Complement C5a receptors in the pituitary gland: expression and function

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Abstract

Communication between the immune and endocrine system is important for the control of inflammation that is primarily mediated through the hypothalamic–pituitary–adrenal axis. The innate immune system rapidly responds to pathogens by releasing complement proteins that include the anaphylatoxins C3a and C5a. We previously reported the existence of C3a receptors in the anterior pituitary gland and now describe the presence of C5a receptors in the gland. C5a and its less active derivative (C5adR) can bind to its own receptor and to another receptor called C5L2. Using RT-PCR and immunocytochemistry, C5a receptors and C5L2 were demonstrated in the rat anterior pituitary gland and in several rodent anterior pituitary cell lines. Western blotting analysis showed that C5a stimulated the phosphorylation of

MAPK and AKT but not p38; C5adR on the other hand, had no effect on any of the signal molecules investigated. The effects of C5a and C5adR on the secretion of the inflammatory molecule, macrophage migration inhibitory factor (MIF) were investigated by ELISA. Both compounds showed a dose-dependent inhibition of MIF release, 30–40% inhibition at around 35–70 nM agonist with IC₅₀ values of around 20 nM. C5a and C5adR also stimulated ACTH secretion (up to 25%) from AtT-20DV16 cells. These data show that functional C5a receptors (C5a and C5L2) are present in the anterior pituitary gland and they may play a role in dampening down inflammation by inhibiting the release of MIF and stimulating the release of ACTH.

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Introduction

The hypothalamic–pituitary–adrenal (HPA) axis is a major regulator of immunity and inflammation via its secretion of glucocorticoids that suppress the immune activation of leukocytes and inhibit proinflammatory mediators. ACTH is the major regulator of glucocorticoids, yet during inflammation, cytokines such as IL1, IL6, tumour necrosis factor- α and leukaemia inhibitory factor are able to activate the HPA axis, and regulate the secretion of pituitary hormones that, in turn, modulate the function of immune cells (Besedovsky & del Ray 1996, Sternberg 1997). The crosstalk between the immune and endocrine systems is critically important for homeostatic control; dysregulation within this system has been implicated as a contributor to a wide range of acute and chronic inflammatory conditions including septic shock and rheumatoid arthritis (Chrousos 1995, Beishuizen *et al.* 2001, Polito *et al.* 2007). Such diseases are associated with increased cytokine production from immune cells leading to altered activity of the HPA axis (Spangelo & Gorospe 1995, Bijlisma *et al.* 2005).

The innate immune system is also the source of the complement family of molecules that provide the principal effector mechanism of immunity (Gasque *et al.* 2000). Complement comprises a cascade of about 20 proteins that

recognise and eliminate a variety of noxious substances and pathogens. When complement is activated, the components C3 and C5 are proteolytically cleaved to liberate the fragments C3a and C5a that are potent peptide anaphylatoxins and leukocyte chemoattractants that stimulate and modulate the inflammatory response by binding to specific receptors expressed on a wide variety of cell types (Hugli 1990, Monk *et al.* 2007). Both C3a and C5a are rapidly cleaved by serum carboxypeptidases to release the terminal arginine moiety; these desarginated (dR) have distinctly different patterns of activity from the parent molecules. C5adR has little proinflammatory activity (Bokisch & Muller-Eberhard 1970) due probably to its lower (100-fold less) binding affinity for the classical C5a receptor, C5aR (Burgi *et al.* 1994). Similarly C3adR has no detectable binding affinity for the C3a receptor, C3aR. In some assays, however, C3adR appears to have comparable activity to C3a where it has been shown to have anti- rather than proinflammatory properties (Kildsgaard *et al.* 2000). For example C3a and C3adR are equally effective in influencing IL6 secretion from human peripheral mononuclear cells and B cells from the tonsil (Fischer & Hugli 1997, Takabayashi *et al.* 1998). C3adR also stimulates triglycerol synthesis in human adipocytes by binding to a receptor that is distinct from C3aR (Cianflone *et al.* 1989).

C3aR is, as expected, expressed on cells of the myeloid lineage, yet expression is now also known to occur in the

central nervous system and the pituitary and adrenal glands suggesting additional roles for maintaining homeostasis (Gasque *et al.* 1998, Francis *et al.* 2003). We have recently showed that C3a receptors are expressed in the majority of the cell types (corticotrophs, lactotrophs, somatotrophs, thyrotrophs and folliculostellate cells) within the anterior pituitary gland. C3a either *in vivo* or *in vitro* caused a rapid release of ACTH, prolactin and GH but not TSH. Surprisingly, C3adR showed similar activity but the inclusion of pertussis toxin inhibited the action of C3a but not that of C3adR, suggesting the latter may be working through a different, non-G protein coupled receptor (Francis *et al.* 2003).

Although we have demonstrated the presence of C3aR in the anterior pituitary gland there is no information on the expression of C5aR although C5a may also have a role in the activation of the HPA axis (Crane & Buller 2007). C5a, in addition to binding to the C5aR can also bind to an additional receptor, C5L2 that has been recently identified in human and rodent tissues (Cain & Monk 2002, Okinaga *et al.* 2003, Gao *et al.* 2005, Chen *et al.* 2007). C5L2 is also a seven transmembrane domain receptor but appears to be unable to signal through G proteins due to the absence of key G protein-coupling motifs, such as the replacement of leucine by arginine in the so-called DRY motif at the intracellular face of the third transmembrane domain. Human C5L2 binds to both C5a and C5adR with high affinity but rodent C5L2 appears to preferentially bind to C5adR. Conflicting reports of the binding of C3a or C4a to C5L2 remain to be resolved (Cain & Monk 2002, Okinaga *et al.* 2003) and the precise role of this receptor is still unknown; it may serve as a decoy receptor for the removal of excess C5a/C5adR, as has been observed in sepsis patients or it may serve as a mediator of acylation-stimulating protein (C3adR) stimulation of triglyceride synthesis (Kalant *et al.* 2005).

In this report, we show that rat anterior pituitary and several anterior pituitary cell lines express both the C5aR and C5L2. C5a but not C5adR stimulated the activation of signalling molecules ERK/MAPK and AKT. On the other hand, C5a and C5adR both inhibited the secretion of the inflammatory molecule, macrophage migration inhibitory factor (MIF) yet stimulated the secretion of ACTH. These data suggest that C5a and C5adR may act to dampen down inflammatory responses by stimulating the anterior pituitary gland and activating the adrenal gland and inhibiting the secretion of MIF.

Materials and Methods

Cell culture materials and reagents were obtained from Invitrogen, Autogen Bioclear (Calne, UK), Sarstedt Ltd (Leicester, UK) and Sigma-Aldrich. Rat anterior pituitary tissue was taken from male Wistar rats (150–200 g) after cervical dislocation. Rodent pituitary GH3 (GH and prolactin secreting), MMQ (prolactin secreting; Judd *et al.* 1988), RC-4B/C (pituitary adenoma producing gonadotrophin and prolactin; Berault *et al.* 1990, Hurbain-Kosmath

et al. 1990, Polkowska *et al.* 1991) and AtT-20DV16 (corticotrophin secreting) cell lines are in-house and TtT/GF (folliculostellate) cells were kindly provided by Professor Kinji Inoue (Department of Regulation Biology, Saitama University, Urawa, Japan). Recombinant mouse and rat C5a and C5adR were prepared in-house and described previously (Paczkowski *et al.* 1999). Specific polyclonal antisera to the C5a receptor and to mouse and rat C5L2 were prepared in-house, as described (Kalant *et al.* 2005). Co-incubation of the antisera with the appropriate C5a or C5L2 peptides that had been used to immunise the rabbits, markedly reduced their binding capacity to cells. Human/rat CRH was from Sigma-Aldrich. Vectastain ABC kits (Vector Laboratories, Peterborough, UK) were used for immunocytochemistry. Molecular biology reagents, except TRIzol (from Invitrogen), were obtained from Promega. Western blotting reagents were from GE Healthcare (Chalfont St Giles, UK).

Cell culture

GH3, and MMQ cells were cultured in Ham's F12, 15% (v/v) horse serum and 2.5% (v/v) foetal bovine serum (FBS) and TtT/GF and AtT-20DV16 in respectively DMEM 10% (v/v) heat-inactivated (HI) FBS and DMEM 10% (v/v) FBS. RC-4B/C cells were cultured in DMEM/ α MEM (1:1) and 10% (v/v) HI FBS. For immunocytochemistry, cells were cultured on 'Thermanox' coverslips (Invitrogen). For experimental purposes MMQ cells were seeded into poly-L-lysine (70–150 kDa, 0.1 mg/ml) coated dishes or coverslips.

Immunocytochemistry

Immunocytochemical procedures were carried out on paraformaldehyde fixed rat pituitary tissue and acetone-fixed anterior pituitary cell-line monolayers. Antigen retrieval in paraformaldehyde-fixed tissue sections was performed by heating in 10 mM citrate buffer pH6 for 30 min in a microwave oven. Incubations with specific antisera (in house) were carried out either overnight at 4 °C or for 1 h. at room temperature (in the case of β -actin, (from Santa Cruz Biotechnology, Santa Cruz, CA, USA)) at the following concentrations: anti-C5a receptor (1:200), affinity purified anti-mouse and anti-rat C5L2 (respectively 0.1 and 0.04 mg/ml) and β -actin (1:2000). Sections were then incubated for 90 min in the appropriate second antibody coupled to biotin and then visualised with streptavidin-fluorescein. Nuclei were stained with DAPI. For negative controls, non-immune sera were used in place of the specific antisera.

RT-PCR

Total cellular RNA was prepared using TRIzol reagent and treated with RQ1 ribonuclease-free DNase. 0.2 μ g RNA was reverse transcribed using oligodeoxythymidilic acid [oligo(dT)₁₅] for 1 h at 37 °C and the cDNA generated was subjected to PCR amplification using primers specific for rat

and mouse C5a receptor, C5L2 and MIF. Primer sequences (shown in Table 1) were designed using the Primer 3 software programme and gene sequences obtained from GenBank. For the PCR 30–40 cycles were carried out as follows: 94, 65 and 72 °C for respectively 30 s, 1 min and 1 min and a final extension step of 72 °C for 10 min. Amplified products were electrophoresed in 2% (w/v) agarose and visualised with ethidium bromide.

Western blotting analysis

Western blotting analysis was used to investigate the effect of C5a and C5adR on the phosphorylation of p44/42 MAPK, p38 MAPK and AKT in GH3, MMQ and TtT/GF cells. The cells were plated out at a density of 1 (GH3 and MMQ) and 0.5×10^6 (TtT/GF) cells/well in six-well multidishes and incubated overnight. The serum containing media were replaced with serum-free media, again overnight, and then for a further 3 h with fresh serum-free media. Cells were exposed to 70 nM C5a or C5adR for 0, 1, 5, 15, 30 and 60 min, rinsed $3 \times$ with 1 mM sodium orthovanadate in PBS and then lysed in 200 μ l RIPA buffer containing 1 mM sodium orthovanadate, 0.1 mg/ml phenylmethylsulphonyl fluoride and chymostatin, leupeptin, antipain and pepstatin A (all at 10 μ g/ml). Lysates after centrifugation were stored at -80 °C. Fifteen micro litre aliquots from each treatment were mixed with an equal volume of electrophoresis buffer, boiled and electrophoresed in 10% (w/v) polyacrylamide. Proteins were transferred onto PVDF membranes and incubated overnight at 4 °C with antisera to the phosphorylated and total forms of p44/42 MAPK and AKT (Cell Signaling Technology, Beverly, MA, USA) and p38 (Santa Cruz Biotechnology). Antisera were used at the concentrations indicated in the data sheets. Secondary anti-rabbit IgG conjugated to HRP, at 1:5000, were applied for 1 h at room temperature and proteins were visualised with ECL Plus reagent.

Table 1 Primer sequences for rat and mouse (Mo) C5a receptor, C5L2 and MIF

Target	Primer sequence (5'–3')	Amplicon (bp)	Accession numbers
Rat/Mo.C5aR			
F	AGCATTGCTCCTCACCATTTC	248	Y09613 (rat)
R	TCACCACTTTGAGCGTCTTG		NM007577 (mo)
Rat C5L2			
F	TTGCAGTGGCTTCTTCACAC	192	AY600435
R	GATACCTTGGTCACGGCACT		
Mo.C5L2			
F	TTTGCTGGACCCCTTATCAC	244	AK053187
R	GATACCTTGGTCACCGCACT		
Rat MIF			
F	CCATGCCTATGTTTCATCGTG	242	U62326
R	CAGCAGCTTGCTGTAGTTGC		
Mo. MIF			
F	GTGCCAGAGGGGTTTCTGT	206	U20156
R	AGGCCACACAGCAGCTTACT		

Macrophage MIF

MIF secretion was measured in GH3, MMQ, TtT/GF and AtT-20DV16 cells after exposure to rat or mouse C5a, as appropriate. C5adR was also used in some experiments. Briefly, cells (GH3 and MMQ, 0.15×10^6 cells/cm², AtT-20DV16 and TtT/GF, 0.2×10^6 cells/cm²) were plated into 48 well multidishes in their respective culture media for 48 h and then exposed to C5a or C5adR (7, 35 and 70 nM) in media (0.15 ml) containing 1% FCS for 1 h. Conditioned media was centrifuged to remove cell debris and stored at -20 °C. MIF was determined by ELISA (R&D Systems, Abingdon, UK).

ACTH

ACTH secretion was measured in AtT-20DV16 cells (0.025×10^6 /cm²) treated with C5a, C5adR and CRH for 1 h as for the MIF experiments. ACTH was measured by RIA (Peninsula Laboratories, San Carlos, CA, USA).

Statistical analysis

Experiments were performed 2–4 times (*n*) with six replicates for each treatment. Results are expressed as mean $\pm$ S.E.M. and compared by ANOVA and the Tukey multiple comparison test. $P < 0.05$ is deemed to be significant.

Results

Expression of C5a and C5L2 receptors

Immunostaining for the C5a receptor and for C5L2 showed strong expression in the rat anterior pituitary gland; C5a receptor but not C5L2 positive cells were also present in the intermediate and posterior lobes (Figs 1 and 2). There was also clear cytoplasmic staining for the C5a receptor and C5L2 in all of

Figure 1 C5aR immunostaining in (A) rat pituitary, (B) AtT-20DV16, (C) GH3 and (D) RC4/B anterior pituitary cell lines ($\times 100$). A negative control with non-immune rabbit serum (1:50) is shown in (D). (Ant, anterior lobe; Int, intermediate lobe; Post, posterior lobe). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0110>

Figure 2 C5L2 immunostaining in (A) rat pituitary, (B) MMQ, (C) GH3 and (D) TtT/GF anterior pituitary cell lines ($\times 100$). A negative control with non-immune rabbit serum (1:50) is shown in (D). (Ant, anterior lobe; Int, intermediate lobe; Post, posterior lobe). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0110>

the pituitary associated cell lines tested (Figs 1 and 2) demonstrating wide distribution within different pituitary (corticotrophs, lactotrophs/somatotrophs and folliculostellate) cell types. The specificity of staining with C5L2 anti-sera was confirmed by co-incubation with the immunising peptides, which considerably reduced the level of antibody binding to the pituitary cell lines. RT-PCR studies (Fig. 3) demonstrated the presence of C5a receptor and C5L2 mRNA in pituitary cells and cell lines consistent with the immunocytochemistry findings. All amplicons were of the expected size and sequencing confirmed their identities (not shown).

Activation of signal molecules

To test whether the C5a receptor is functional, the effect of C5a and C5adR on the phosphorylation of p44/42 MAPK (T202/Y204), p38 and AKT (Ser 473) was investigated in TtT/GF, MMQ and GH3 cells. Figure 4A shows the time-course effect of rat C5a on p44/42 phosphorylation and total p44/42 in MMQ cells; phosphorylated p44/42 was detectable after 15 min and reached a plateau after 30 min of exposure. Similar findings were found in both the GH3 and TtT/GF cell lines (data not shown). C5adR, over the same time frame had no effect on p44/42 phosphorylation. C5a but not C5adR had similar effects on AKT phosphorylation with similar plateau time points of 30 min in both MMQ and TtT/GF cells (Fig. 4B). There was no clear effect of C5a on p38 MAPK phosphorylation in any of the cell lines (data not shown).

MIF secretion

The anterior pituitary gland is a major source of MIF and its expression has been demonstrated in corticotrophs and thyrotrophs. We have used RT-PCR to show that MIF is synthesised in many cell types within the anterior pituitary gland; these include lactotrophs/somatotrophs and folliculostellate cells (Fig. 5). Both C5a and C5adR inhibited, in a dose-related manner, the secretion of MIF in MMQ, GH3 and AtT-20DV16 cells over a 1 h incubation time period (Fig. 6). MIF secretion was inhibited by 30–40% in the presence of 35–70 nM C5a or C5adR with IC₅₀ values of around 20 nM. Interestingly, C5adR was at least as potent as C5a in this assay. No significant inhibitory or stimulatory effects were observed beyond 1 h. We were unable to carry

Figure 3 Detection of C5aR and C5L2 mRNA by RT-PCR in anterior pituitary cells. (Pit, rat anterior pituitary; GH, rat GH3; MM, rat MMQ; AtT, mouse AtT-20DV16; TtT, mouse TtT/GF cells). The 100 bp ladder is shown on the left.

Figure 4 Western blotting analysis of (A) phosphorylated (P) and total (T) p44/42 MAPK and (B) phosphorylated (P) and total AKT (T) in MMQ cells after incubation for up to 1 h. with 70 nM rat C5a. (Cell lysates were electrophoresed in 10% acrylamide and probed with antisera to phosphorylated and total forms of p44/42 MAPK and AKT). A representative blot (of three experiments) is shown.

out these experiments in TtT/GF cells as basal levels of secreted MIF were very low and barely detectable in our ELISA system.

ACTH secretion

The corticotroph tumour cell line AtT-20DV16 was used to assess the effect of murine C5a and C5adR on ACTH secretion. The natural agonist for ACTH secretion, CRH, was used for comparison. Figure 7 shows that both C5a and C5adR slightly stimulated (up to 25%) ACTH secretion over the 1 h time period, these effects were however, comparable with that for CRH (up to 40%) at similar concentrations.

Discussion

The process of inflammation leads to rapid activation of the complement system and release of two anaphylatoxin molecules C3a and C5a. C3a and C5a mediate their actions via specific receptors; these actions include chemotaxis of neutrophils, eosinophils and monocytes and smooth muscle contraction and vasodilatation (Monsinjon *et al.* 2003). Recent studies however, have shown that C3aR and C5aR

Figure 5 Detection of MIF mRNA by RT-PCR in anterior pituitary cells. (Pit, rat anterior pituitary; GH, rat GH3; MM, rat MMQ; RC, rat RC4/B; AtT, mouse AtT-20DV16; TtT, mouse TtT/GF cells). The 100 bp ladder is shown on the left.

Figure 6 Effect of C5a and C5adR on the secretion of MIF from (A) GH3, (B) AtT-20DV16 and (C) MMQ cells. (Replicate cultures (six in each experiment) were treated with recombinant rat or mouse C5a or C5adR for 1 h; MIF was measured by ELISA in the conditioned media). $N=2-4$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ when compared with the respective basal (in the absence of agonist) value.

expression is much more widespread and C5aR are now known to be expressed on endothelial and epithelial cells as well as in non-myeloid cells of the liver, lung and brain (Monsinjon *et al.* 2003). C3aR also have a similar distribution and in addition have also been described in the adrenal and pituitary gland (Francis *et al.* 2003, Monsinjon *et al.* 2003). We have recently shown that both C3a and C3aR are functionally active in the anterior pituitary gland by virtue of its stimulatory actions on hormone secretion and involvement in immunoprotection (Francis *et al.* 2003). The use of cell lines, in our current experiments, show that C5aR, perhaps not unexpectedly, is expressed in corticotrophs, lactotrophs, somatotrophs, gonadotrophs and folliculostellate cells. The second C5a receptor, C5L2 had a similar

Figure 7 Effect of C5a, C5adR and CRH on the secretion of ACTH from AtT-20DV16 cells. (Replicate cultures (four in each experiment) were treated with recombinant mouse C5a and C5adR or rat CRH for 1 h; ACTH was measured by RIA in the conditioned media). $N=2$ and 3. * $P<0.05$, ** $P<0.01$, when compared with the basal (in absence of agonist) value.

distribution to the C5aR but the C5L2 was not detectable (at least by immunocytochemistry) in the intermediate or posterior lobes. Immunostaining for C5aR and C5L2 appeared to be both surface and cytoplasmic. In the case of C5aR, this is rather surprising as it is known to be primarily located at the cell surface whereas C5L2 is known to be primarily intracellular (manuscript in preparation).

The C5aR can transduce signals via phosphorylation of p44/42 MAPK, AKT and p38 MAPK (Monsinjon *et al.* 2003, Chiou *et al.* 2004, Riedemann *et al.* 2004a). Our data are consistent with these findings and also indicate that C5adR is not an activator of these signals, presumably due to its low activity at the C5aR. On the other hand, both C5a and C5adR inhibited the secretion of MIF from pituitary cells with similar potencies. Other researchers have shown that C5a stimulates the secretion of MIF from eosinophils (Rossi *et al.* 1998) and neutrophils (Riedemann *et al.* 2004b). The inhibitory actions on MIF secretion that we observed were likely to be due to effects on preformed MIF rather than on MIF gene transcription. The presence of MIF in unstimulated pituitary cells suggests there must be some storage in the cytoplasm and this has also been observed in neutrophils (Riedemann *et al.* 2004b). Similarly, in eosinophils, phorbol myristate acetate stimulated MIF release is only inhibited 50% by prior incubation with cycloheximide (Rossi *et al.* 1998) suggesting that some MIF is pre-made and stored for rapid release.

C5a and C5adR also stimulated ACTH secretion to a similar level to that seen when similar concentrations of CRH were used. This suggests that anaphylatoxin activation of the anterior pituitary gland can independently lead to a release of corticosteroids from the adrenal gland. It is also conceivable that anaphylatoxin molecules may act in concert with other ACTH-releasing molecules from the anterior pituitary gland to regulate the release of corticosteroids and to dampen down stress and inflammation.

Apart from the inflammatory actions of C5a and C3a, there have been several reports on all three receptors also having an anti-inflammatory role (Kildsgaard *et al.* 2000, Bhatia *et al.* 2001, Gavriluyuk *et al.* 2005, Gerard *et al.* 2005, Chen *et al.* 2007, Crane & Buller 2007); this is consistent with their presence in the HPA axis and its regulation of inflammation and immunity. The anti-inflammatory properties of C3a and C5a may be due, at least in part, to interactions with cytokines and chemokines (Kohl 2001, Guo & Ward 2005). Our findings on ACTH and MIF secretion further support an anti-inflammatory role for C5a and C5aR in the anterior pituitary gland. The mechanism of action of C5aR in our experiments, however, is not known and needs further investigation. Nevertheless, the presence of C5a receptors in the anterior pituitary gland further supports interactive communication between the immune and endocrine systems.

Declaration of interest

The authors declare there is no conflict of interest that would prejudice the article's impartiality.

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