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Inhibition of C5a-Induced Neutrophil Chemotaxis and Macrophage Cytokine Production *In Vitro* by a New C5a Receptor Antagonist

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ABSTRACT. A cyclic peptide, Phe-[Orn-Pro-D-Cyclohexylalanine-Trp-Arg] (F-[OPdChaWR]), was recently shown *in vitro* to antagonise the binding of C5a to its receptor (CD88) on human polymorphonuclear leukocytes (PMNs) and *in vivo* to inhibit the neutropenia associated with septic shock induced by lipopolysaccharide (LPS) in rats. The aim of this study was to investigate whether F-[OPdChaWR] inhibits C5a-mediated chemotaxis of human PMNs using a modified Boyden chamber and C5a-stimulated release of cytokines from human monocytes *in vitro*. Approximately 50% of the chemotactic activity induced by 10 nM C5a was inhibited by 76 nM F-[OPdChaWR]. This correlated with inhibition of C5a-induced polarisation of PMNs by F-[OPdChaWR]. C5a alone failed to induce release of the inflammatory cytokines interleukin(IL)-1β, tumour necrosis factor (TNF)-α, and IL-6 from human monocytes at concentrations up to 100 nM. However, in the presence of low concentrations of LPS (50 ng/mL), both IL-1β and TNF-α were stimulated by 1 nM C5a. This co-stimulation was inhibited by F-[OPdChaWR] with IC_{50}s of 0.8 and 6.9 nM for release of TNF-α and IL-1β, respectively. No agonist activity was detected for F-[OPdChaWR] in either the chemotaxis or cytokine release assays at concentrations up to 50 μM. These results show that F-[OPdChaWR] inhibits several important inflammatory activities of C5a and suggest that C5a receptor antagonists may be effective in the treatment of inflammatory diseases mediated by C5a. BIOCHEM PHARMACOL **60**;5:729–733, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. C5a; C5a antagonist; CD88 antagonist; chemotaxis; tumour necrosis factor-α; interleukin-1β

Anaphylatoxins produced following the activation of the complement system are associated with a variety of pathologies. The anaphylatoxin C5a is generated via either classical or alternative pathways by the cleavage of the α-chain of the fifth component of complement (C5) by the serine protease known as C5 convertase. C5a has been shown to be an important factor stimulating many processes of acute inflammation. Complement factors were initially shown to be chemotactic for leukocytes by Boyden, who noted the appearance of a heat-labile chemotactic factor for polymorphonuclear leukocytes in serum following the addition of immune complexes [1]. Recently, C5a has been shown to have an immunoregulatory role and may alone [2, 3], or in combination with other factors [4, 5], stimulate mediators of both chronic and acute inflammation.

The significance of C5a in several inflammatory diseases is demonstrated by the fact that agents that blocked the action of C5a also suppressed inflammation in several animal models [6–10]. Most of these studies used blocking antibodies raised against C5a [7, 9] or recombinant proteins that are receptor antagonists and analogues of C5a [6, 10]. However, there are many problems associated with the use of such proteins to treat human patients. Immunogenicity is a common problem and proteins are expensive to manufacture, very susceptible to degradation by proteases in serum or the gastrointestinal tract, and generally display poor pharmacokinetic properties. Therefore, smaller molecules that are more stable, cheaper to make, with fewer side effects and better bioavailability are more attractive drug candidates for treating human diseases mediated by C5a.

This study was designed to investigate several possible anti-inflammatory activities of a novel synthetic C5a receptor antagonist *in vitro*. A recent study demonstrated that this antagonist, F-[OPdChaWR]¶ (Fig. 1), was very effective in an animal model at inhibiting the neutropenia characteristic of septic shock, but its mode of action is yet

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¶ *Abbreviations:* F-[OPdChaWR], Phe-[Orn-Pro-D-cyclohexylalanine-Trp-Arg]; PMN, polymorphonuclear leukocytes; fMLP, *N*-formylmethionine leucyl-phenylalanine; LPS, lipopolysaccharide; IL-1β, interleukin-1beta; and TNF-α, tumor necrosis factor-alpha.

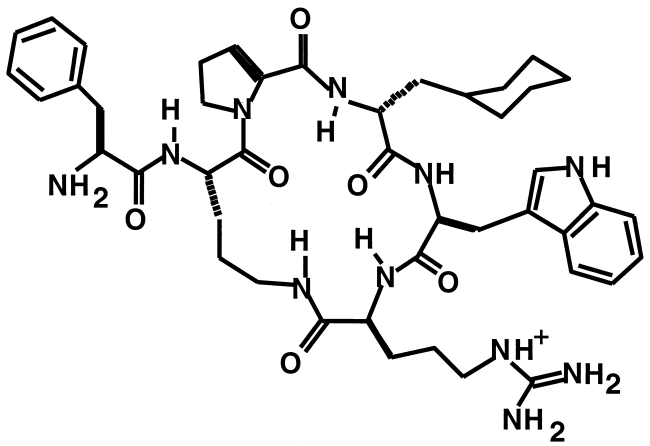

FIG. 1. Chemical structure of F-[OPdChaWR].

to be defined at the level of cellular responses [8]. The effects on two inflammatory activities of C5a were investigated in the present study. Firstly, the effect of F-[OPdChaWR] on the migration of PMN was determined by investigating its ability to inhibit induction of the formation of motile forms of PMN as well as PMN chemotaxis using a modified Boyden chamber. Secondly, its ability to inhibit production of inflammatory cytokines by human monocytes prestimulated by C5a was also investigated. Inhibition by F-[OPdChaWR] of the action of such C5a-mediated activities that are associated with both acute and chronic inflammation would help to further establish potential therapeutic roles for C5a receptor antagonists in human inflammatory diseases.

MATERIALS AND METHODS

Materials

C5a was purchased from Sigma Chemical Co. F-[OPdChaWR] was synthesised as previously described [8]. The water-soluble receptor antagonist features six amino acids (L-phenylalanine, L-ornithine, L-proline, D-cyclohexylalanine, L-tryptophan, L-arginine) connected in the sequence F-O-P-dCha-W-R with the C-terminus of the arginine residue condensed onto the side chain amine of the ornithine (Fig. 1). The key receptor-binding residues of the resulting cyclic peptide F-[OPdChaWR] (F, dCha, W, R) are restrained in a turn conformation as demonstrated by a solution structure determination using NMR spectroscopy [11, 12]. Using competition experiments with ^{125}I-C5a, the apparent receptor-binding affinity (IC_{50}) of F-[OPdChaWR] for human PMNs was recently reported to be 60 nM [12], and for membranes from human umbilical artery containing C5a receptor-bearing macrophages to be 100 nM [13].

Chemotaxis

The chemotaxis experiments using human blood PMNs were carried out using a modified Boyden chemotaxis chamber as previously described [14]. All individual experiments were carried out in triplicate. In the first series of experiments, the number of PMNs passing through the 3.0 μM sparse-pore membrane in response to C5a was determined over a concentration range of 0.1–1000 nM. A concentration of 10 nM C5a was shown to give approximately 50% of the maximum chemotactic response induced by C5a and was chosen as the concentration to be used to test chemotaxis inhibition by F-[OPdChaWR]. C5a at 10 μM induced very similar levels of chemotaxis to that exhibited by fMLP at a concentration of 10^{-8} M FMLP (10^{-8} M) and was used as a positive control for chemotaxis. In addition, F-[OPdChaWR] at concentrations between 0.5 and 50 μM did not affect the chemotactic activity of 10^{-8} M fMLP.

Polarisation

The morphological response of PMNs to C5a was studied according to previously published procedures [15]. The effects of F-[OPdChaWR] were examined by pretreating cells for 10 min with 1, 10, or 100 nM of the C5a antagonist prior to addition of stimuli. The morphologies of fixed cells were observed using differential interference contrast optics (DIC; Nomarski) and classified into the subtypes, described in Fig. 3. At least one hundred cells were classified per treatment level.

Cytokine Production

Human blood monocytes were obtained from healthy donors as previously described [16]. In the first set of experiments, monocytes were incubated with various concentrations of C5a (0 and 0.005–5000 nM) and LPS (0 and 0.5–5000 ng/mL) (*Escherichia coli* B114, Sigma Chemical Co.) for 24 hr, and the levels of the cytokines IL-1β, IL-6, and TNF-α released were measured by ELISA, using commercially available matched antibodies purchased from R&D Systems. From these experiments, it was shown that C5a did not stimulate any of the cytokines in the absence of LPS. IL-6 release stimulated by LPS was not further stimulated by C5a. However, stimulation of the release of IL-β and TNF-α by low levels of LPS was enhanced by C5a. From these results, it was decided to use a concentration of 50 ng/mL of LPS and 1 μM C5a to test the ability of F-[OPdChaWR] to inhibit cytokine release.

RESULTS

Chemotaxis

C5a strongly stimulated PMN chemotaxis (see Methods). The C5a antagonist was tested over a range of concentra-

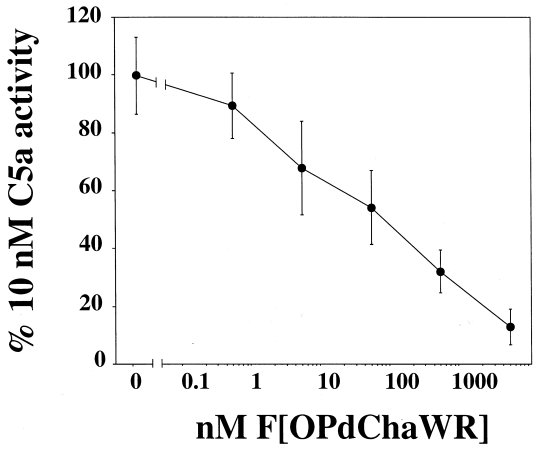

FIG. 2. Inhibition of C5a induced chemotaxis of PMN by F-[OPdChaWR]. Data are means ± SEM of 5 experiments carried out in triplicate.

tions from 0.5 to 5000 nM with ~90% of the inhibition of chemotactic activity observed at a concentration of 5000 nM. The IC_{50} for the inhibition of 10 nM C5a was 75 nM of the antagonist (Fig. 2). Agonist activity of F-[OPdChaWR] was tested over a wide range of concentrations (0.5–50 μM), but no agonist activity was detected at any concentration examined.

Polarisation

The effects of F-[OPdChaWR] on the morphological responses of neutrophils to C5a and fMLP are summarised in Fig. 3. Pretreatment of cells with up to 100 nM F-[OPdChaWR] did not alter the normal response of PMNs to fMLP, indicating that the antagonist does not bind to the fMLP receptor. However, F-[OPdChaWR] had a dramatic inhibitory effect on the shape changes induced by 1 nM C5a. Pretreatment of cells with an equimolar (1 nM) concentration of F-[OPdChaWR] reduced the response to C5a by approximately 50%, based on relative numbers of spherical and fully polarised cells. Total inhibition (>95% spherical cells) was observed with a 10-fold higher concentration of F-[OPdChaWR].

Cytokine Production

Figure 4 shows that F-[OPdChaWR] inhibited both IL-1β and TNF-α production stimulated by C5a in the presence of 50 ng/mL of LPS. C5a alone at concentrations up to 500 nM did not stimulate detectable levels of cytokines (<8 pg/mL). LPS at 50 ng/mL resulted in 196–66 ng/mL of IL-1β and 640–57 ng/mL of TNF-α. The IC_{50} values were calculated to be the concentration of F-[OPdChaWR] that inhibited 50% of the additional cytokines released by C5a

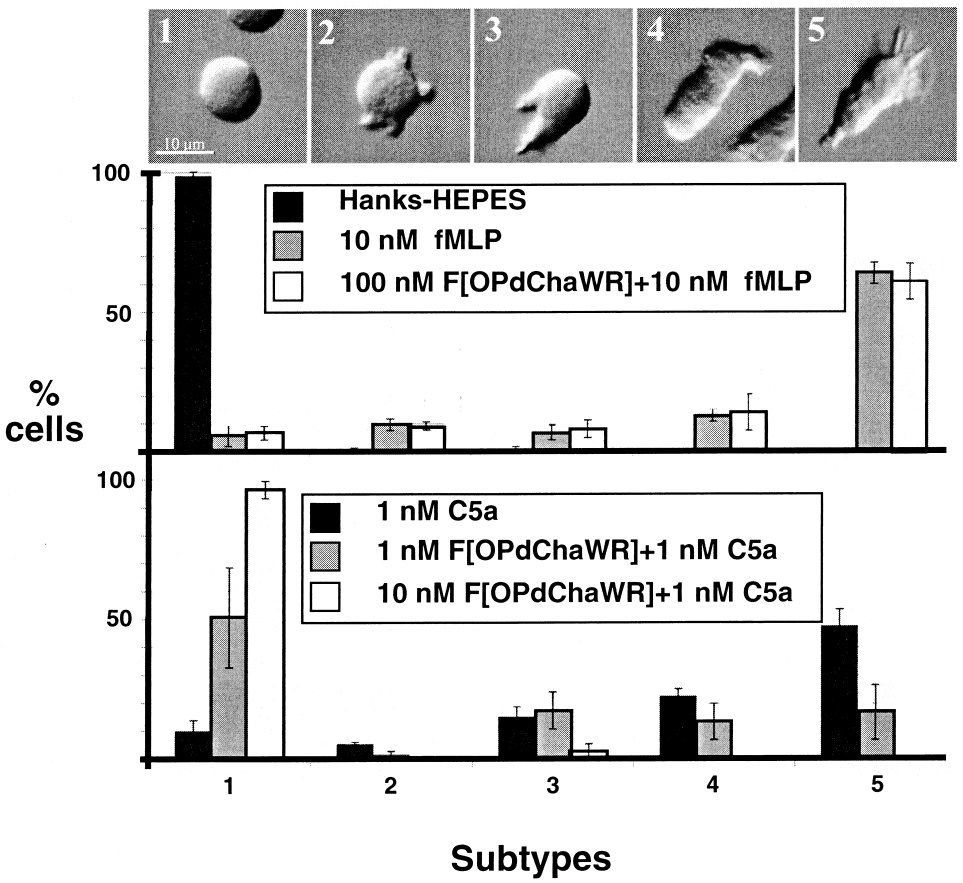

FIG. 3. Specific inhibition of C5a-induced polarisation by F-[OPd ChaWR]. PMN were preincubated for 10 min with or without F-[OPdChaWR], then incubated for a further 30 min in the presence of either C5a (1 nM) or fMLP (10 nM). The morphology of the suspended cells was then classified according to their degree of polarity as illustrated in the photographs. Data are means ± SEM of 3 experiments.

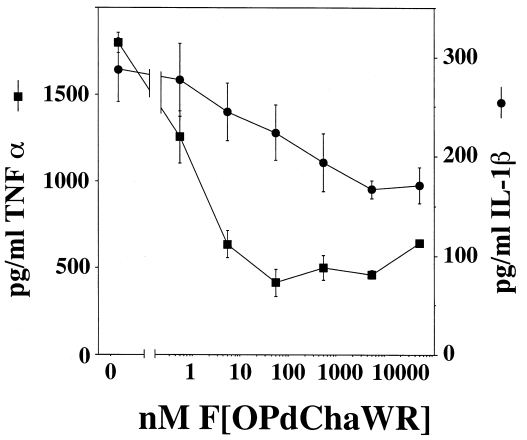

FIG. 4. Inhibition of IL-1β and TNF-α release induced by C5a in the presence of 50 ng/mL of LPS by F-[OPdChaWR]. Data are means ± SEM of 5 experiments carried out in duplicate.

(in the presence of LPS above the levels released with LPS treatment alone). The IC_{50} of F-[OPdChaWR] for TNF-α was 0.8 nM and for IL-1β was 6.9 nM. F-[OPdChaWR] at concentrations ranging from 0.5 to 50 μM did not affect the release of any cytokines either alone or in the presence of 50 ng/mL of LPS.

DISCUSSION

Many inflammatory responses are characterised by the accumulation of PMNs at an inflammatory site. Where local inflammation is triggered by infection, trauma, or immune complex deposition, C5a is likely to be an important chemokine. The locomotory response of PMN to C5a, like those to other chemotactic factors, begins with a rapid change in cell morphology that is most clearly studied in cell suspensions [15]. Changes in cell shape correlate with those required for optimal migration across a substrate and can therefore be used as a sensitive assay for measuring activation of the internal motile apparatus [17]. Moreover, factors that directly affect the internal motile apparatus can be distinguished from those that alter migration by simply affecting adhesion. In the present study, F-[OPdChaWR] potently inhibited C5a-induced chemotaxis, and the polarisation data indicate that this inhibition was due to a direct effect of blocking C5a receptors rather than to a change in cell adhesion molecule expression *per se*. The lack of detectable agonist effects of F-[OPdChaWR], in a wide range of concentrations, suggests that the compound is a pure antagonist, which is in good agreement with *in vivo* studies demonstrating that the compound does not cause neutropenia when administered intravenously [8].

It is well established that C5a can stimulate both IL-1β and TNF-α production *in vitro* [2–5]. These cytokines share many activities, including the ability to induce fever and shock syndrome when injected into animals [18]. In addition, they may both stimulate production of, and synergise with, each other to amplify many of the effects seen in the local Schwartzman reaction [19] and septic shock [18]. C5a may be an important initial trigger in the damaging inflammatory cascade in these pathologies. The concept that C5a is upstream of the cytokines in the cascade of events is supported by studies showing that blocking TNF-α in experimental sepsis inhibits several markers of inflammation but not complement activation [20].

It is reported that C5a can induce IL-1 and TNF-α production in mononuclear cells in the absence of other factors [2, 3]. However, our findings are in agreement with more recent reports [4, 5] showing that C5a requires a co-stimulation with LPS to induce release of these cytokines. One reason for this difference might be that this, as well as the more recent studies, have used recombinant C5a rather than native C5a purified from serum. However, we found that recombinant C5a was active in our chemotaxis assay by itself. Other factors might have been present in samples of C5a used previously, such as LPS contamination or interferon gamma [4, 5], which may synergise with C5a. There is evidence that C5a primes cells for the production of IL-1β and TNF-α by stimulating transcription, and a second stimulus, such as LPS, is required before these cytokine proteins are synthesised and released by cells [21].

F-[OPdChaWR] was designed to inhibit the activity of C5a by competing with it for binding to the human C5a cell surface receptor but, unlike C5a, F-[OPdChaWR] does not activate the receptor when it binds. It appears from our reported studies that F-[OPdChaWR] binds equally well to C5a receptors on human PMNs [12] and mononuclear cells such as macrophages or monocytes [13]. At a molecular level, our studies show that 7–8 molecules of F-[OPdChaWR] inhibited the PMN chemotactic activity of 1 molecule of C5a, while approximately 1 molecule of F-[OPdChaWR] inhibited the stimulation of TNF-α by 1 molecule of C5a. This high efficiency of inhibition by F-[OPdChaWR] may be in part due to the way C5a receptors are expressed on the cell surface. Unbound receptors present on the cell surface are rapidly internalised once they bind to C5a [22]. It is likely that F-[OPdChaWR] binding masks C5a receptors on the cell surface and effectively blocks stimulation by C5a.

In conclusion, we have found that the C5a antagonist, F-[OPdChaWR], effectively blocks two proinflammatory activities of C5a for human PMNs and monocytes. C5a is likely to be the initial trigger of the cascade of inflammatory events that leads to many local and systemic inflammatory pathologies such as the Schwartzman reaction and septic shock. Stable and effective molecular antagonists of C5a such as F-[OPdChaWR] may be important drug candidates for development of treatments of a variety of diseases that involve C5a, such as infections, trauma, and pathologies associated with immune complex formation.

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