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HUMAN C5a-RELATED SYNTHETIC PEPTIDES AS NEUTROPHIL CHEMOTACTIC FACTORS

Dennis E. Chenoweth, Bruce W. Erickson and Tony E. Hugli

From the Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037 and Rockefeller University, New York, New York 10021

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SUMMARY

The pentapeptide L-methionyl-L-glutaminyl-L-leucyl-glycyl-L-arginine, which mimics the C-terminal sequence of human C5a anaphylatoxin, and two additional N-formylmethionyl derivatives of this peptide have been assessed for their ability to simulate C5a-related biological activities. Only the N-formylated peptides display chemotactic activity or induce lysosomal enzyme release when assayed with human neutrophils. Additional studies indicate that the active peptides, although designed after the C-terminal structure of the human C5a molecule, were apparently active because of their interaction with the N-formylmethionyl peptide receptor rather than the C5a receptor on neutrophils.

INTRODUCTION

The human complement-derived anaphylatoxin C5a and its des Arg74 derivative are both potent neutrophil chemotactic factors (1,2). Following elucidation of the primary amino acid sequence of human C5a (3) our goals are now to define regions of the linear structure which may play a role in the expression of this particular biological activity. Experiments employing peptide analogs of human C3a have already successfully demonstrated that the spasmogenic activity of the native molecule can be ascribed to the C-terminal region (4) . We elected to begin our C5a structure-function studies with the pentapeptide MQLGR[1], a peptide that mimics the C-terminus of human C5a. There were several reasons for selecting this particular peptide. First, based on previous C3a

[1]Abbreviations used: MQLGR, L-methionyl-L-glutaminyl-L-leucyl-glycyl-L-arginine; N-f-MQLGR, N-formyl-L-methionyl-L-glutaminyl-L-leucyl-glycyl-L-arginine; N-f-MQLG, N-formyl-L-methionyl-L-glutaminyl-L-leucyl-glycine; N-f-MF, N-formyl-L-methionyl-L-phenylalanine; HBSS, Hanks' balanced salt solution without phenol red; DMSO, dimethyl sulfoxide; EDTA, (ethylenedinitrilo)-tetraacetic acid; CI, chemotactic index and ED_{50}, concentration of peptide required to stimulate a half maximal response.

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peptide studies (5), it was felt that five residues would provide the minimum size required for a significant biological activity. Secondly, this peptide contains the single methionyl residue in C5a and hence could provide indirect evidence for the involvement of this residue in governing expression of chemotactic activity. The latter point is deemed particularly important in view of the fact that various N-formylmethionyl peptides are potent chemotactic factors (6). Along these lines, we also examined the biological properties of two formylated C5a derivatives, N-f-MQLGR and N-f-MQLG.

MATERIALS AND METHODS

MQLGR was synthesized by the solid-phase method (7,8) using a previously published protocol (4). N-f-MQLGR was purchased from Peninsula Laboratories (San Carlos, Calif.). N-f-MQLG was prepared by digestion of N-f-MQLGR with 5% (w/w) carboxypeptidase B (Worthington) for 1 hr at 37° in 0.05M $NaHCO_3$, pH 8.5. N-formyl-L-methionyl-L-phenylalanine (N-f-MF) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Peptides were dissolved either directly in Hank's balanced salt solution without phenol red (HBSS), or in the case of N-f-MF, in DMSO prior to dilution with HBSS. The final concentration of each peptide was 10^{-2}M.

Homogeneous human C5a was obtained from yeast cell-activated serum containing 1M/ϵ-aminocaproic acid as described by Fernandez and Hugli (9). Radiolabeled (^{125}I) C5a was prepared by the method of Bolton and Hunter (10), with free ^{125}I being removed by dialysis. Specific activity of ^{125}I-C5a was 10 μCi/μg.

Peptide and C5a compositions and concentrations were obtained by total amino acid analysis after acid hydrolysis (11) employing a Beckman amino acid analyzer (Model 121M). In the case of N-f-MQLG, complete liberation of the C-terminal arginine from N-f-MQLGR by carboxypeptidase B was established by direct amino acid analysis (See Table I).

Highly purified human neutrophils were prepared from EDTA-anticoagulated venous blood obtained from healthy male donors as described by Boyum (12). Contaminating erythrocytes were largely removed by sedimentation in the presence of 1.5% Dextran T500 with residual erythrocytes removed by hypotonic lysis. Typical preparations contained 95±2% neutrophils and cell viability was 97 ± 2% as judged by trypan blue dye exclusion.

Neutrophil chemotaxis was assessed by a serum-free modification of the chemotaxis under agarose method (13). Results are expressed as the chemotactic index (A/B), i.e., the linear distance of neutrophil migration toward the test factor (A) divided by the distance of migration toward an appropriate buffer control (B). Thus a chemotactic index of greater than one indicates that the test substance possesses chemotactic activity for human neutrophils.

The ability of peptides to induce lysosomal enzyme release from cytochalasin B-treated neutrophils was determined by previously described methods (14). Typically, neutrophils in HBSS (4×10^6/ml) were incubated with cytochalasin B (10μg/ml) for 15 min at 37° prior to the

TABLE I

CHEMICAL CHARACTERIZATION OF SYNTHETIC C5a PEPTIDES

A. Amino Acid Compositions of Synthetic C5a Peptides

	MQLGR (mols/mol)	N-f-MQLGR (mols/mol)
Arginine	1.03	0.94
Glutamic acid [a]	1.01	0.99
Glycine	0.99	0.95
Methionine	0.95	0.92
Leucine [b]	1.00	1.00

[a] Glutamine determined as glutamic acid.
[b] Molar ratios were normalized based on leucine recovery.

B. Carboxypeptidase B Conversion of N-f-MQLGR to N-f-MQLG

Conditions	Arginine Released (mols/mol)
Digestion of N-f-MQLGR pancreatic carboxypeptidase B for 1 hr at 37° in 0.05M $NaHCO_3$, pH 8.5	0.92

addition of an equal volume of the peptide stimulus. After incubation for an additional 30 min at 37°, cells were removed by centrifugation and the supernatants analyzed for extracellular enzyme activity. Activity of the lysosomal marker enzyme β-glucuronidase (E.C. 3.2.1.31) was determined using phenolphthalein glucuronidate as substrate after 20 hrs of incubation at 37° (15). Activity of the cytoplasmic marker enzyme lactate dehydrogenase (E.C. 1.1.1.27) was determined with pyruvate as substrate (16).

Peptide inhibition of ^{125}I-C5a binding to intact neutrophils was determined as previously described (17). A simultaneous addition method was employed, i.e., a fixed concentration of ^{125}I-C5a (2.5×10^{-9}M) and various concentrations of the peptides were mixed and then cells were added to the mixture. Following incubation for 30 min at 24°, the amount of cell-bound ^{125}I-C5a was determined.

RESULTS AND DISCUSSION

The three synthetic peptides were first examined to determine if they could promote human neutrophil chemotaxis in the agarose assay. As shown in Fig. 1, only the N-formyl-methionyl derivatives N-f-MQLGR and N-f-MQLG displayed chemotactic activity. Based on the ED_{50} values, calculated by probit analysis (18), N-f-MQLG (3.3×10^{-5}M; correlation coefficient, .99) was found to be about 10-fold more active than its parent peptide N-f-MQLGR (3.2×10^{-4}M; correlation coefficient, .99). Both of these active peptides displayed a typical dose-response profile, stimulating cellular migration at low concentrations

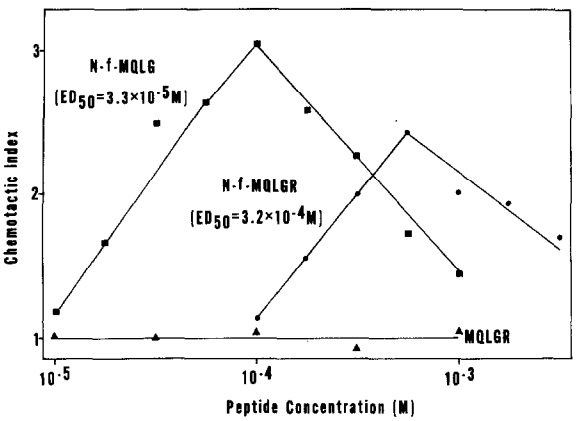

Figure 1 - Human neutrophil chemotactic response to varying concentrations of human C5a-related peptides. Human neutrophils (2.5 x 10^5 cells/10 µl) were exposed to varying concentrations of peptide chemotactic factor (10 µl) for 2 hrs at 37° with HBSS (10 µl) serving as the buffer control. Following fixation, the linear distance of cellular migration toward the peptide (A) and the control buffer (B) were determined and the chemotactic index (A/B) calculated. Peptides employed: (▲) MQLGR, (●) N-f-MQLGR and (■) N-f-MQLG. Each data point represents the mean value of triplicate determinations in a single experiment. SEM: MQLGR, ± 7.0%; N-f-MQLGR, ± 5.0%; and N-f-MQLG, ± 5.4%.

The pentapeptide MQLGR was devoid of any measurable activity even at a concentration as great as 1 mM. Absence of a characteristic desensitization-type cellular pattern (13) at any concentration of MQLGR tested clearly ruled out the possibility that an excessive concentration of the peptide had been employed.

An excellent correlation was observed between the ED_{50} values for chemotaxis and those obtained when each peptide was assessed for its ability to induce the release of β-glucuronidase from cytochalasin B-treated cells. As shown in Fig. 2, N-f-MQLG and N-f-MQLGR were active in this regard, while MQLGR was inactive. Again N-f-MQLGR was approximately 10-fold more active than N-f-MQLGR as judged by the ED_{50} values of 5.1 x 10^{-5}M (correlation coefficient, 0.96) and 3.3 x 10^{-4}M (correlation coefficient, 0.99), respectively. Release of β-glucuronidase was specifically induced by the two peptides and was not the result of cytolysis, as judged by the fact that the cytoplasmic marker enzyme lactate dehydrogenase was not released in excess of that released from cells exposed to buffer alone.

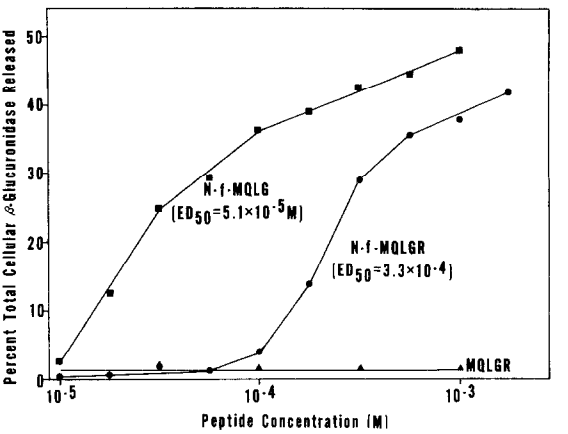

Figure 2 - Release of β-glucuronidase from cytochalasin B-treated human neutrophils induced by varying concentrations of human C5a-related peptides. Human neutrophils (5×10^5 cells in a total volume of 250 µl) were incubated with peptide for 30 min at 37° and then removed by centrifugation. The amounts of β-glucuronidase present in the supernatant were determined and activity is expressed as the percentage of the total enzyme activity liberated from cells after disruption with 0.2% Triton X-100 (6.4 µg phenolphthalein/10^6 cells/hr). Peptides employed: (▲) MQLGR, (●) N-f-MQLGR and (■) N-f-MQLG. Each data point represents the mean value of duplicate determinations in a single experiment. SEM: MQLGR, 13.2%; N-f-MQLGR, 6.2%; and N-f-MQLG, 5.4%.

Results from both chemotaxis and enzyme release measurements in human neutrophils clearly indicate that formylation of the N-terminus is required for expression of biological activity. These results suggest that N-f-MQLG and N-f-MQLGR function by resembling the N-formylmethionyl peptides (6) rather than C5a. Since it has been established that the N-formylmethionyl peptides (19) and C5a (17) interact with separate neutrophil receptors, we attempted to determine if N-f-MQLGR was indeed interacting with receptors for the N-formylmethionyl peptides as opposed to the C5a receptors. In one set of experiments, neutrophils were incubated with either HBSS, C5a, N-f-MQLGR or the model N-formylmethionyl peptide N-f-MF. In each instance, the respective chemotactic factor was introduced to the incubation mixture at a concentration which is known to desensitize the cell for chemotactic responsiveness (2). In the case of C5a, it is known that this desensitizing concentration corresponds with the concentration required for saturation of the cellular C5a receptor (17). Following this incubation, the cells were then challenged to respond chemotactically to either the same or to different chemotactic

TABLE II

NEUTROPHIL CHEMOTACTIC RESPONSE FOLLOWING INCUBATION WITH VARIOUS CHEMOTACTIC FACTORS

Factor Added to Neutrophils (Concentration, M)[a]	Chemotactic Stimulus (Concentration, M) C5a (1.2×10^{-8}) CI[b]	N-f-MF (5×10^{-6}) CI	N-f-MQLGR (7.5×10^{-4}) CI
HBSS (control)	2.5 ± 0.1	3.7 ± 0.2	3.5 ± 0.2
C5a (1.2×10^{-7})	1.0 ± 0.1	2.9 ± 0.1	2.6 ± 0.1
N-f-MF (1×10^{-4})	1.7 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
N-f-MQLGR (1×10^{-2})	2.1 ± 0.2	1.5 ± 0.1	1.0 ± 0.1

[a] Neutrophils allowed to incubate with added factor for 10 min at 37° prior to being employed in the chemotaxis assay.

[b] Chemotactic index (± SEM, N=3). A CI value of unity indicates that directed migration did not occur.

factors, each factor being employed at a concentration known to stimulate a maximal chemotactic migratory response. As shown in Table II, when neutrophils are incubated with HBSS alone, they respond chemotactically to N-f-MQLGR, N-f-MF and C5a in the expected fashion. When incubated with C5a, the cells are still capable of migrating in response to N-f-MQLGR or N-f-MF, but not to C5a. On the other hand, exposure of the neutrophils to N-f-MF or N-f-MQLGR abolishes or markedly impairs their chemotactic response to either of these peptides while leaving the cells free to response to C5a. These findings are consistent with those of Aswanikumar et. al.(20) who have similarly demonstrated the individual nature of the receptors for N-formylmethionyl peptides and those for C5a. The fact that N-f-MQLGR does not interfere with C5a-mediated chemotaxis, but does greatly supress neutrophil chemotaxis mediated by N-f-MF suggests a specific interaction of N-f-MQLGR with the N-formylmethionyl peptide receptor.

A second type of experiment also indicated the N-f-MQLGR was acting through the N-formylmethionyl peptide receptor. In these experiments, MQLGR and N-f-MQLGR were assessed for their ability to inhibit the specific binding of ^{125}I-C5a to intact neutrophils. Neither peptide at concentrations up to 1 mM, influenced binding of 2.5×10^{-9}M ^{125}I-C5a. Compared to buffer controls, ^{125}I-C5a binding in the presence of 1 mM MQLGR was 100.4 ±

2.8% (SEM, N=6) and in the presence of 1 mM N-f-MQLGR was 100.3 ± 1.4% (SEM, N=6). Thus neither the functionally inactive peptide MQLGR nor the chemotactically active, formylated derivative was capable of inhibiting C5a binding to intact neutrophils.

The pentapeptide MQLGR, which duplicates the C-terminal portion of the human C5a primary structure, was found to be devoid of biological activities normally ascribable to the intact parent molecule. Even at concentrations as great as 1 mM, there was no evidence that MQLGR could promote human neutrophil chemotaxis, induce β-glucuronidase release from cytochalasin B-treated cells or could inhibit the specific binding of ^{125}I-C5a to intact cells. This particular peptide possesses less than 0.002% of the spasmogenic activity of native C5a, since 1.1 μmole of MQLGR was unable either to contract a guinea pig ileal strip or to desensitize the strip to C5a-induced contraction (4). Therefore, MQLGR as a peptide analog of C5a differs significantly from the corresponding pentapeptide analog of C3a which is known to simulate C3a-related spasmogenic activity (4). On the other hand, the present results are consistent with our conceptual model for the neutrophil C5a receptor which implies a requirement for binding interactions between both the internal disulfide-crosslinked portion and the C-terminal portion of C5a for expression of biological activity (2). On this basis, it would seem that the pentapeptide, although duplicating the C-terminal C5a sequence, is of insuficient length to promote active binding to the cellular receptor. This hypothesis can be further tested by expanding the series of C-terminal synthetic C5a peptides to include polypeptides of greater length.

Of great interest was the finding that chemotactic and lysosomal enzyme releasing activity could be imparted to MQLGR simply by N-formylation of the N-terminal methionine residue. In addition, removal of the C-terminal Arg from N-f-MQLGR to produce N-f-MQLG actually increased chemotactic and enzyme releasing activities some 10-fold. In contrast, $C5a_{des\ Arg}$ is approximately 30-fold less active than intact C5a for promoting chemotaxis or lysosomal enzyme release (2) and inactive as a spasmogen. It remains to be determined whether the increased activity of N-f-MQLG relative to N-f-MQLGR is the result of peptide length or the increased hydrophobicity associated with loss of the cationic arginyl side chain. It seems clear that both

N-formylmethionyl peptides, although based structurally on the human C5a sequence, are expressing their biological activity because of an interaction with the N-formylmethionyl receptor rather than with the C5a receptor on human neutrophils.

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