide production, degranulation, chemotaxis and phagocytosis (Buchta *et al.*, 1987a). However no effect on neutrophil function could be obtained with a 15 amino acid synthetic peptide (residues 173–187) containing Thr-Lys-Pro at positions 9–11 (Buchta *et al.*, 1987a). Since the biological activity of our peptides, derived by lysosomal enzyme degradation of CRP, corresponds to the biological activity of the synthetic peptides of residues 181–187, 51–58 and 37–58 prepared by Buchta *et al.* (1987a) it is possible that peptides produced biologically from CRP have similar C-terminal or N-terminal residues. Further fractionation followed by amino acid sequencing is required to ascertain the exact structure of the bioactive peptides.

It can be concluded that in the event of CRP being intralysosomally processed, soluble peptides can be produced which exhibit marked suppression of neutrophil superoxide production, chemotaxis, degranulation and phagocytosis. Cell biological studies of CRP degradation by intact neutrophils will be essential to confirm the biological significance of these findings.

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