Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 100-105

PEPTIDES FROM THE AMINO-TERMINUS OF RANTES CAUSE CHEMOTAXIS OF HUMAN T-LYMPHOCYTES

Timothy N.C. Wells[1], Florence Guye-Coulin, and Kevin B. Bacon[2]

Glaxo Institute for Molecular Biology, 1228 Plan-les-Ouates, Geneva, Switzerland

Received April 5, 1995

SUMMARY: RANTES is a potent chemotactic and activating agent for a variety of leukocytes including T-lymphocytes. To identify the region of the molecule responsible for this activity, we have made overlapping 10 amino acid peptides scanning the protein. A micro-Boyden chamber chemotaxis assay showed the most efficacious peptides came from at the N-terminus. EC_{50} values of 8 nM (± 2.2 nM), 3.7 nM (± 2.0 nM) and 3.1 nM (± 2.0 nM) were calculated from dose response curves for the peptides (1-10), (3-12), and (5-14). Control peptides from other regions are not active. In THP-1 cells, none of the peptides give a Ca^{2+} response. The active peptides are shown to be principally chemotactic rather than chemokinetic by a checker board analysis. The results imply that the principal region of RANTES responsible for chemotaxis is located in the amino terminus.

One of the principle events in inflammatory diseases such as atopic dermititis and asthma is an influx of T-lymphocytes and eosinophils. Several cytokines have been implicated in this process, one of which is the protein RANTES (1). This is a member of a growing set of small proteins, known as chemokines (2). The best characterised member of this chemokine superfamily to date is interleukin-8 (IL-8), for which extensive three dimensional structural studies and mutagenesis has been carried out (3). Although the sequence identity is only 21.2 % between IL-8 and RANTES, the sequence identity within the chemokine superfamily is good enough to predict that the two proteins may share essentially the same subunit three dimensional protein structure. Mutagenesis and peptide synthesis studies with IL-8 have shown that three residues play an essential part in the bioactivity of the protein, and that these are the residues Glu^4-Leu^5-Arg^6 at the N-terminus of IL-8 (4, 5). Previous studies had attempted to define this essential region

[1] To whom correspondence should be addressed. Fax: + 41 22 794 69 65.

[2] Current Address: DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA.

ABBREVIATIONS:

DMF, N,N-Dimethyl formamide; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid.

0006-291X/95 $5.00

or pharmacophore of IL-8 by making synthetic peptides (6, 7), but the data had been ambiguous. One argument against using peptides to try define the pharmacophore is that they lack the three dimensional conformation that is essential for biological activity. However, structural studies of IL-8 by NMR (8) and X-ray crystallography (9) show that the three dimensional location of the amino terminal region is not completely defined in any case. We therefore hypothesised that it may be possible to find peptides which mimic the action of chemokines, provided that a systematic approach to the peptide synthesis was carried out. We have therefore synthesised overlapping peptides which scan the sequence of RANTES, starting with every second amino acid, and have found peptides which are potent chemoattractants of human T-cells.

MATERIAL AND METHODS

Reagents. The cleavable peptide kit and synthesis design program were purchased from Cambridge Research Biochemicals (Cheshire, UK). All the chemicals for peptide synthesis were from Flucka (Buchs, Switzerland). Recombinant human RANTES standard was obtained from PeproTech (London, UK). The selected peptides were resynthesized on a 25 mg scale by Neosystem (Strassbourg, France) and then HPLC purified before use. The 48-well micro-well Boyden Chamber and the filters were from Neuro Probe Inc. (Cabin John, MD, USA). Ficoll-Paque was obtained from Pharmacia (Dubendorf, Switzerland), and sheep erythrocytes for rosetting, from BioMerieux SA (Marcy-l'Etoile, France). Medium RPMI 1640 was obtained from Seromed (Berlin, Germany), FCS, Glutamine and Hepes were purchased from Gibco (Paisley, Scotland). Field's stains were obtained from BDH (Poole, UK). VIDAS, the Image Analysis software, was from Zeiss (Zürich, Switzerland). The data were analysed with Excel 4.0 (Microsoft, USA) and Grafit 3.01 (Erithacus Software, Staines, UK) on a Compaq prolinea 4/33 PC.

Peptide synthesis. The Pin Technology cleavable peptide kit software was used to design the protocol to produce a set of 30 overlapping 10-amino acid peptides, which scan the sequence of human RANTES, starting every second amino acid. To prevent polymerisation the cysteine residues were replaced by alanines. Thus, the first peptides were SASPYSSDTT, SPYSSDTTPA, YSSDTTPAAF etc., as far as REYINSLEMS. The peptides were synthesised using Fmoc protected amino acids, according to the manufacturer's instructions. The final peptides were N-terminally acetylated by incubation for 90 min at 30 ^{o}C in a DMF:acetic anhydride:triethylamine mixture (5:2:1 V/V/V). After side chain deprotection in TFA containing scavengers (10) for 2 h at room temperature, the peptides were cleaved from pins using 100 mM phosphate buffer pH 7.0. Authenticity of peptide synthesis was checked by analysing duplicate peptides by electrospray mass spectrometry.

Lymphocyte preparation. Human T-lymphocytes, extracted from fresh human blood, were purified from mixed mononuclear cells by density gradient centrifugation on Ficoll-Paque (11, 12), followed by rosetting with sheep red blood cells (13). Following purification, T-cells were resuspended in RPMI 1640 medium containing 10% heat-inactivated FCS, 25 mM Hepes and 2 mM L-Glutamine, and were used immediately.

Chemotaxis assay. Chemotaxis of T-Lymphocytes following RANTES stimulation, was analysed using a 48-well micro-well Boyden chamber. For the initial studies 25 µl of

RANTES or peptide diluted in medium was placed in the lower wells of the chemotaxis chamber, covered with an 8 μm pore-size polyvinylpyrrolidone-free polycarbonate filter, and overlaid with 50 μl cell suspension (2 x 10^6/ml) in the upper wells of the apparatus. Following 1 h incubation at 37 °C, the filter was removed, the upper surface wiped free of sedimented cells, and cells which had migrated onto the lower surface of the filter fixed in methanol, stained with Field's stain and counted using a Zeiss Axiophot microscope and image analysis system. A minimum of five high powered fields were scanned for each well. The number of migrated cells were then normalised by comparison with the medium controls and expressed as a relative chemotactic index.

Calcium mobilisation studies. Mobilisation of intracellular calcium in RANTES stimulated T-cells was assessed using by loading cells with Fura-2 dye (5 μM), and incubating for 30 minutes at 37 °C. Cells were washed after loading, and kept in the dark until analysis. Fluorescence detection was carried out using a Jasco FP777 spectrofluorimeter. For THP-1 cells, IL-8 (10^{-7} M) was used as a positive control.

RESULTS AND DISCUSSION

The chemokines are a superfamily of similar protein sequences of around 70-100 amino acids in length, all involved in the recruitment and activation of cells during the inflammatory response or wound healing (3). The chemokine superfamily have been shown to bind to a series of membrane bound receptors of the 7-transmembrane alpha helix type. A receptor for several of the CC chemokines, which binds RANTES (14,15) and MIP-1α has also recently been reported. The first question in attempting to understand the relationship between structure and function of such ligands, is to ask whether all of the molecule is required for function, by making truncated versions of the molecule. Previous studies on IL-8 (6,7) had attempted to answer this question using synthetic peptides, but the results were ambiguous. We attributed this to the experimental variability observed between different laboratories, and therefore decided to make a single collection of peptides which scanned the sequence of RANTES, and test them all in the same experiment.

The ability to synthesise microgram quantities of peptides using pin based technology, and diketopiperazine based cleavable linkers, gave us enough peptide to measure T-cell chemotaxis in the presence of 10^{-7} M of each of the peptides (Fig.1). This result shown is typical of the three experiments (all carried out in triplicate) in that the majority of the chemotaxis is in the N-terminal region. We therefore resynthesised peptides from the N-terminus, and also amino acids 23-32 and 57-66, since these regions (containing the "inner" beta sheet and part of the C-terminal alpha helix) also appeared to be active. On retest, all three of the peptides from the amino terminus were active, showing EC_{50} values in the nanomolar range: 1-10, 8.9 ±2.2 nM; 3-12, 3.7±2.0 nM and 5-14,3.1±2.1 nM. There was also a difference in the efficacy of the peptides (as judged from the maximal chemotactic signal) with the peptide 3-12 showing an efficacy almost equivalent to RANTES (Fig. 2). The heptamer peptide 5-11 is also active, indicating that the region of importance is the amino-terminal region before the first disulphide

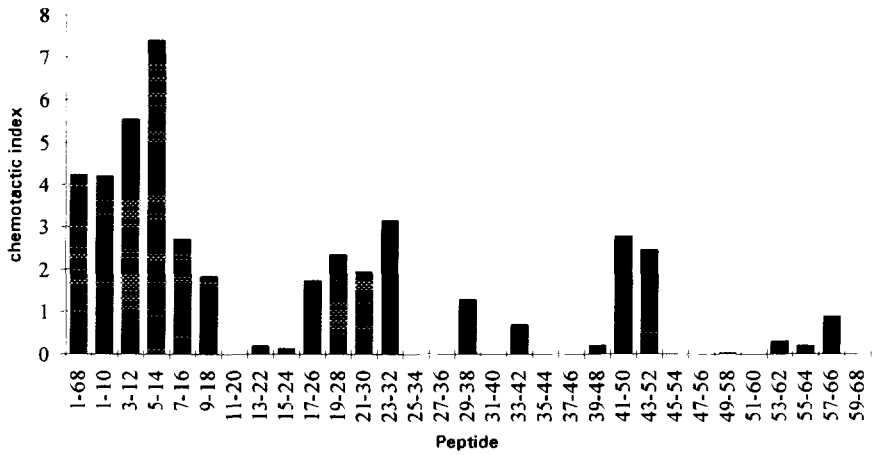

Fig. 1. Chemotaxis of T-lymphocytes by overlapping peptides produced based on the sequence of human RANTES. The peptides were added at a concentration of 2 10^{-8} M, and the underside of the filters analysed after an incubation of 1 h. The data have been normalised using the number of cells which migrate in the presence of RPMI 1640 medium alone.

bridge (assuming that RANTES and IL-8 have similar subunit folds). The peptides 23-32 and 57-66 were both inactive even at concentrations as high as 10^{-6} M when material from the larger scale synthesis was used. The differences between the two batches of peptides indicates the dangers of trying to interpret chemotactic data when very small chemotactic indices. We investigated whether the peptides could cause mobilisation of cellular calcium. None of the peptides, nor even RANTES itself, would mobilise calcium in T-cells, consistent with the literature. On the other hand, calcium responses

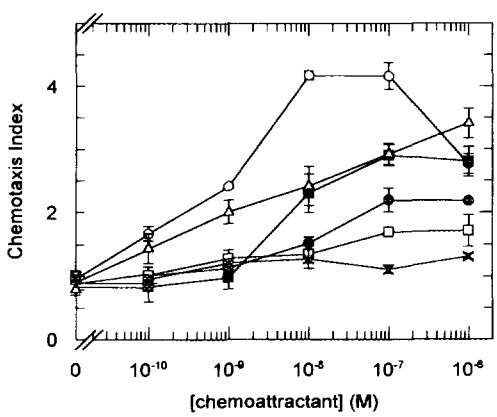

Fig. 2. Dose response curves for chemotaxis of T-lymphocytes, using peptides selected on the basis of activity in the initial peptide scan. Peptides used were ● (1-10), Δ (3-12), ■ (1-8), □ (5-14), x (23-32), and ○ recombinant human RANTES.

		Concentration above the filter (M)			
		0	10^{-9}	10^{-8}	10^{-7}
Concentration	0	1 ± 0.03	1.18 ± 0.43	1.02 ± 0.23	0.95 ± 0.12
below the	10^{-9}	2.3 ± 0.46	1.76 ± 013	1.53 ± 0.34	1.08 ± 0.12
filter (M)	10^{-8}	2.9 ± 0.16	2.2 ± 0.37	2.12 ± 0.06	1.72 ± 0.41
	10^{-7}	2.95 ± 0.10	2.5 ± 0.43	2.53 ± 0.10	1.79 ± 0.35

Fig. 3. Checkerboard analysis of the chemotactic/chemokinetic properties of RANTES (1-8) peptide on human T-lymphocytes, using the micro-Boyden chamber system.

were seen in THP-1 cells treated with RANTES (10^{-7} M) (16,17). However none of the peptides were able to induce a calcium response in T-cells, even at concentrations as high as 10^{-6} M. These data indicate that the chemotactic effects of RANTES and the synthetic peptides are not directly coupled to an observable calcium signal.

Checkerboard analysis was carried out on the peptide (3-12) to determine if the activity on T-cells was chemokinetic or chemotactic (Fig. 3). The principle effect of the peptide is chemotactic, consistent with data seen for RANTES (not shown). However, there is a two fold stimulation of chemotaxis on the addition of chemokine to the upper chamber, indicating that the effect does have a chemokinetic component.

The finding that the N-terminal region is important for the chemotactic activity of RANTES is consistent with the literature data on the relationship between protein structure and function in the chemokine superfamily. In the case of IL-8, the demonstration that the N-terminal region Glu^4-Leu^5-Arg^6 was essential for the correct functioning of the ligand required site directed mutagenesis or peptide synthesis of an almost complete peptide chain (4,5). Synthetic peptides such as Glu-Leu-Arg or Ac-Glu-Leu-Arg-NH_2 were not chemotactic (Timothy N. C. Wells unpublished data). This may be because they were unable to obtain the correct conformation, or that more of the IL-8 protein is required to interact with its receptor. In this study we have shown using a series of overlapping peptides that the flexible N-terminal region of RANTES, before the first disulphide bridge, is important for inducing the chemotactic response in T-cells. In this case the peptides give a chemotactic response without the rest of the molecule to restrict their conformation. The individual amino acids responsible for this interaction can now be determined by mutagenesis.

REFERENCES

1. Schall, T. J., Jongstra, J., Dyer, B. J., Jorgensen, J., Clayberger, C., Davis, M. M. and Krensky, A. M. (1988). J. Immunol. 141, 1018-1025
2. Schall. T. J. (1991) Cytokine 3, 165-183
3. Oppenheim, J. J., Zachariae, C. O., Mukaida, N. and Matsushima, K. (1991) Ann. Rev. Immunol., 9, 617-48
4. Hébert, C. A., Vitangcol, R. V. and Baker, J. B. (1991) J. Biol. Chem. 266, 18989-18994

5. Clark-Lewis, I., Schumacher, C., Baggiolini, M., and Moser, B. (1991) J. Biol. Chem. 266, 23128-23134
6. Schraufstatter, I. U. and Cochrane, C. (1990). J. Cell Biol. 111, 48a (Abstr. 248)
7. Suzuki, K., Koshio, O., Ishida-Okawara, A., Shibata, M., Yamakawa, Y., Tagawa, M., Ota, H., Kuramoto, A. and Mizuno, S. (1989) Biochem. Biophys. Res. Commun. 163, 1298-1305
8. Clore, G. M., Apella, E., Yamada, M., Matsushima, K. and Gronenborn, A. M. (1990) Biochemistry, 29, 1689-1696
9. Baldwin, E. T., Weber, I. T., St. Charles, R., Xuan, J.-C., Apella, E., Yamada, M., Matsushima, K., Edwards, B. F. P., Clore, G. M., Gronenborn, A. M. and Wlodawer, A. (1991) Proc. Natl. Acad. Sci. USA, 88, 4498-4502
10. King, D. S., Fields, C. G. and Fields, G. B., (1990), Int. J. Pept. Protein Res., 36, 225-229
11. Boyum A., (1968) Scand. J. Clin. Invest 21, suppl. 97, 77
12. Boyum A., (1968) Scand. J. Clin. Invest 21, 97
13. Kaplan, M. E. and Clark, C., (1974), J. Immun. Meth. 5, 131- 135
14. Neote, K., DiGregorio, D., Mak, J. Y., Horuk, R. and Schall, T. J. (1993) Cell, 72, 415-425
15. Gao, J.-L., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U. and Murphy, P. M. (1993) J. Exp. Med. 177, 1421-1427
16. Wang, J. M. McVicar, D. W., Oppenheim, J. J. and Kelvin, D. J. (1993) J. Exp. Med. 177, 699-705
17. Riper, G. V., Sicilliano, S., Fischer, P. A., Meurer, R., Springer, M. S. and Rosen, H. (1993) J. Exp. Med. 177, 851-856