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SYNTHETIC MACROPHAGE ACTIVATING PEPTIDES DERIVED FROM THE N-TERMINUS OF HUMAN MCF[1]

C. Michael Jones*, Carlos J. Reyes*, Richard G. Cook+,
George A. Krudy*, and Paul R. Rosevear*

*Departments of Medicine and Biochemistry,
University of Texas Medical School in Houston, Houston, Texas

+Department of Microbiology, Baylor University College of Medicine, Waco, Texas

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Recently, we described the purification and N-terminal sequencing of a novel cytokine termed MCF (Monocyte Cytotoxicity Inducing Factor) (1,2). In order to study the interaction of this cytokine with monocytes, we synthesized a nona-peptide GAAVLEDSQ corresponding to the N-terminus of MCF: two truncated peptides, GAAVL and LEDSQ; and the substituted peptide, GAAVLENSQ. The authentic N-terminal peptide is biologically active in the nanomolar range, while substitution of asparagine for aspartic acid at position 7 diminishes biological activity. Biological activity was observed from the C-terminal fragment LEDSQ, but the N-terminal pentapeptide (GAAVL) was devoid of biological activity. Scatchard analysis revealed a single class of saturable high affinity sites. These studies indicate that the N-terminus of MCF is important in interacting with the binding site on monocytes and it may be possible to design synthetic activators and inhibitors of monocyte/macrophage cytotoxicity.

The receptor binding domains for a number of immunologically active molecules are contained within short peptide sequences (3-8). In particular, the N-terminus of IFNγ and C3A and IL-8 participate in binding to their respective receptors and in eliciting their biological response. Synthetic peptides spanning both the C and N-terminus of C3A were found to be 10^3 times more potent than the native molecule (4). In contrast, synthetic nona-peptides derived from IL-1ß have been reported to have some of the immunostimulatory actions of IL-1, but lack pyrogenic activity (5-6). These same peptides do not show saturable binding to monocytes, and presumably do not function through an IL-1 receptor (6). Synthetic peptides corresponding to the N-terminal nine amino acids of MCF were found to interact with specific high affinity saturable cellular binding sites

[1] Supported by CA 39441 to CMJ and GM 41232 to PR.

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and activate macrophages for cytotoxicity. Moreover, residues 5-9 appear to be absolutely necessary for this activation.

Materials and Methods

Monocyte Isolation and Cytotoxicity

Human monocyte-enriched leukopaks were prepared with a COBE Spectra cell separator. The protocol was approved by the Committee for the Protection of Human Subjects, University of Texas Health Science Center. Mononuclear cells were prepared by centrifugation over Ficoll-paque. Monocyte monolayers were prepared by adherence to 96-well flat-bottom plates previously coated with human serum as described by Golightly et al (9). The monocyte cytotoxicity assay was performed according to the method of Fischer et al (10) with some modifications (1) as previously described. Monocytes were adjusted to a concentration of 1 x 10^7/ml and were allowed to adhere to the serum-coated plates for 15 to 30 minutes followed by vigorous washing. The effector (monocytes) to target (K562 tumor cells) ratio was 30:1. Spontaneous release averaged 15% (7 to 20%) in more than 50 experiments. LPS-free RPMI 1640 (M.A. Bioproducts, Walkersville, MD) with 10% heat inactivated AB-negative human serum (Flow Laboratories, Arlington, VA) was used throughout the assay procedure. Specific release from ^{111}In labelled targets calculated as described by Wiltrout et al (11).

Peptide Synthesis

Peptides were synthesized with either FMOC or BMOC chemistries using an Applied Biosystems Model 430 synthesizer, cleaved from the resin and purified over an Aqua pore C18 column using a 5-90% acetonitrile gradient in 0.1% trifluro acetic acid (TFA)/water. The peptide GAAVLEDSQ eluted in 45% acetonitrile. Fidelity of synthesis was determined by N-terminal sequence analysis using an Applied Biosystems Model 477A sequencer.

Radiolabelling of Peptides and Determination of Specific Activity

One milligram of stock synthetic peptide GAAVLEDSQ was suspended in an equal volume of 0.1M molar borate buffer pH 8.5 and added directly to the dry prelabeled Bolton-Hunter reagent (Amersham) and incubated for 1 hour. The reaction was quenched with the addition of 100 microliters of .2 molar glycine in 0.1 molar borate buffer pH 8.5 for 15 minutes (12). In some experiments unlabeled Bolton and Hunter reagent was obtained from Pierce and iodinated directly using anhydrous reagents as described (13). Percent incorporation of ^{125}I into peptide was 73.9% $\pm$ 6.9%. Specific activity was 1.89 +/- .775 x 10^6 cpm/nM.

Binding Analysis

Binding of radiolabeled peptide and displacement with cold peptide was performed at both 4° and 25°C. Both purified human monocytes and the monocytic cell line U937 were employed for these studies at cell densities of 1 x 10^6/ml. Binding was performed under serum free conditions using RPMI 1640 medium. Cell-bound peptide was separated from the free peptide in the supernatant by centrifugation over a 3:2 mixture of dibutyl and dioctyl phthalate oils (Eastman Chemicals, Rochester, NY) as described by Finbloom et al (14). Initially, kinetic studies were performed to determine optimum time for the measurement of binding and displacement. Binding constants were calculated using the Ligand program of Munson and Rodbard (15) (Biosoft software) and the Kaleidagraph package (Synergy Software). Non-specific binding was determined using the B/F limiting ratio as described by Chamness and McGuire in the presence of 10^3-fold molar excess of unlabeled peptide (16).

NMR Studies

Proton NMR studies on approximately 10mM GAAVLEDSQ were performed at 500 MHz in 90% H_2O/10% ^2H_2O at pH=5.5. One-dimensional NMR spectra were collected with 256 transients, each

with a spectral width of 5024 Hz, 16K data points, a 90° pulse, and a 3 sec recycle time. Proton chemical shifts were referenced to the dioxane signal at 3.7ppm. Coupling constants, ^{3}JNH-CαH, were measured at 25°C. Amide proton exchange rates were determined over the temperature range of 5 to 45°C. Phase-sensitive NOESY spectra were collected with 150-, and 300-ms mixing times as 512 t_1x2048 t_2 complex data points. Two dimensional NMR data were processed with the FELIX software package (Hare Research Inc.).

Results

Peptides having the sequence GAAVLENSQ (P1), GAAVLEDSQ (P2), GAAVL (P3), LEDSQ (P4) were synthesized and purified as described. The data in Figure 1 are representative mean and standard deviations derived from eight individual donors in eight experiments. P2 demonstrated a peak of biological activity at 6.5nM/10^6 cells, while P1 induced maximal activation of monocytes between 25 and 50nM. P3, whose sequence corresponds to residues 1-5 of MCF, did not exhibit significant biological activity (data not shown). However, P4, whose sequence corresponds to residues 5-9 of MCF, was able to activate monocyte monolayers, albeit in the micromolar range (Figure 2). Overnight activation of monocytes with the peptides did not affect their viability nor were the peptides directly toxic to K562. P2 is, however, toxic to the human melanoma cell line A 375[2].

Since P3 had no biological activity and the addition of tyrosine to the C-terminus of P2 decreased biological activity (data not shown); labelling of the N-terminal amino group of the peptide P2 was carried out using the Bolton-Hunter reagent. Labelling of the N-terminus did not interfere with biological activity (data not shown).

Figure 3 demonstrates that binding of the P2 to monocytes and U937 was saturable. 10^6 monocytes were then pre-incubated for 1 hour with 8 x 10^{12} nanomoles of P2 followed by the addition of excess unlabeled P2. The data summarized in Figure 4, demonstrate that cold peptide was able to compete labeled peptide from both human blood monocytes (and the monocytic cell line U937). Data from these sets of experiments were then plotted by the method of Scatchard as shown in Figures 5,6. Nonspecific binding to U937 cells was 5.7% in three experiments and 10% for human peripheral blood monocytes in five experiments at 4°C. The Kd (at 4°C) for monocytes and U937 cells was 5.57 x 10^{-12} $\pm$4.88 and 1.92 x 10^{-12} $\pm$.87, respectively. Monocytes had on their surface 6,234 $\pm$ 5,430 (range 814-11,634) high affinity binding sites per cell while U937 had 1860 $\pm$ 713

[2] Personal communication Stewart Lyman (Immunex).

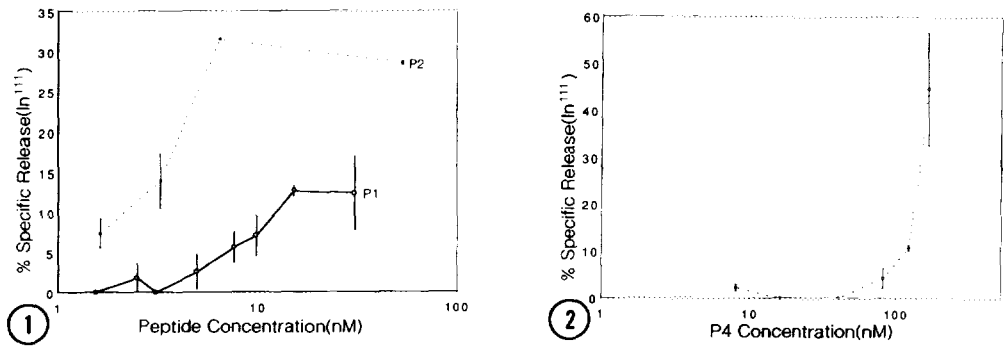

Figure 1. **Induction of Cytotoxicity by Synthetic Peptides.**
Monocytes were incubated for 18 hours with the peptide before its removal and the addition of labelled targets. The results are from eight experiments and represent the mean and ± standard error from eight individual donors.

Figure 2. **Induction of Cytotoxicity by Synthetic Peptide P4.**
Monocytes were incubated for 18 hours with the peptide before its removal and the addition of targets. The results are from two experiments using two different individual donors and represent the mean ± standard error.

(range 1147-2572). The wider range seen with monocytes was due to variation among the 8 individual human donors. At 24°C, the Kd and sites per cells for monocytes and U937 were 2.6 x 10^{-12}, 970 and 1.42 x 10^{-11}, 3250 respectively (mean of two experiments). Although specific saturable binding to U937 could be demonstrated, these cells were not cytolytic for K562.

In order to determine if P2 displayed alpha-helical structure in solution, the stability of the amide protons was measured by determining their change in chemical shift as a function of temperature.

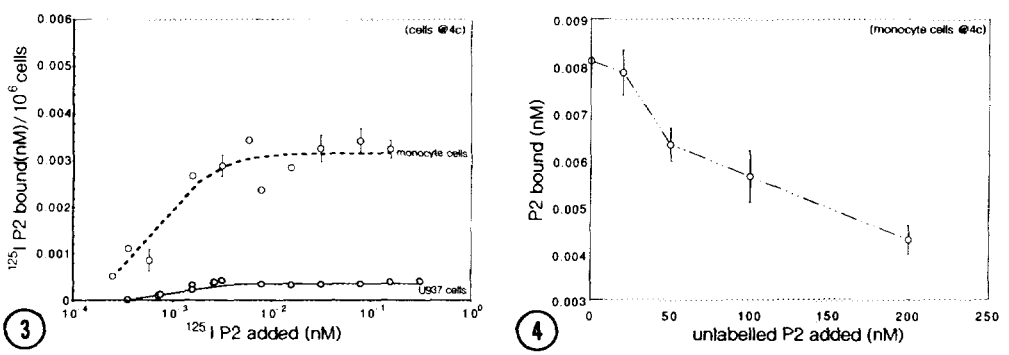

Figure 3. **Saturable Binding of Labelled P2.**
Monocytes and U937 cells were incubated with increasing doses of labelled peptide, +/- 100 fold molar excess cold peptide. Results presented are for specific binding and are the mean and standard deviation from three experiments.

Figure 4. **Competitive Displacement of P2.**
Monocytes were incubated with labelled peptide (P2) alone for 60 minutes followed by addition of unlabelled peptide (mean ± standard deviation). Results are from four experiments and represent total binding.

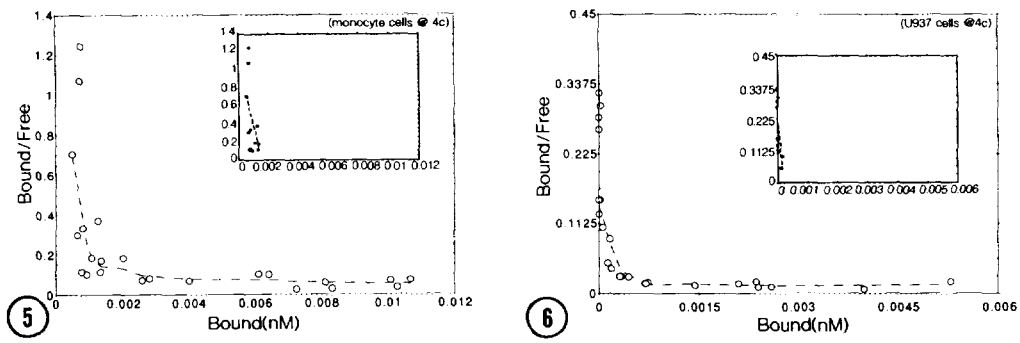

Figure 5. **Scatchard Analysis of P2 Binding to Monocytes.**

Monocytes were isolated and results calculated as in Materials and Methods. Results for the 4°C binding are from six separate experiments. The large graph represents total binding while the inset represents specific binding linearized using the Ligand program.

Figure 6. **Scatchard Analysis of P2 Binding to U937 cells.**

Results for the 4°C binding are from three experiments. The large graph represents total binding while the inset represents specific binding linearized using the Ligand program.

All 8 amide protons were found to have temperature coefficients, $-\Delta\delta/\Delta T$ between 6.6 and 9.4ppb/K. A large temperature coefficient is consistent with rapid exchange and the absence of strong hydrogen bonding. In addition, amide proton coupling constants are most consistent with random coil conformations. This data and the lack of strong CαH to NH NOEs in a NOESY spectrum of the peptide are not consistent with dimerization of the peptide at concentrations up to approximately 10mM.

Discussion

The structures of several cytokines have recently been determined and receptor binding domains identified which correspond to relatively small portions of the intact molecule (3-8).

We synthesized N-terminal peptides corresponding to human MCF based on the hypothesis that this regions was involved in receptor interaction. These peptides were able to activate monocytes for tumor cytolysis. While P2, the nona-peptide with the N-terminal sequence identical to MCF, was active in the 6.5nM/10^6 cells, P1 which contains a single amino acid substitution at Position 7, was only biologically active in the 25 - 50 nanomolar range. Moreover, the maximal response at the 50 nanomolar dose was lower with P1 ($p = 0.02$). The fragment P4 corresponding to residues 5-9 (LEDSQ), was active in the .1 - 1 micro molar range, while the N-terminal pentapeptide displayed

no biological activity. Taken together these data indicate that the residues EDSQ are necessary for biological activity and charged residues are likely to be important in receptor interaction, but it is unlikely that they contain the entire receptor binding domain (17,18), and do not display the full biological activity seen with the intact molecule. $-\Delta\delta/\Delta T$ values and amide proton coupling constants ^{3}JNH-CαH, were consistent with the absence of strong hydrogen bonding and conformational flexibility (19,20). Nonetheless, these peptides have helped us identify residues important in the amino terminus for binding to the cellular receptor and subsequent activation of monocytes.

Note

These data were presented in part at the meeting of the American Association of Immunologists/Clinical Immunology Society, Denver, Colorado, May, 1993.

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