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# Effect of partially modified retro-inverso analogues derived from C-reactive protein on the induction of nitric oxide synthesis in peritoneal macrophages

F. Arcoleo, S. Milano, P. D'Agostino, G. Misiano, *S. Cappelletti, †G. Gromo, *F. Marcucci, *F. Leoni & [1]E. Cillari

Institute of General Pathology, University of Palermo, Faculty of Medicine, Corso Tukory, 211, 90134 Palermo; *Italfarmaco Research Centre, via Dei Lavoratori, 54, 20092 Cinisello Balsamo (MI), Italy and †Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285, U.S.A.

**1** The ability of three modified tetrapeptides, representing fragments of the C-reactive protein (CRP) sequence and stabilized in the first peptide bond by retro-inverso modification, to affect the secretion of nitric oxide (NO) was studied in macrophages of BALB/c mice.

**2** These tetrapeptides, resembling the aminoacid sequence of tuftsin (CRP I, H-gThr-(R,S)mLys-Pro-Leu-OH, ITF 1192; CRP II, H-gGly-(R, S)mLys-Pro-Arg-OH, ITF 1127; CRP III, H-gThr-(R,S)mLys-Pro-Gln-OH, ITF 1193), were able to induce NO synthesis by peritoneal macrophages in a dose-dependent manner; the most stimulating dose was 1000 ng $ml^{-1}$ for CRP II and 100 ng $ml^{-1}$ for CRP I and CRP III. NO synthesis was not strictly dependent on lipopolysaccharide (LPS) activation.

**3** The enhanced effect of retro-inverso CRP-related analogues on the expression of iNOS (inducible NO synthase) was confirmed by higher levels of iNOS activity in the cytosol and by the increase in iNOS protein, as evaluated by Western blot analysis, in macrophages stimulated by CPR compared with untreated ones.

**4** The production of NO by retro-inverso CRP-peptide analogues was significantly inhibited by dexamethasone (20 $\mu$M), $N^G$-monomethyl-L-arginine (L-NMMA) (500 $\mu$M) and pyrrolidine dithiocarbamate (PDTC) (100 $\mu$M).

**5** Retro-inverso CRP-peptide analogues stimulated macrophages to produce high levels of interleukin-1 (IL-1) and tumour necrosis factor-$\alpha$ (TNF-$\alpha$) in the presence of LPS.

**6** Retro-inverso CRP-peptide analogues stimulated NO synthesis by the enhancement of endogenously produced IL-1 and TNF-$\alpha$, as the treatment of peritoneal macrophages with LPS in the presence of neutralizing anti-IL-1 and anti-TNF monoclonal antibodies (mAbs) reduced retro-inverso analogue-induced NO secretion. Data indicate a predominant role for IL-1$\alpha$ in the induction of NO secretion by retro-inverso analogues.

**7** These results suggest that retro-inverso CRP derived analogues act as costimulators of NO and cytokine synthesis in macrophages. The mechanisms by which they cause iNOS induction appear to be strongly dependent on the activation of nuclear factor-$\kappa$B (NF-$\kappa$B).

**Keywords:** Nitric oxide; nitric oxide synthase; tumour necrosis factor-$\alpha$ (TNF-$\alpha$); interleukin-1 (IL-1)

## Introduction

C-reactive protein (CRP), a protein with a pentameric organization of identical subunits arranged as a single annular pentagonal disc (pentraxin) (Pepys & Baltz, 1983), is an acute-phase reactant that increases by as much as 2000 fold above its normal level during the inflammatory process (Morley & Kushner, 1982; Steel & Whitehead, 1994). CRP acts as an opsonin for phagocytic cells (Mortensen *et al*., 1976), activates the classical pathway of complement (Ballou & Kushnar, 1992), modulates platelet activation (Fiedel, 1988) and enhances natural-killer cell activity and macrophage tumouricidal activity (Steel & Whitehead, 1994).

The proteolysis of CRP leads to the release of bioactive CRP peptides with potent immunomodulating activity (Robey *et al*., 1987; Fiedel, 1988; Heuertz *et al*., 1996). These peptides have homology with the macrophage activating tetrapeptide, (H-Thr-Lys-Pro-Arg-OH) tuftsin (Najjar & Nishioka, 1970; Fridkin & Gottlieb, 1981), the homologues of which are repeated multiple times in each CRP subunit (Robey *et al*., 1987). Tuftsin is an important stimulator of natural immune responses as it activates macrophages, potentiating phagocytosis, motility, pinocytosis and chemotaxis (Najjar & Nishioka, 1970; Fridkin & Gottlieb, 1981; Babcock *et al*., 1983). Furthermore, tuftsin increases the bactericidal and tumouricidal activities of macrophages (Nishioka *et al*., 1981; Catane *et al*., 1983) and enhances nitric oxide (NO) synthesis by murine peritoneal macrophages (Cillari *et al*., 1994).

NO is one of the most potent antimicrobial agents (Hibbs *et al*., 1990). NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine (Palmer *et al*., 1988; Sakuma *et al*., 1988) by an NADPH-dependent enzyme, NO synthase (NOS). There are at least two distinct isoenzymes: the constitutive $Ca^{2+}$-dependent enzyme (cNOS), responsible for basal NO synthesis in both the endothelium and nervous system, and the $Ca^{2+}$-independent cytokine-inducible enzyme (iNOS) (Palmer *et al*., 1988; Hibbs *et al*., 1990).

As the enzymatic degradation of tuftsin strongly reduces its stimulating effect on immunocompetent cells by producing inhibitory tripeptides (H-Thr-Lys-Pro-OH and H-Lys-Pro-Arg-OH) (Spirer *et al*., 1975; Verdini *et al*., 1991), several attempts have been made to produce stabilized peptides. Thus, analogues with aminoacid substitutions, protected N- and C-terminal elongated chains and derived sugar moieties have been prepared (Fridkin & Najjar, 1989). More recently, an analogue of tuftsin was prepared, in which the first peptide bond was stabilized by the inversion of its direction, i.e. from -CONH- to -NHCO- (retro-inverso-tuftsin) (Verdini *et al*., 1991).

[1] Author for correspondence.

Since Robey *et al*. (1987) demonstrated that three tetrapeptides, resembling the aminoacid sequence of tuftsin (Thr-Lys-Pro-Leu, Gly-Lys-Pro-Arg and Thr-Lys-Pro-Gln), evenly distributed throughout the human C-reactive protein (CRP), have potent immunomodulating activities, we were interested in studying the biological activities of their retro-inverso analogues. Thus, we have investigated the ability of the retro-inverso analogues, CRP I (H-gThr-(R,S)mLys-Pro-Leu-OH = ITF 1192), CRP II (H-gGly-(R,S)mLys-Pro-Arg-OH = ITF 1127) and CRP III (H-gThr-(R,S)mLys-Pro-Gln-OH = ITF1193) to affect macrophage secretion of NO and those cytokines (tumour necrosis factor $\alpha$ (TNF-$\alpha$) and interleukin-1 $\alpha$ (IL-1$\alpha$)) capable of stimulating its synthesis (Drapier *et al*., 1988; Beasley *et al*., 1991).

Since transcription nuclear factor-$\kappa$B (NF-$\kappa$B) is crucial for the inducible expression of multiple cellular genes involved in immune and inflammatory responses including the production of IL-1$\alpha$, TNF-$\alpha$ and iNOS (Lowenthal *et al*., 1989; Muller *et al*., 1993; Baeuerle & Henkel, 1994; Baldwin, 1996) it seemed of interest to determine whether the NF-$\kappa$B inhibitor, pyrrolidine dithiocarbamate (PDTC), was able to inhibit retro-inverso analogue-induced NO synthesis.

## Methods

### *Mice*

Ten to twelve week-old female BALB/c mice were obtained from Charles River Italia (Calco, Lecco, Italy).

### *Retro-inverso analogues*

The modified peptides were synthesized at the Peptide Chemistry Department at Italfarmaco. No attempts were made to separate the two diastereomers originated by the presence of (**R**, **S**) malonyl lysine residue thus all the analogues are a 1 : 1 mixture of isomers. The synthesis was carried out as described previously (Verdini *et al*., 1991).

The compounds were free of endotoxin at 0.03 eu ($<10$ ng ml$^{-1}$) by the Limulus amebocyte lysate assay kit (Whittaker Bioproducts, Walkersville, MD, U.S.A.).

### *Peritoneal cells*

Peritoneal exudate cells were collected from mice injected i.p. 3 days previously with 3 ml of a 2% sterilized hydrolysed starch solution (BDH Chemicals, Poole, U.K.) by injecting 5 ml of cold culture medium per mouse into the peritoneal cavity under sterile conditions.

### *Induction of NO synthase activity, NO synthesis and cytokines*

Murine peritoneal macrophages were cultured for 2 h at 37°C in an atmosphere of 5% $CO_2$ in 24-well Costar plates at $2 \times 10^6$ cells in 2 ml of culture medium. Non-adherent cells were then removed by washing and the adherent cells were cultured with various stimuli as detailed in the figure legends. At various time intervals, culture supernatants were collected for NO and cytokine measurement and the cells were washed and treated with extraction buffer (250 $\mu$l of 0.1 M HEPES, pH 7.4, with 1 $\mu$M dithio-threitol, Sigma). The cells were frozen and thawed three times, scraped and transferred into Eppendorf tubes, centrifuged at 10,000 *g* for 30 min at 4°C and the supernatants of the cells extracts were assayed for NOS activity.

### *Measurement of NO and NO synthase activity*

NO production, measured in the culture supernatants as $NO_2^-$, was determined by the Griess reaction (Ding *et al*., 1988). Briefly, 100 $\mu$l/well of sample were incubated with an equal volume of Griess solution (1% sulfanilamide in 5% phosphoric acid + 1% $\alpha$-naphthylamine in distilled water) at room temperature for 10 min. The absorbance was evaluated with a Titertek ELISA reader (Flow, Rockville, MD) at 492 mm. The level of $NO_2$- reflected NO synthesis.

NO synthase activity in the supernatants was evaluated by measuring the conversion of L-[$^3$H]-arginine to L-[$^3$H]-citrulline, as described by Salter *et al*. (1991). Briefly, 20 $\mu$l of lysate were incubated with $KH_2PO_4$ (50 mM), valine (59.8 mM), $MgCl_2$ (2 mM), $CaCl_2$ (0.4 mM), EDTA (1.2 mM), DTT (0.8 mM), NADPH (0.2 mM), L-arginine (38 $\mu$M), L-citrulline (2 mM), $TH_4$biopterin (50 mM, 50 $\mu$l), FAD (1 mM, 500 $\mu$l) and [$^3$H]-arginine (25 $\mu$Ci) (Amersham, Milan, Italy). In some tubes L-NMMA, a NOS inhibitor, (1 mM) was added to the other reagents. After 15 min incubation at 37°Cm the reaction was stopped by adding 0.5 ml HEPES-Na (20 mM), pH 6, containing 2 mM EDTA. The whole reaction mixture was applied to 1 ml columns of Dowex. The radioactivity corresponding to [$^3$H]-citrulline content in 450 $\mu$l eluate was measured by liquid scintillation counting (Beckman, Milan, Italy). The protein content of the cytosol was determined by the Coomassie blue dye binding method according to the manufacturer's recommendations (Pierce Chemicals, Rockford, IL). The results are expressed as pmol of NO generated mg$^{-1}$ protein min$^{-1}$.

### *SDS–PAGE analysis and Western blot*

Adherent peritoneal cells were rapidly rinsed with PBS, lysed in boiling lysis solution (1% SDS-10 mM Tris pH 7.4) and then boiled for an additional 5 min. Protein concentration was measured by the BCA method (Pierce, Rockford, IL, U.S.A.).

SDS–PAGE was performed as described by Laemmli (Laemmli, 1970). with 7.5% (w/v) polyacrylamide gel. An appropriate amount of total protein from the cell lysate was loaded onto the gel with 6 × SDS sample buffer (Tris-Cl pH 7.8 0.35 M, glycerol 30% v/v, SDS 10% w/v, $\beta$-mercaptoethanol 6% v/v, bromophenol blue 0.012% w/v). After electrophoresis the proteins were transferred onto an immobilon membrane (Millipore, Bedford, MA, U.S.A.) by use of a wet electrotransfer system (Biorad, Hercules, CA, U.S.A.). The membrane was then blocked in TBS-T (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% dried non-fat milk and subsequently incubated with a mouse monoclonal anti-iNOS antibody (1.5 $\mu$g) (Transduction Laboratories, Lexington, KY), diluted in TBS-T. An anti-mouse HRP labelled antibody was used as second antibody (Amersham, Little Chalfont, Buckinghamshire, U.K.). Bands were visualized by autoradiography with the ECL detection system (Pierce) following the manufacturer's instructions. A mixture of different colour proteins was used as protein molecular weight markers (Sigma, St. Louis, MO).

### *Cell viability assay*

A modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay of Mosmann (1983) was used. Cultures were incubated for 24 h and pulsed with 100 $\mu$l of 0.2 mg ml$^{-1}$ MTT reagent for 2 h at 37°C, followed by 15 min incubation at 37°C with 100 $\mu$l DMSO. After this period, microtitre plates were read at 595 nm in an ELISA plate reader. Results are expressed as absolute OD readings. The s.e.mean never exceeded 15% of the mean OD value and is omitted.

### *Cytokine assay*

IL-1$\alpha$ and TNF-$\alpha$ were determined by enzyme-linked immunosorbent assay (ELISA) commercial kits (Genzyme, Kocklight Ltd., Hatfield, U.K.) which employ the multiple antibody sandwich principle.

### *Materials*

Lipopolysaccharide (LPS), CRP, acetate salt tuftsin, $N^G$-monomethyl-L-arginine (L-NMMA) and PDTC were obtained from Sigma (Poole, U.K.). Dexamethasone was purchased from MSD (Rahway, N.J.). Anti-IL-1α and TNF-α monoclonal antibodies (mAbs) were purchased from Genzyme. Tissue culture medium consisted of RPMI-1640 (Flow Laboratories, Herts., U.K.) supplemented with glutamine (2 mM), antibiotics, 2-mercaptoethanol (50 μM) plus 5% or 10% foetal calf serum (FCS).

### *Statistics*

All experiments were carried out three or four times on different days and each was made in triplicate. The results are expressed as the mean ± s.e.mean. Some data are presented as the mean ± s.e.mean of three or four individual experiments, others as the mean ± s.e.mean of a single representative experiment, because in some experiments the level of NO was variable probably due to the different state of activation of the starch-stimulated macrophages. However, within each single experiment, the s.e. was within 10%. Standard deviations and s.e.mean were calculated and statistical significance was tested by use of Student's *t* test by variance analysis (Student-Newmann-Keuls test).

## Results

### *Biochemical data on retro-inverso analogues*

The characteristics of retro-inverso analogues of tuftsin-like peptides are presented in Table 1. CRP II represents Gly residue in position 1 in place of the Thr of tuftsin. CRP III and CRP I represent Gln and Leu residues respectively in position four in place of Arg. Stability was determined in RPMI-1640 culture medium in the presence of 10% FCS at 37°C and against murine plasma. Tuftsin was almost completely hydrolysed in less than 90 min in cell culture medium RPMI and in 8 min in mouse serum. Retro-inverso-peptide analogues showed no significant hydrolysis after 2 h of incubation (data not shown).

### *Effect of retro-inverso analogues on NO synthesis*

Peritoneal macrophages were incubated with different doses of retro-inverso peptide analogues (1–10,000 ng $ml^{-1}$) in the presence of suboptimal amounts of LPS for 48 h and the supernatants tested for NO synthesis. Analogues induced NO production in a dose-dependent manner. The optimal amount of LPS inducing significant NO production was determined after accurate titration experiments with different times and doses of analogues. The most significant cell stimulations were obtained with 100 ng $ml^{-1}$ CRP I or CRP III and 1,000 ng $ml^{-1}$ of CRP II (Figure 1; $P<0.05$). Analogues alone were not effective in stimulating significant NO production. Analogues alone gave extremely variable results; in some experiments they activated NO synthesis (between 5–20 nmol of NO $ml^{-1}$) whereas in others they were ineffective. The variability appeared to be dependent on the basal state of activation of macrophages during their preparation (Novogrodsky *et al.*, 1994) rather than on LPS contamination. However, the cultures and materials used were screened for endotoxin (<10 ng $ml^{-1}$) and LPS given at low concentration (10 ng $ml^{-1}$) did not cause any significant production of NO with respect to RPMI alone (13.4±4 vs 8.5±3 nmol $ml^{-1}$ of nitrite). We have observed that authentic C-reactive protein was able to modulate iNOS induction, as attested by its ability to potentiate NO synthesis (Table 2).

**Table 1** Structure of retro-inverso synthetic peptides containing tuftsin-related sequences

| *1,2 Retro-inverso tetrapeptides* | *Amino acid sequences* |
|---|---|
| CRP I | H-gThr-NHCO[a]-(R,S)m-Lys-Pro-Leu-OH[b] |
| CRP II | H-gGly-NHCO[a]-(R,S)m-Lys-Pro-Arg-OH[c] |
| CRP III | H-gThr-NHCO[a]-(R,S)m-Lys-Pro-Gln-OH[d] |
| Tuftsin | H-Thr CONH-Lys-Pro-Arg-OH |

[a]Retro-inverso modification: -CO-NH- > > -NH-CO-.
[b]Sequence 27–30 of CRP.
[c]Sequence 113–116 of CRP.
[d]Sequence 200–203 of CRP.

### *Sensitivity of retro-inverso analogue-induced NO synthesis to treatment by dexamethasone and L-NMMA*

Peritoneal exudate cells were incubated with retro-inverso analogues, in the presence of 20 μM dexamethasone or 500 μM L-NMMA, to investigate whether the effects of the compounds on NO synthesis are mediated by the activation of NOS. The induction of NO synthase and NO production by retro-inverso analogues were significantly inhibited by dexamethasone and L-NMMA treatment (Table 3).

### *Effect of CRP II analogue on iNOS protein content*

To confirm the ability of analogues to increase the expression of iNOS we studied the expression of iNOS by SDS–PAGE and Western blot (Figure 2). The expression of iNOS protein was increased in peritoneal cells stimulated with CRP II plus LPS compared with controls, as determined by Ultroscan XL laser densitometer evaluation (Table 4).

### *Effect of retro-inverso analogues on IL-1α and TNF-α synthesis*

To determine whether the analogues stimulated the production of other cytokines, peritoneal cells were treated with different

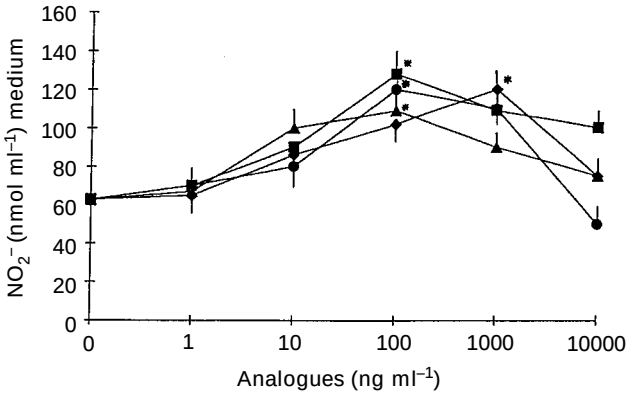


**Figure 1** Levels of $NO_2^-$ produced by peritoneal macrophages after being cultured for 48 h with different doses of retro-inverso analogues together with 1 μg $ml^{-1}$ LPS. CRP I (■), CRP II (◆), CRP III (▲), tuftsin alone (●). Vertical lines show s.e.mean ($n=4$). Results are representative of three experiments. *$P<0.05$ compared with untreated cells.

**Table 2** Effect of authentic CRP on NO synthesis induced by LPS

| | *CRP* (μg $ml^{-1}$) | | |
|---|---|---|---|
| *Control* | *0.1* | *1* | *10* |
| 60±9 | 80±3 | 103±13* | 70±10 |

[a]Data shown are mean amounts of $NO_2^-$ (nmol $ml^{-1}$ medium) ±s.e.mean of 3 experiments.
*$P<0.05$ significantly different from untreated control.

doses of CRP II at the times of maximal stimulation in the presence of LPS (1 $\mu$g ml$^{-1}$) and culture supernatants were collected for cytokine measurement and the determination of nitrite concentrations. CRP II induced very high levels of IL-1$\alpha$ cytokine synthesis compared with untreated control cells at doses of 0.1 and 1.0 $\mu$g ml$^{-1}$, whereas 10 $\mu$g ml$^{-1}$ was ineffective (Figure 3a). Similar data were obtained for TNF-$\alpha$ (Figure 3b). The concentrations of CRP II used in this experiment were also effective in stimulating NO synthesis (Figure 3c).

**Table 3** Sensitivity of retro-inverso synthetic peptide-induced NO-synthesis and NOS activity to dexamethasone and L-NMMA

| | *Stimulus* | | | |
|---|---|---|---|---|
| *Treatment* | – | *CRP I* | *CRP II* | *CRP III* |
| Control | 61±4 | 90±9* | 124±15* | 99±8* |
| | (70±8) | (100±11)* | (150±10)* | (110±12)* |
| Dexamethasone | 15±4 | 20±6 | 30±4 | 22±7 |
| (20 $\mu$M) | (8±2) | (10±5) | (12±4) | (11±4) |
| L-NMMA | 2±0.5 | 2±1 | 10±2 | 8±1 |
| (500 $\mu$M) | (<0.1) | (<0.1) | (<1) | (<0.5) |

Data shown are means±s.e.mean of three experiments and are expressed as nmol $NO_2^-$ ml$^{-1}$ or (in parentheses) as pmol iNOS mg$^{-1}$ protein min$^{-1}$. Peritoneal exudate cells ($10^6$ ml$^{-1}$) were stimulated with the compounds (100 ng ml$^{-1}$ CRP I or CRP III and 1 $\mu$g ml$^{-1}$ CRP II) for 48 h plus 1 $\mu$g ml$^{-1}$ of LPS.
*$P<0.05$ significantly different from untreated group.

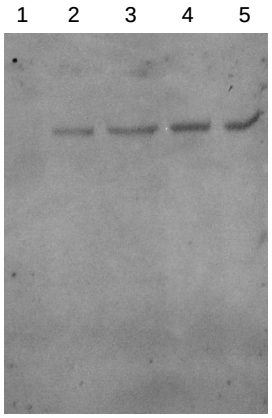


**Figure 2** Effect of CRP II on iNOS protein. Starch-elicited macrophages were cultured in medium alone or stimulated with LPS (1 $\mu$g ml$^{-1}$) with or without three different doses of CRP II for 12 h. Total cell lysates from macrophages were processed for determination of iNOS enzyme by SDS–PAGE and Western blot analysis, as described in the Methods section. Lane 1, RPMI alone; lane 2, LPS; lane 3, 10 $\mu$g ml$^{-1}$ CPR II plus LPS; lane 4, 1 $\mu$g ml$^{-1}$ CPR II plus LPS; lane 5, 100 ng ml$^{-1}$ CPR II plus LPS. The areas, from lane 1 to lane 5, as scanned by Ultroscan XL laser densitometer, were respectively: 0.00, 0.230, 0.238, 0.462, 0.545. (→) Position of typical iNOS band (ca. 130 Kd). Results are representative of four experiments.

### *Retro-inverso analogues stimulate NO synthesis preferentially by the enhancement of endogenously produced IL-1α and TNF-α*

The effect of enhanced endogenous production of IL-1$\alpha$ and TNF-$\alpha$ by retro-inverso analogues on NO production by macrophages was evaluated by quantifying $NO_2^-$ levels produced by peritoneal macrophages activated with these compounds together with LPS (1 $\mu$g ml$^{-1}$), in the presence or absence of neutralizing anti-IL-1$\alpha$ and anti-TNF-$\alpha$ monoclonal antibodies (mAbs). A representative experiment with the analogue CRP II is shown in Table 5. In both the presence and absence of LPS plus anti-IL-1$\alpha$ and anti TNF-$\alpha$ mAbs, CRP II-stimulated macrophages produced lower levels of NO than those produced in the absence of mAbs. This indicates that endogenously produced IL-1$\alpha$ and TNF-$\alpha$, whose secretion was enhanced by CRP II, are critically involved in the induction of NO secretion.

**Table 4** Effect of the analogue CRP II on iNOS protein content evaluated by Western blot

| | *CRP II* ($\mu$g ml$^{-1}$)[a] | | |
|---|---|---|---|
| *Control* | *0.1* | *1* | *10* |
| 0.179±0.03 | 0.372±0.09* | 0.290±0.09* | 0.178±0.03 |

Means±s.e.mean of four individual experiments are shown.
[a]Ultrascan XL laser densitometer evaluation.
*$P<0.05$ significantly different from untreated (control) group.

**Table 5** Effect of anti-IL-1$\alpha$ and anti-TNF-$\alpha$ mAbs on LPS-induced NO synthesis in the presence of CRP II

| *Group* | *Stimulus* | *Additional stimulus* | *$NO_2^-$* (nmol ml$^{-1}$) |
|---|---|---|---|
| 1 | RPMI | – | 3±0.5 |
| 2 | LPS | – | 110±9 |
| 3 | CRP II | LPS | 172±12 |
| 4 | LPS | Anti-IL-1$\alpha$ | 35±7* |
| 5 | CRP II | Anti-IL-1$\alpha$+LPS | 50±10* |
| 6 | LPS | Anti-TNF-$\alpha$ | 80±9 |
| 7 | CRP II | Anti-TNF-$\alpha$+LPS | 140±12 |

Means±s.e.mean of three experiments are shown. Peritoneal cells were cultured at $10^6$ cells ml$^{-1}$ for 48 h with medium alone or with LPS (1 $\mu$g ml$^{-1}$) with or without 1 $\mu$g ml$^{-1}$ CRP II. Anti-IL-1$\alpha$ and anti-TNF-$\alpha$ mAbs were used at a final concentration of 40 $\mu$g ml$^{-1}$.
*$P<0.01$, compared with corresponding control.

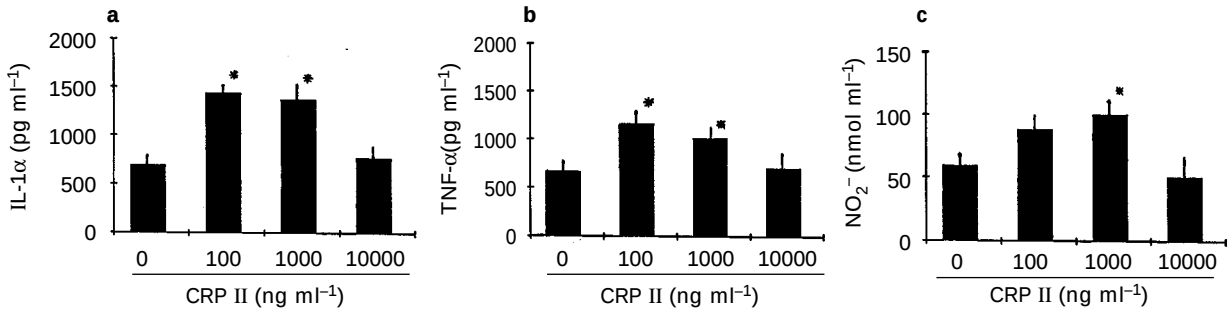


**Figure 3** Levels of (a) interleukin-1$\alpha$ (IL-1$\alpha$), (b) tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and (c) $NO_2^-$ produced by peritoneal macrophages activated with different doses of CRP II (for 48 h) in the presence of LPS (1 $\mu$g ml$^{-1}$). *$P<0.05$ compared with untreated cells.

*Sensitivity of retro-inverso analogue-induced NO synthesis to treatment with PDTC*

We further evaluated the effect of the NF-$\kappa$B inhibitor, PDTC, in reducing NO synthesis induced by CRP II and LPS (1 $\mu$g ml$^{-1}$). As shown in Figure 4a, 10 $\mu$M PDTC reduced NO synthesis in CRP II treated and untreated cells by 60%, whereas treatment with 100 $\mu$M PDTC resulted in its complete inhibition. At the concentrations of PDTC used in these experiments, the numbers of living cells remained constant for at least 24 h, as attested by the colorimetric MTT reduction assay (Figure 4b), indicating that the inhibitory effect of PDTC on NO synthesis induced by CRP II plus LPS or by LPS alone is not due to toxic activity.

## Discussion

Tuftsin is a well documented stimulator of macrophage functions *in vitro* (Najjar & Nishioka, 1970; Nishioka *et al*., 1981; Babcock *et al*., 1983; Catane *et al*., 1983; Cillari *et al*., 1994) and *in vivo* (Martinez & Winternitz, 1981; Nishioka *et al*., 1986; Smith *et al*., 1986). However, its use is limited by its instability and sensitivity to enzymatic degradation, which reduces its stimulating effect on immunocompetent cells (Spirer *et al*., 1975) and its use *in vivo*.

In this study we showed that three CRP-related analogues of tuftsin, prepared by inverting the 'direction' of the first peptide bond between Thr and Lys replacing Thr and Lys respectively with gem-diamine and malonyl residues (Verdini *et al*., 1991), acquire very significant peptidase stability and become less susceptible than their parent molecule to hydrolysis by proteolytic enzymes (Goodman & Chorev, 1979; Bonelli *et al*., 1984; Groggia *et al*., 1985).

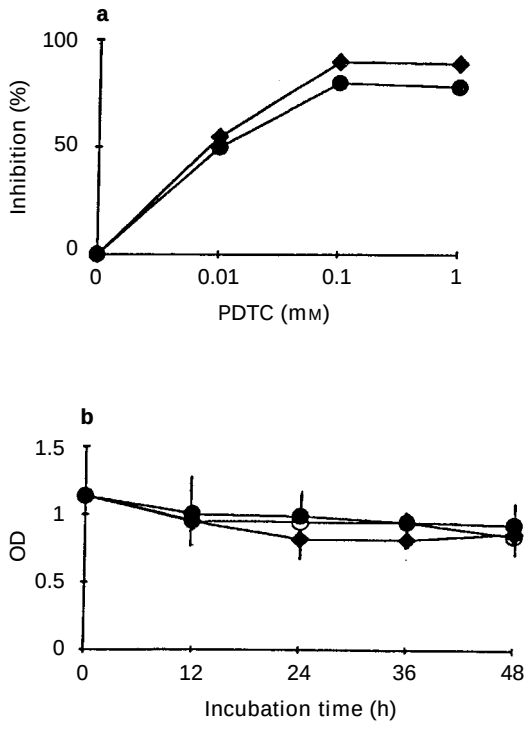


**Figure 4** (a) Effect of pyrrolidine dithiocarbamate (PDTC) on CRP II analogue-induced NO synthesis. Murine macrophages were incubated with LPS (1 $\mu$g ml$^{-1}$) (●) or LPS (1 $\mu$g ml$^{-1}$) plus CRP II (◆) in the presence of various doses of PDTC (0.01 – 1 mM), which were added in cultures 2 h before the addition of CRP II and/or LPS. The inhibitory effect of PDTC on the NO-induced synthesis is expressed as % inhibition of NO synthesis. (b) Effect of PDTC on cell viability. Murine macrophages cultured for various times in the presence of the most effective dose of PDTC (0.1 mM) were stimulated with LPS (●) or LPS plus CRP II (1 $\mu$g ml$^{-1}$) (◆) and viability was evaluated by MTT reduction assay. LPS without PDTC (○). Data are expressed as OD and are mean of three experiments; vertical lines show s.e.mean.

Furthermore, these retro-inverso CRP-related analogues display potent immunostimulating activities. In particular, retro-inverso CRP analogues enhanced NO production induced by suboptimal amounts of LPS (1 $\mu$g ml$^{-1}$) and increased iNOS activity in peritoneal macrophages as observed for tuftsin (Cillari *et al*., 1994). The enhanced effect of retro-inverso CRP-related analogues on the expression of iNOS is highlighted both by higher levels of iNOS activity and by the increase of protein mass for iNOS, evaluated by Western blot analysis in stimulated macrophages. Further evidence of their stimulating activity on iNOS is provided by the observation that iNOS synthesis and NO production, mediated by retro-inverso CRP-related analogues, are inhibited by the addition of dexamethasone. The induction of NO synthesis by these compounds is also sensitive to the action of L-NMMA, the substrate analogue inhibitor of NOS.

The observed stimulating effects on NO synthesis by authentic CRP could indicate a novel mechanism by which CRP participates in the acute phase response to tissue damage (Baumann & Gauldie, 1994). The inducible transcription factor of Rel family NF-$\kappa$B plays a central role in coordinately controlling gene expression during monocyte/macrophage activation (Baeuerle & Henkel, 1994; Baldwin, 1996). Between the various genes induced with the help of NF-$\kappa$B are those of IL-1, TNF and iNOS (Lowenthal *et al*., 1989; Muller *et al*., 1993; Baeuerle & Henkel, 1994; Baldwin, 1996). On the other hand, TNF and IL-1 may be critical in initiating the events leading to NF-$\kappa$B activation via the degradation of (I$\kappa$Ba) (Lowenthal *et al*., 1989; Osborn *et al*., 1989; Weigmann *et al*., 1994; Baldwin, 1996). Furthermore, although NF-$\kappa$B transcriptionally regulates the iNOS gene, the production of NO may inhibit the activation of NF-$\kappa$B (Peng *et al*., 1995; Zeiher *et al*., 1995) thus NO may block its own production through the inhibition of NF-$\kappa$B (Baldwin, 1996). Our finding that the NF-$\kappa$B inhibitor PDTC prevents NO synthesis by CRP analogues, could indicate that one of the mechanisms by which CRP analogues induce iNOS activation is the activation of NF-$\kappa$B. However, it was not possible to exclude the possibility that CRP analogues could activate IL-1 and TNF synthesis in turn inducing post-translationally the active nuclear form of NF-$\kappa$B which could activate the iNOS gene and, hence, NO synthesis. Consistent with this hypothesis is the demonstration that CRP peptides are very effective in enhancing LPS-induced IL-1 and TNF synthesis and that, in the presence of anti-IL-1 and anti-TNF mAbs, they were ineffective in stimulating significant NO secretion by peritoneal macrophages stimulated with LPS (Table 5).

So far the effects on macrophages by short peptide chains have been only marginally investigated. CRP I and CRP III are more similar to each other, than they are to CRP II. Although CRP I and CRP III differ in amino acid position 4, both possess Thr, whose presence has already been demonstrated to be crucial for the activity of natural tuftsin, since its replacement drastically reduces immunostimulating activity (Fridkin & Gottlieb, 1981). On the other hand, Thr together with Phe are the critical residues of CRP which mediate cell attachment *in vitro* (Mullinex *et al*., 1994). CRP II is devoid of Thr and its two residues (Gly and Arg) are different from those of the other two compounds. Nevertheless, CRP II is very active in stimulating peritoneal cells and could bind strongly to the surface membrane of these cells, because it contains two of the three amino acids of the cell adhesion motif RDG (Arg-Gly-Asp) that are recognised by several membrane integrins (D'Souza *et al*., 1991).

CRP-analogues downregulated cytokine and NO synthesis at higher concentrations, producing biphasic dose-response curves. These bell-shaped curves are common to other peptides (Rovati & Nicosia, 1994) and to antioxidants (Schreck *et al*., 1992). It is conceivable that peptides acting on receptors used by other substances behave as 'partial agonists' with lower

efficacy compared with 'full agonists' (Rovati & Nicosia, 1994; Pliska, 1994). The possibility that the peptides interact as full agonists with two receptors that mediate opposite effects cannot be excluded (Rovati & Nicosia, 1994; Pliska, 1994).

Studies are in progress to characterize better the effect of single amino acid substitutions on the biological activity of these linear peptide chains and the potential immunostimulation *in vivo* of such molecules, more stable than tuftsin, on immunocompetent cells.

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