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SPECIAL REPORT

Response-selective C5a agonists: differential effects on neutropenia and hypotension in the rat

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Some *in vivo* activities of two complement C5a agonist analogues have been evaluated by measuring changes in blood pressure and neutropenia in the rat and comparing the results with their receptor affinities in peritoneal macrophages and polymorphonuclear leucocytes (PMNs). *In vitro* C5a receptor (C5aR) binding experiments showed that YSFKPMPLaR and YSFKD(NMeNle)PlaR had similar affinities for the macrophage C5aR (IC_{50} 0.2, 0.1 μM respectively). In PMNs, the affinity of YSFKPMPLaR (IC_{50} 0.1 μM) was similar to that in macrophages, whereas the affinity of YSFKD(NMeNle)PLaR for the PMN C5aR was >100 μM. Given i.v., YSFKD(NMeNle)PLaR had similar activity to YSFKPMPLaR on blood pressure but did not cause neutropenia.. These results demonstrate selectivity of a new C5a agonist *in vitro*, which is paralleled *in vivo*. The results suggest the possibility of developing selective agonists of C5a for *in vivo* use in humans.

Keywords: C5a; C5a agonist; neutropenia; hypotension

Abbreviations: C5a, complement factor 5a; C5aR, C5a receptor; PMNs, polymorphonuclear leukocytes

Introduction The inflammatory mediator derived from activation of the complement system, C5a, has a number of actions within the body. It is a chemoattractant for polymorphonuclear leukocytes (PMNs), monocytes and macrophages. It activates these cells, causing them to release other inflammatory mediators and cytokines. Immunostimulatory events have also been attributed to C5a, as it enhances the *in vitro* production of antibodies by T cells (Morgan, 1986). While the C5a receptor (C5aR) has been traditionally thought to be myeloid-cell specific, it has now been shown to be present on a number of different cell types including endothelial (Schieferdecker *et al*., 1997), glial (Gasque *et al*., 1997), and human T-cells (Nataf *et al*., 1999).

C5a agonists have been developed from the C-terminal portion of C5a to investigate the interaction between C5a and its receptor (Ember *et al*., 1992). We have developed panels of peptides which we have shown to be potent agonists on human PMNs, as well as being spasmogenic in human umbilical artery (Finch *et al*., 1997; Sanderson *et al*., 1995). In this tissue, the contractile effects of C5a (Marceau *et al*., 1990) and C5a agonists (Taylor *et al*., 1994) are blocked by indomethacin, indicating that the contractile effects are mediated by prostanoids, thought to be released from intramural macrophages containing C5aRs (Marceau *et al*., 1990). These structure-activity studies have shown that there are major differences in the selectivity of analogues between the vascular preparation and PMNs. The agonists are all more potent in the vascular preparation than in PMNs, but their relative selectivities between the two tissues varies markedly. The reasons for this selectivity are not yet known.

We have recently shown that one of these potent C5a agonists, YSFKPMPLaR, can act as an immunoadjuvant *in vivo* when conjugated to otherwise non-immunogenic peptides (Tempero *et al*., 1997). Although the mechanism of this effect has not yet been elucidated, a likely scenario concerns the binding of the conjugated C5a agonist-epitope to C5aRs on antigen-presenting cells, internalization of the receptor-ligand construct, processing by the major histocompatibility complex and the generation of cytokine signals from these cells to stimulate helper T-cells (Tempero *et al*., 1997). The *in vivo* administration of C5a agonist peptides has the attendant risk of activation of inflammatory C5aRs and unwanted side effects. The development of C5a agonist peptides for use as immunoadjuvants in human clinical medicine will require a more specific ligand for immunologically relevant C5aRs.

In a new structure-activity study on C5a agonists, we discovered that one compound, YSFKD(NMeNle)PLaR, was equipotent to its parent homologue, YSFKPMPLaR, on human umbilical artery, but was considerably less potent on human PMNs, with a very high selectivity ratio ($\sim$3000) between the two systems (Paczkowski, unpublished). These *in vitro* findings opened the possibility of determining if this selectivity could be expressed *in vivo*. In this study we demonstrate in the rat that the new peptide, YSFKD(NMeNle)PLaR, unlike the standard agonist YSFKPMPLaR used in the immunization studies, is capable of causing changes in blood pressure at doses that spare the activation of PMNs *in vivo*, and that this *in vivo* selectivity is paralleled by its respective affinities for macrophage and PMN C5aRs.

Methods *Peptide synthesis* The agonist peptides YSFKPMPLaR and YSFKD(NMeNle)PLaR were synthesized using methods previously described (Finch *et al*., 1997).

Receptor binding assays PMNs and peritoneal macrophages were isolated from rats as previously described (Mosier, 1984; Short *et al*., 1999). C5a (human recombinant, Sigma) or peptides, cells (100,000) and ^{125}I-C5a (50 pM, New England Nuclear, MA, U.S.A.) in 100 μl binding buffer (50 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% bacitracin, 0.5% bovine serum albumin, 100 μM phenylmethylsulphonyl fluoride) were incubated in 0.6 ml polypropylene tubes (60 min, 4°C). An additional 200 μl buffer was added to each tube, followed immediately by centrifugation (11,000 $\times$ *g*, 30 s). The supernatant was discarded, and the tubes containing the

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remaining pellet were measured for radioactivity in an LKB γ counter.

In vivo *measurements of blood pressure and neutropenia* Female Wistar rats (200–240 g) were anaesthetized with i.p. ketamine (80 mg kg^{-1}) and xylazine (12 mg kg^{-1}). A polyethylene catheter was inserted in the femoral vein for peptide administration and blood sample collection. The pulse was recorded using a pfiez finger pulse transducer (ADI Instruments) placed caudally on the tail and blood pressure readings recorded using a pressure transducer connected to a computerized chart recording system (MacLab/8).

Rats were dosed i.v. with agonist peptides (3 mg kg^{-1}) or saline, infused over 1 min. Heparinized blood samples and blood pressure measurements were collected 10 min prior to agonist challenge and periodically over a 90 min observation period. Blood samples (0.2 ml) were layered onto an equal volume of Histopaque-Ficoll solution, PMNs isolated, and cell number was counted on an haemocytometer. PMN concentrations and systemic blood pressure values are presented as mean percentage $\pm$ s.e.mean of the values obtained immediately prior to peptide challenge.

Results *Receptor binding* The affinities of C5a, YSFKPMPLaR and YSFKD(NMeNle)PLaR for the C5aR were measured in rat PMNs and peritoneal macrophages (Figure 1). C5a had similar affinity for the C5aR on both PMNs and macrophages ($-\log IC_{50} = 9.8 \pm 0.1$, 10.2 ± 0.2, respectively, $n = 4-5$). YSFKPMPLaR bound to PMNs and macrophages also with similar affinities ($-\log IC_{50} = 7.1 \pm 0.4$, 6.8 ± 0.4, respectively, $n = 3-4$). Conversely, YSFKD(NMeNle)PLaR had relatively low affinity for the PMN receptor ($-\log IC_{50} < 4$, $n = 3$) compared to the macrophage receptor ($-\log IC_{50} = 7.0 \pm 0.2$, $n = 4$, Figure 1). These marked differences in affinities for macrophages and PMN C5aRs suggested that differences in the PMN- and macrophage-dependent activities *in vivo* might be seen following peptide administration. Accordingly, both neutropenia and blood pressure parameters were measured following i.v. administration of C5a and both peptides to the rat.

Effects on blood pressure and neutropenia Circulating PMN concentrations and systemic blood pressure were measured at regular time points over a 90 min observation period. Intravenous injection of saline controls caused no significant change in either circulating PMN concentrations or systemic blood pressure at any time point when compared to values obtained at 0 min (data not shown). Intravenous administration of C5a (2 μg kg^{-1}) caused a significant decrease in circulating PMNs and systemic blood pressure, reaching a minimum value at 5 min to $16 \pm 6\%$ and $68 \pm 5\%$ of initial values respectively (Figure 2). Intravenous administration of YSFKPMPLaR and YSFKD(NMeNle)PLaR (3 mg kg^{-1}) caused a similar hypotensive response, reaching a minimum value at 15 min to $62 \pm 5\%$ and $46 \pm 7\%$ of initial values respectively (Figure 3a). The same challenge of 3 mg kg^{-1} YSFKPMPLaR also caused a rapid and transient neutropenia, which reached a minimum value to $35 \pm 8\%$ of initial values 15 min after injection, and returned to baseline within 90 min (Figure 3b). Conversely, the same i.v. challenge of 3 mg kg^{-1} YSFKD(NMeNle)PLaR caused no significant neutropenia at any time point ($P > 0.05$, one-way ANOVA) (Figure 3b).

Discussion Intravenous administration of C5a or C5a agonist peptides causes hypotension and neutropenia. The hypotension is largely abolished by pretreatment with cyclo-oxygenase inhibitors, indicating the involvement of prostanoids (Lundberg *et al*., 1987), whereas neutropenia is unaffected by this treatment (Lundberg *et al*., 1987). Neutropenia is caused by the rapid and transient expression of the ICAM, CD11, on PMNs interacting with P-selectin expressed on endothelial cells; both events occur rapidly following activation of C5aRs (Issekutz *et al*., 1999; Ward, 1996). However, both C5a and

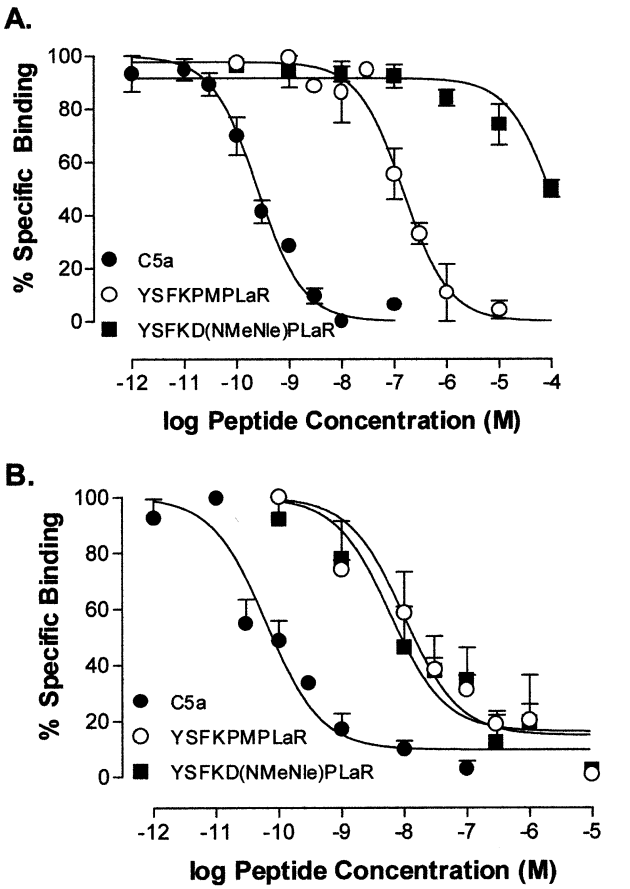

Figure 1 Inhibition of ^{125}I-C5a binding to isolated rat PMNs (A) and peritoneal macrophages (B) with increasing concentrations of human recombinant C5a and agonist peptides. Peptide, cells and ^{125}I-C5a were incubated (4°C, 60 min), and centrifuged before the supernatant was removed, and the remaining pellet counted. All values represent mean $\pm$ s.e.mean ($n \geqslant 3$).

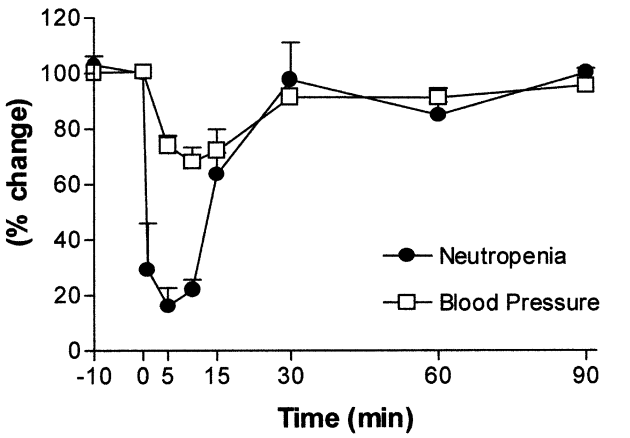

Figure 2 Effects of recombinant human C5a (2 μg kg^{-1} i.v.) on mean blood pressure and circulating PMNs. Data are shown as per cent of pretreatment value, and represent the mean $\pm$ s.e.mean ($n \geqslant 5$).

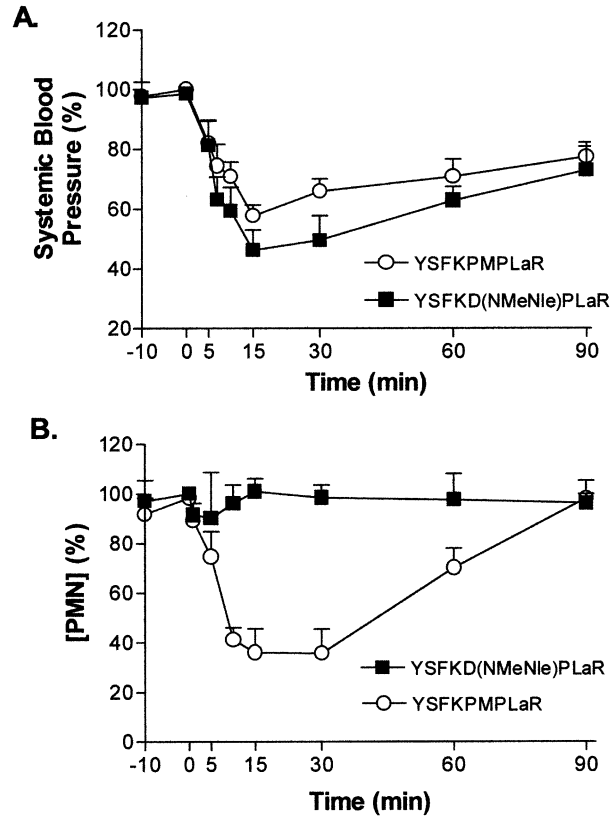

Figure 3 Effects of C5a agonist peptides on mean blood pressure (A) and circulating PMN concentrations (B) in the rat. Rats were anaesthetized and agonist peptides (3 mg kg^{-1} i.v.) administered. Data shown as mean ± s.e.mean ($n \geqslant 3$) for each group.

C5a agonist peptides preferentially cause neutropenia, with higher doses required to affect blood pressure (Drapeau *et al*., 1993). We have confirmed these observations in the rat, demonstrating that C5a (2 μg kg^{-1} i.v.) causes a profound degree of neutropenia, with smaller changes in blood pressure.

C5a causes either constriction or relaxation of isolated blood vessels, depending on the species or type of blood vessel assayed, but in all cases eicosanoids largely mediate the effect (Marceau & Hugli, 1984). Tissue macrophages are thought to be the source of the vasoactive prostanoids released (Marceau *et al*., 1990). In peritoneal macrophages, the C5aR binding affinities of the two agonists were the same, whereas in PMNs, YSFKD(NMeNle)PLaR did not bind to the receptor except in very high concentrations, while YSFKPMPLaR bound to PMN C5aRs with similar affinity as in macrophages.

The present study was performed for several reasons. The *in vitro* binding data in rat PMNs and peritoneal macrophages showed that there were major differences in the affinities of the two peptides for the C5aRs in these cells. Assuming both that macrophages are the source of blood pressure-lowering prostanoids released by C5a or C5a agonists, and that the vascular effects of C5a or C5a agonists *in vitro* are related to their blood pressure changes *in vivo*, it was predicted first that both peptides would be approximately equiactive as hypotensive agents. Based on the PMN binding data, it was next predicted that YSFKPMPLaR would be effective as a neutropenic agent, while YSFKD(NMeNle)PLaR would be essentially inactive. The results obtained confirmed these expectations, and have demonstrated that certain C5a-mediated events *in vivo* can be selectively modulated by low molecular weight C5a agonist peptides. The low affinity of YSFKD(NMeNle)PLaR for the PMN receptor explained its inability to cause neutropenia, while the equivalent binding affinities of both peptides in macrophages correlated well with the blood pressure data. The present results therefore suggest a link between activation by C5a agonists of C5aRs on macrophages and changes in blood pressure.

The basis for the selectivity of C5a peptide analogues for different C5aRs is not yet known. Only one gene coding for the C5aR has been described (Gerard *et al*., 1993), and receptor subtypes for C5a have not yet been clearly delineated. In part, this is due to the paucity of selective agonists and antagonists at C5aRs for receptor characterization. We have previously described the wide variation in C5aR selectivities for C5a agonists in human umbilical artery and PMNs (Sanderson *et al*., 1995; Finch *et al*., 1997). Differential chemotaxis of monocytes and PMNs has been shown by a newly discovered molecule, ribosomal protein S19 homodimer, which had high affinity for the C5aR and cross-reactivity with a monoclonal anti-C5a antibody (Nishiura *et al*., 1998). In this study, the molecule was an agonist at the C5aR on monocytes and macrophages but acted to inhibit C5a-induced chemotaxis of PMNs. Future work with more selective agonists and antagonists may lead to the need for classification of functional C5aR subtypes on the basis of agonist and antagonist selectivities.

The development of C5a agonists for *in vivo* use is foreshadowed by the ability of at least one peptide analogue, YSFKPMPLaR, to act as a molecular adjuvant or immunogen *in vivo* (Tempero *et al*., 1997). For their prospective use in humans, it is desirable to develop agents that are potent, metabolically stable, and selective activators of antigen-presenting cells. Our standard C5a agonist peptide fulfils the first two criteria, but its activity on inflammatory cells such as PMNs is significant, whereas the other peptide described in this study has much less activity on PMNs. This ability to modulate the activation of different C5aRs could eventually lead to the development of agents which are highly selective for C5aRs on antigen-presenting cells such as dendritic cells. The present study supports the feasibility of this notion.

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References

DRAPEAU, G., BROCHU, S., GODIN, D., LEVESQUE, L., RIOUX, F. & MARCEAU, F. (1993). Synthetic C5a receptor agonists. Pharmacology, metabolism and in vivo cardiovascular and hematologic effects. *Biochem. Pharmacol.*, **45,** 1289–1299.

EMBER, J.A., SANDERSON, S.D., TAYLOR, S.M., KAWAHARA, M. & HUGLI, T.E. (1992). Biologic activity of synthetic analogues of C5a anaphylatoxin. *J. Immunol.*, **148,** 3165–3173.

FINCH, A., VOGEN, S., SHERMAN, S., KIRNARSKY, L., TAYLOR, S. & SANDERSON, S. (1997). Biological active conformer of the effector region of human C5a and modulatory effects of N-terminal receptor binding determinants on activity. *J. Med. Chem.*, **40,** 877–884.

GASQUE, P., SINGHRAO, S.K., NEAL, J.W., GOTZE, O. & MORGAN, B.P. (1997). Expression of the receptor for complement C5a (CD88) is up-regulated on reactive astrocytes, microglia, and endothelial cells in the inflamed human central nervous system. *Am. J. Pathol.,* **150,** 31–41.

GERARD, N.P., BAO, L., XIAO, P.H., EDDY, R.J., SHOWS, T.B. & GERARD, C. (1993). Human chemotaxis receptor genes cluster at 19q13.3-13.4. Characterization of the human C5a receptor gene. *Biochem.,* **32,** 1243–1250.

ISSEKUTZ, A.C., ROWTER, D. & SPRINGER, T.A. (1999). Role of ICAM-1 and ICAM-2 and alternate CD11/CD18 ligands in neutrophil transendothelial migration. *J. Leukoc. Biol.,* **65,** 117–126.

LUNDBERG, C., MARCEAU, F. & HUGLI, T.E. (1987). C5a-induced hemodynamic and hematologic changes in the rabbit. Role of cyclooxygenase products and polymorphonuclear leukocytes. *Am. J. Pathol.,* **128,** 471–483.

MARCEAU, F., DEBLOIS, D., LAPLANTE, C., PETITCLERC, E., PELLETIER, G., GROSE, J.H. & HUGLI, T.E. (1990). Contractile effect of the chemotactic factors f-Met-Leu-Phe and C5a on the human isolated umbilical artery. Role of cyclooxygenase products and tissue macrophages. *Circ. Res.,* **67,** 1059–1070.

MARCEAU, F. & HUGLI, T.E. (1984). Effect of C3a and C5a anaphylatoxins on guinea-pig isolated blood vessels. *J. Pharmacol. Exp. Ther.,* **230,** 749–754.

MORGAN, E.L. (1986). Modulation of the immune response by anaphylatoxins. *Complement,* **3,** 128–136.

MOSIER, D.E. (1984). Separation of macrophages on plastic and glass surfaces. *Methods Enzymol.,* **108,** 294–297.

NATAF, S., DAVOUST, N., AMES, R.S. & BARNUM, S.R. (1999). Human T cells express the C5a receptor and are chemoattracted to C5a. *J. Immunol.,* **162,** 4018–4023.

NISHIURA, H., SHIBUYA, Y. & YAMAMOTO, T. (1998). S19 ribosomal protein cross-linked dimer causes monocyte-predominant infiltration by means of molecular mimicry to complement C5a. *Lab. Invest.,* **78,** 1615–1623.

SANDERSON, S.D., KIRNARSKY, L., SHERMAN, S.A., VOGEN, S.M., PRAKASH, O., EMBER, J.A., FINCH, A.M. & TAYLOR, S.M. (1995). Decapeptide agonists of human C5a: the relationship between conformation and neutrophil response. *J. Med. Chem.,* **38,** 3669–3675.

SCHIEFERDECKER, H.L., ROTHERMEL, E., TIMMERMANN, A., GOTZE, O. & JUNGERMANN, K. (1997). Anaphylatoxin C5a receptor mRNA is strongly expressed in Kupffer and stellate cells and weakly in sinusoidal endothelial cells but not in hepatocytes of normal rat liver. *Febs. Lett.,* **406,** 305–309.

SHORT, A., WONG, A.K., FINCH, A.M., HAAIMA, G., SHIELS, I.A., FAIRLIE, D.P. & TAYLOR, S.M. (1999). Effects of a new C5a receptor antagonist on C5a- and endotoxin- induced neutropenia in the rat. *Br. J. Pharmacol.,* **126,** 551–554.

TAYLOR, S.M., FINCH, A.M., HERON, A.E., BROWN, L.C. & FLORIN, T.H. (1994). Reversibility of tachyphylaxis to C5a in guinea pig tissues, perfused human placental lobule, and umbilical artery. *Inflammation,* **18,** 645–657.

TEMPERO, R.M., HOLLINGSWORTH, M.A., BURDICK, M.D., FINCH, A.M., TAYLOR, S.M., VOGEN, S.M., MORGAN, E.L. & SANDERSON, S.D. (1997). Molecular adjuvant effects of a conformationally biased agonist of human C5a anaphylatoxin. *J. Immunol.,* **158,** 1377–1382.

WARD, P.A. (1996). Role of complement, chemokines, and regulatory cytokines in acute lung injury. *Ann. N. Y. Acad. Sci.,* **796,** 104–112.

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