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Cloning and preliminary pharmacological characterization of the anaphylatoxin C5a receptor in the rabbit

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1 The rabbit receptor for C5a was cloned from a genomic library and found to be 79.5% identical to the human homologue, the highest degree of similarity found so far in nonprimate laboratory animals.

2 The rabbit C5a receptor stably expressed in RBL cells binds human ^{125}I-C5a (2 nM). Unlabelled C5a and the C-terminal analogue N-acetyl-Tyr-Ser-Phe-Lys-Pro-Met-Pro-Leu-D-Ala-Arg (Ac-YSFKPMPLaR) were found to be competitors of that binding, the peptide analogue retaining approximately 0.1% of the affinity of human C5a.

3 The order of potency human C5a > Ac-YSFKPMPLaR was conserved in bioassays based on rabbits (relaxation of the isolated portal vein and pulmonary artery; acute *in vivo* neutropenia), but with a decreasing potency gap between the two compounds, a likely consequence of the resistance to peptidases of the analogue.

4 The molecular definition of the rabbit C5a receptor evidenced a high preservation degree of sequence and pharmacologic properties relative to the human ortholog receptor, thus defining a set of molecular tools for the investigation of the role of C5a in physiologic and pathologic models based on the rabbit (e.g. atherosclerosis, inflammation).

Keywords: Rabbit C5a receptor; gene cloning; PCR; anaphylatoxin C5a; radioligand binding; isolated rabbit blood vessels; polymorphonuclear leukocytes

Abbreviations: C5a, complement 5a; cDNA, complementary DNA; MoAb, monoclonal antibody; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; TM, transmembrane domain

Introduction

The anaphylatoxin C5a, a 74 amino acid proteolytic fragment of the serum complement protein C5, is now regarded as the major chemotactic factor from complement and as a powerful inflammatory mediator (Ember & Hugli, 1997). Within the multifunctional complement system, anaphylatoxin C5a plays a prominent role as a pharmacologically active fragment of C5 that activates various leukocyte functions. Mast cells and basophils release histamine and other secondary mediators in response to C5a. In phagocytic leukocytes, namely neutrophils and monocytes/macrophages, several distinct activation pathways are stimulated by C5a; there is a chemotactic and chemokinetic response, an increased eicosanoid formation, a release of reactive radicals and of granule content (Ember & Hugli, 1997), an increased adherence to cultured endothelium (Tonensen *et al*., 1984), and a potentiation of the synthesis of cytokines in the monocyte (Schindler *et al*., 1990). All these functions are dependent on a type of G protein coupled receptor present on the surface of several leukocyte subtypes. The pharmacologic effects of C5a are mimicked by analogues of the C-terminal sequence of C5a, a possible basis for future drug development (Drapeau *et al*., 1993; Kawatsu *et al*., 1996; Finch *et al*., 1997).

The cloning of the C5a receptor from human cells (Gerard & Gerard, 1991; Boulay *et al*., 1991) was followed by its characterization in several other mammals (mouse, Gerard *et al*., 1992; rat, Akatsu *et al*., 1997; Sayah *et al*., 1997; dog, Perret *et al*., 1992; guinea-pig, Fukuoka *et al*., 1998; several nonhuman primates, Alvarez *et al*., 1996). A relatively low homology level has been observed across mammalian species (identity with the human sequence: mouse 65%, rat 70.8%, dog 68%, guinea-pig 67%, but 95–99% in the nonhuman primates).

Knowledge of receptor structure has had several implications to better understand the role of anaphylatoxin C5a. Structure-function studies of the receptor have been initiated, notably with the recent characterization of C-terminal determinants for ligand-receptor complex internalization (Naik *et al*., 1997) and with several other structural findings (reviewed by Ember & Hugli, 1997). The gene-targeted disruption of the C5a receptor in mice is associated with a high susceptibility to pulmonary infection and to a remarkable resistance to inflammation induced by immune complexes (Höpken *et al*., 1996; 1997). This gene is a possible model for tissue-specific receptor gene expression, as few biological responses to C5a may be dependent on receptors expressed by cells of nonmyeloid lineages (critically discussed by Marceau, 1996; Schiefferdecker *et al*., 1997); however, there is evidence of extramyeloid receptor expression in some cases (e.g. in cultured rat astrocytes, Sayah *et al*., 1997). The predicted human sequence allowed the production of polyclonal (Morgan *et al*., 1993) and monoclonal antibodies (MoAbs) against extracellular N-terminal epitopes; two of the MoAb, designated S5/1 and W17/1, define the CD88 cluster of differentiation antigen (Oppermann, 1995). The first one corresponds to an aspartate rich sequence in the N-terminal

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domain of the receptor. Although initially believed to be ineffective to detect C5a receptors in laboratory animals (Oppermann, 1995), the MoAb S5/1 was used to identify lesional macrophages as the C5a responding cells in atherosclerotic rabbit aortas (Petitclerc *et al*., 1996), suggesting a close homology in the receptor region that binds the antibody between the two species.

Thus, the cloning of the rabbit C5a receptor will be useful for comparative studies with the human receptor, and will allow the study of its regulated expression in models of pathology involving this laboratory animal.

Methods

Materials

Radiolabeled nucleotides, [α-^{32}P] dCTP (3000 Ci mmol^{-1}) and [^{35}S]-dATP (1000 Ci mmol^{-1}) were obtained from New England Nuclear (Mississauga, ON, Canada). Oligodeoxynucleotide primers were purchased from Life Technologies (Toronto, ON, Canada). Restriction enzymes, dideoxy-sequencing and oligolabelling kits were from Pharmacia (Baie d'Urfé, QC, Canada). The rabbit genomic library was obtained from Clontech (Palo Alto, CA, U.S.A.). The TA cloning kit was purchased from Invitrogen (San Diego, CA, U.S.A.).

Isolation of genomic clones containing the rabbit C5a receptor gene

A specific human C5a receptor DNA fragment was generated by PCR using human genomic DNA as a template. The primers used were 5′-AATGCCCTGGTGGTCTGGGTGAC-3′ (human C5a receptor sense nucleotide 163–185) and 5′-CTACACTGCCTGGGTCTTCTGGG-3′ (human C5a receptor anti-sense nucleotide 1031–1053; Gerard & Gerard, 1991). The amplified PCR fragment was gel-eluted, cloned in the TA cloning vector (Invitrogen) and upon sequencing it showed an identical sequence with the coding region of the human C5a receptor (nucleotides 163–1053 from the published human C5a receptor cDNA by Garard & Gerard, 1991).

A rabbit λ genomic DNA library (Clontech) was probed with the 890 bp [^{32}P]-labelled PCR fragment of the human C5a receptor gene. Approximately 1×10^6 bacteriophage plaques were screened on nylon membranes (Hybond-N, Amersham). Hybridization was performed at 65°C in $6 \times$ SSC ($20 \times$ SSC: 3 M NaCl, 0.3 M Sodium citrate pH 7), $5 \times$ Denhardt's ($100 \times$ Denhardt's: 2% Ficoll, 2% Polyvinylpirrolidone, 2% bovine serum albumin), 0.5% sodium dodecyl sulphate (SDS), 100 μg ml^{-1} salmon sperm DNA and 10^6 c.p.m. ml^{-1} of the labelled probe. The membranes were washed twice with $2 \times$ SSC at room temperature, then finally with $1 \times$ SSC, 0.1% SDS at 65°C and were exposed to radiographic film at −70°C with an intensifying screen. The DNAs from the positive lambda clones were digested with different restriction enzymes and Southern-transferred restriction fragments were hybridized with the labelled probe. The positive ones were further sub-cloned into pBluescript KS(+) plasmids (Stratagene) and sequenced in both orientations.

Expression of cloned cDNA in mammalian cells

The coding region of the rabbit C5a receptor cDNA was amplified using specific primers containing additional *Eco*RI sites (5′-CGGAATTCATGGCGCCCATGGAAAATAGCAC-3′ and 5′-GCGAATTCTCACACCGCTTGGCACTTG-3′). The PCR product was subcloned into the *Eco*RI site of the pSFFV-neo expression vector (Fuhlbrigge *et al*., 1988) kindly provided by Dr Ta-Hsiang Chao (The Scripps Research Institute). The rabbit C5a receptor cDNA pSFF-V-neo plasmid was transfected into RBL-2H3 cells (ATCC CRL-2256) using the SuperFect reagent (Qiagen, Chastworth, CA, U.S.A.). Cells were grown in DMEM containing 10% foetal calf serum and 800 μg ml^{-1} of the antibiotic G418 (Life Technologies, Gaithersburg, MD, U.S.A.) for ten days to select the resistant clone and were maintained in DMEM containing 10% foetal calf serum and G418 at 300 μg ml^{-1}.

Synthesis of a C-terminal analogue of C5a

A C5a C-terminal analogue was synthesized by solid phase methodology in order to provide a preliminary pharmacologic profile for the rabbit C5a receptors. General procedures for synthesis, purification and analysis of the synthesized peptide are described elsewhere (Boulanger *et al*., 1996). The tested agonist sequence, N-acetyl-Tyr-Ser-Phe-Lys-Pro-Met-Pro-Leu-D-Ala-Arg-OH, (Ac-YSFKPMPLaR) has been reported previously (Kawatsu *et al*., 1996; Finch *et al*., 1997). Based on assays performed using human leukocytes, the synthetic peptide is predicted to retain as much as 2–10% of the potency of C5a. This peptide has been applied *in vitro* (acute vasomotion) and *in vivo* (acute neutropenia) to estimate its effect and potency on rabbit cells, and to the competition assay for the binding of ^{125}I-C5a to recombinant rabbit receptors (see below).

Binding assay with radiolabelled C5a

The C5a peptide was isolated from expired human plasma essentially as described earlier (Hugli *et al*, 1981). Iodination of C5a with ^{125}I was performed with the Iodobead method (Pierce) as recommended by the manufacturer. The average specific activity of the labelled material was at least 356 Ci mmol^{-1}. For binding assay, 2 nM ^{125}I-C5a was added to $5-10 \times 10^5$ RBL cells stably transfected with the rabbit C5a cDNA in 100 μl of binding buffer (Earle's buffered saline containing 0.5% bovine serum albumin, pH 7.35), in the presence or absence of unlabelled competitors. The binding mixture was incubated on ice for 60 min and unbound labelled material was separated from the cells by centrifugation through a 100 μl phthalate oil cushion in 500 μl polyethylene tubes. The tips of the tubes, containing the cells plus bound radiolabelled ligand, were cut off and radioactivity was measured in a gamma counter (Packard, Cobra Autogamma). The following ligands were used as competitors: serial dilution of unlabelled human C5a in the $10^{-10}-10^{-6}$ M concentration range, and serial dilutions of the C5a analogue peptide described above ($7 \times 10^{-7}-1.2 \times 10^{-3}$ M). Nonlinear regression analysis using the Prizm computer software program (GraphPad, San Diego, CA, U.S.A.) was used to determine the IC_{50} for these competitors.

Isolated blood vessels

Rabbit vascular strips were prepared as described (Petitclerc & Marceau, 1991) and were suspended in 5 ml tissue baths containing Krebs solution (Petitclerc *et al*., 1996). Their responses to agents were recorded using isometric transducers (model 52-9545, Harvard Bioscience, South Natick, MA, U.S.A.). The pulmonary artery and portal vein segments were cut helically and subjected to a baseline tension of 1 and 0.5 g respectively. After an equilibration period of 1 h, tissues were

challenged with one of the chemotactic agents. The response to C5a is predominantly a relaxation (Hugli & Marceau, 1985). In order to record this type of response, tissues were always contracted with a submaximal concentration of phenylephrine (500 nM) for 10–30 min, (the period required to reach a contraction plateau), before injecting the chemotactic peptides (Drapeau *et al*., 1993). The effects of human recombinant C5a (Sigma) or the related peptide analogue were tested up to four times at 90 min intervals, with extensive washings between the tests. Results were expressed as the per cent of relaxation of the phenylephrine-induced plateau.

In vivo *hematologic evaluation of the C5a analogue*

The *in vivo* C5a assay is based on the very rapid fall of the circulating neutrophil count following the intravenous injection of C5a, a phenomenon interpreted as a transient adherence of these responsive cells to the blood vessel walls and later followed by a rebound leukocytosis (Kajita & Hugli, 1990; Drapeau *et al*., 1993). The acute hematologic effect of the novel C5a analogue Ac-YSFKPMPLaR was evaluated using conscious New Zealand white rabbits of either sex (2.69 ± 0.10 kg, $n = 17$). Arterial blood samples were obtained from a small incision of the central ear artery under local anaesthesia (2% lidocaine) 5 min before and 5 min after C5a analogue injection for a determination of the white blood cell counts (in a hemacytometer after lysis of erythrocytes in 1% acetic acid) and a differential leucocyte count (following Wright staining of blood smears). The results of the leukocyte counts are expressed as a per cent of the baseline value (count 5 min before C5a receptor agonist) for neutrophils and lymphocytes; other leukocyte types are infrequent in rabbit peripheral blood.

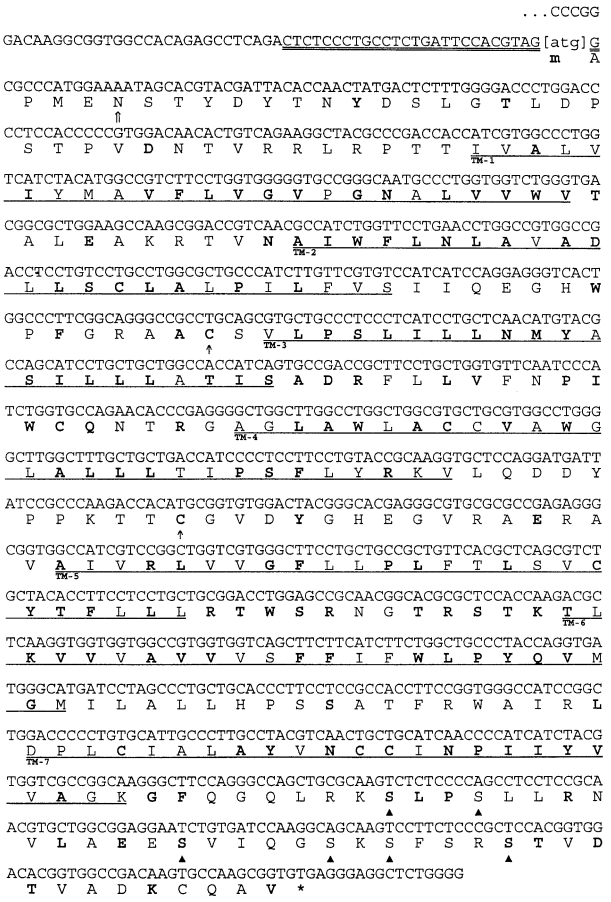

Figure 1 Nucleotide and deduced amino acid sequences of the rabbit C5a receptor. The sequence is deposited in the GenBank database, accession No. AF068680. The methionine (m) start codon (atg) is conventionally inserted into the sequence as it is assumed to be located in another exon (see text for details); the nucleotide sequence upstream of the atg codon represents the 3′ end of the intronic sequence containing a typical splice acceptor site (double-underlined). The seven transmembrane (TM) domains, the conserved putative N-linked glycosylation site (⇑), the intramolecular disulfide bridge (↑) and the major phosphorylation sites at the C terminal (▲) are indicated; all conserved amino acid residues amongst the human and five non-primate species studied are presented in bold.

Results

Isolation and structure of the rabbit C5a receptor gene

A 890 bp PCR fragment containing most of the coding region of the human C5a receptor (positions 163–1053, according to Gerard & Gerard, 1991) was generated by using human genomic DNA as a template, and consecutively used to probe a rabbit λ genomic DNA library. Of approximately 1×10^6 bacteriophage plaques screened, three positive clones were isolated which yielded identical restriction patterns with *Eco*RI, *Sac*I, *Sma*I and *Xba*I, and one of them was subjected to extensive characterization. Southern blotting of restriction digests from this clone identified a 3.2 kb *Sma*I fragment that hybridized specifically with the human C5a receptor probe (data not shown). Upon subcloning and sequencing of the *Sma*I fragment it became evident that almost the entire coding region of the rabbit C5a receptor gene together with the 3′ non-coding end are intronless. Our clone was lacking the 5′ non-translated region of the receptor gene together with the first methionine (initiation) codon. Instead, there was a partial intronic sequence present upstream from the second (Ala) codon with a classical acceptor splice site adjoining it (see Figure 1 for details). A large intron, exactly at the same position (separating the initiation codon from the rest of the intrinless coding region) has also been found in the human and the mouse C5a receptor genes (Gerard *et al*., 1992; 1993), which confirms the conservatism in the genomic organization of this receptor gene in the three species studied. Figure 1 shows the nucleotide and deduced amino acid sequence of the rabbit C5a receptor. The rabbit C5a receptor is 352 amino acids long and showed the typical structure of G protein

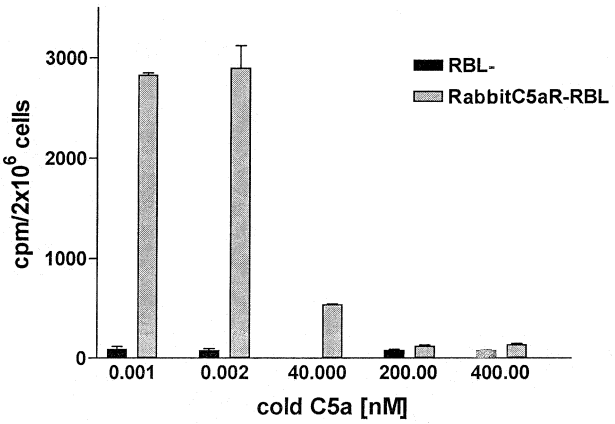

Figure 2 Total binding of ^{125}I-C5a (2 nM) to untransfected RBL cells (solid histograms) or to stable transfectant expressing the putative rabbit C5a receptor (hatched histograms). Unlabelled C5a was added at the indicated concentration to determine nonspecific binding. Values are means ± s.d.mean of duplicate determinations.

coupled receptor with seven transmembrane domains and with conserved sites of possible functional significance similar to its homologues in the other species studied (N-linked glycosylation site, a intramolecular disulphide bridge, C-terminal phosphorylation sites, conserved amino acid residues in all species studied (see for details Figure 1). Alignment of the rabbit C5a receptor peptide sequence with that of the other species reveals an overall identity of 79.5% (human), 72.8% (dog), 71.8% (mouse), 72.7% (rat) and 70.1% (guinea-pig). Thus, the rabbit C5a receptor gene displayed a significantly higher homology to its human homologue compared to the C5a receptors of the other species studied (see also Introduction). This homology is especially pronounced within the transmembrane domains (TMs), reaching 100% for TM3 and 90% for TM4 (for comparison the homology between the transmembrane segments of the C5a receptors of the species studied up to now varies between 46–86% (see Fukuoka *et al*., 1998).

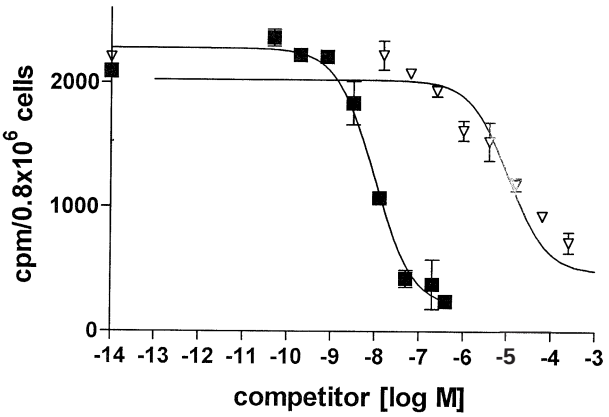

Figure 3 Competition of ^{125}I-C5a binding to rabbit C5a receptors expressed in RBL cells by unlabelled C5a (■) or the synthetic peptide Ac-YSFKPMPLaR modelled on the C-terminal sequence of C5a (▽) (see text). One representative out of three similar experiments is shown. Values are the means±s.d.mean of duplicate determinations.

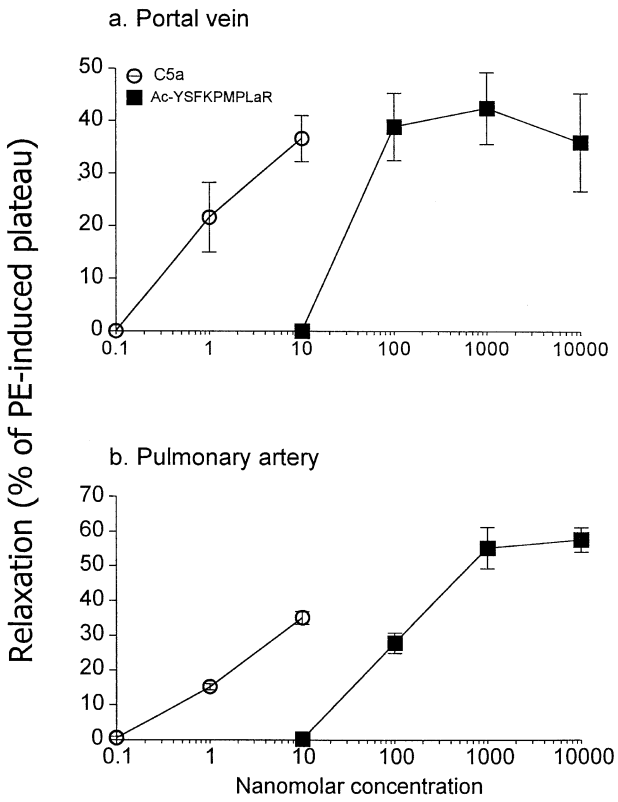

Figure 4 Relaxant effect of human recombinant C5a and of the synthetic analogue Ac-YSFKPMPLaR on the rabbit isolated portal vein (a) and on the rabbit isolated pulmonary artery (b). Values are the means±s.e.mean of 4–6 determinations.

Pharmacological identity of the rabbit C5a receptor in stably transfected RBL cells

Untransfected RBL cells bound a very small quantity of radioligand, when exposed to 2 nM of ^{125}I-C5a, and this binding may not be specific because it remained constant in the presence of an excess of unlabelled human C5a (Figure 2). The stable transfectant cell line which expresses the cloned rabbit receptor for C5a exhibited a much greater binding of the radioligand, most of which appeared to be specific binding, as estimated by competition with cold C5a. The competition of ^{125}I-C5a binding (2 nM) with unlabelled peptides was further examined in transfected cells (Figure 3 shows a representative experiment). The IC_{50} for human C5a was determined to be 12.5±3.5 nM ($n=3$), while the value for peptide Ac-YSFKPMPLaR was in the 10–49 μM range.

Pharmacological profile of the naturally expressed rabbit C5a receptor in vascular tissue and circulating neutrophils

Human recombinant C5a and the synthetic peptide Ac-YSFKPMPLaR were found to elicit biphasic mechanical

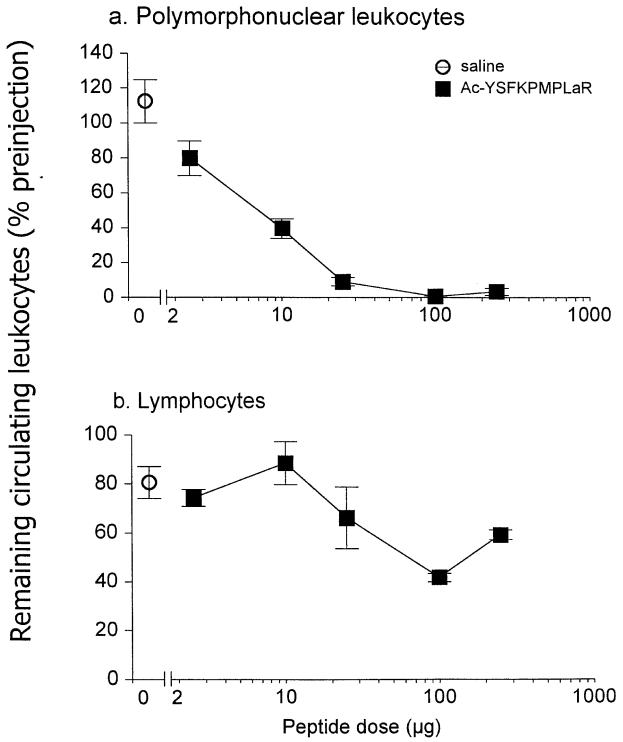

Figure 5 Change of polymorphonuclear leukocyte and lymphocyte counts in peripheral blood of rabbits 5 min after i.v. boluses of the synthetic C5a analogue Ac-YSFKPMPLaR. The effect of the saline vehicle (0.25 ml) is also shown. Results are the means±s.e.mean of three determinations (two for the 250 µg dose level) and are expressed as a per cent of preinjection values. Baseline (pre-injection) polymorphonuclear leukocyte and lymphocyte counts for all groups were, respectively, 3174±293 and 10074±1984 cells per μl of blood ($n=17$).

responses in rabbit isolated portal veins and pulmonary arteries precontracted with phenylephrine; this type of reaction has been described and analysed previously (Hugli & Marceau, 1985; Petitclerc & Marceau, 1991; Drapeau *et al*., 1993). Only the prolonged relaxant response, expressed as a percentage of the contractile plateau, was used in the quantitative analysis of biological activity in the rabbit blood vessels (Figure 4). The peptide analogue Ac-YSFKPMPLaR was found to be 20–30 fold less potent than C5a in these bioassays.

The acute neutropenic response (5 min after i.v. boluses) to Ac-YSFKPMPLaR (reported in Figure 5) was dose-related with a maximal effect essentially amounting to the complete disparition of circulating neutrophils (ED_{50} of about 5 nmol). Human recombinant C5a is only twice as potent as this analogue when tested under similar conditions for acute neutropenic effects in the rabbit (response to human recombinant C5a reported by Drapeau *et al*., 1993). Lymphocyte counts were affected only by doses of Ac-YSFKPMPLaR which were maximal or supramaximal for the acute neutropenic effect (Figure 5), as previously observed with authentic C5a (Drapeau *et al*., 1993).

Discussion

A strategy based on the selection of homologous sequences from a rabbit genomic library allowed the isolation and the characterization of a gene corresponding to a G protein coupled receptor that exhibits the essential features of the C5a receptor. It is highly homologous to the human C5a receptor (79.5% aminoacid identity) and, upon expression, displays a compatible pharmacologic profile (see below). The putative N-glycosylation site and intramolecular disulphide bridge present in the human sequence are also found at the corresponding positions of the rabbit C5a receptor (Figure 1). The rabbit C5a receptor gene shows the same genomic organization as its human and mouse homologues as the 5′ non-translated region and the initiation codon are separated by intron from the rest of the coding sequence and the 3′ non-translated region. Among nonprimate laboratory animals, the sequence homology of the rabbit and human C5a receptors is exceptionally high, perhaps in line with the recent reassessment of the phylogenetic position of the lagomorphs (rabbits and hares). The latter are now believed to be more closely related to the primate stock than to the rodents, carnivora or ruminants (Gruar *et al*., 1996; Penny & Hasegawa, 1997). A definite homology between the human and the rabbit C5a receptors is even present in the N-terminal extracellular domain, which is normally less conserved among the various mammalian species and, therefore, exploited for the development of species-specific C5a receptor antibodies. For instance, the minimal epitope corresponding to the S5/1 MoAb, the human sequence 15–21 (DDKDTLD) (Oppermann *et al*., 1993; Oppermann, 1995), has the highest degree of conservation in the rabbit (DSLGTLD) among several nonprimate laboratory animals, which support previous results based on the utilization of the S5/1 MoAb in rabbit tissues (Petitclerc *et al*., 1996).

The specific binding of a nanomolar concentration of human ^{125}I-C5a on the cloned rabbit receptor supports the role of the molecule as a functional receptor. The recipient cell line, of rat leukaemic origin, did not spontaneously express receptors for C5a, based on the lack of specific radioligand binding (Figure 2). The binding of ^{125}I-C5a to the rabbit C5a receptor was competed efficiently by Ac-YSFKPMPLaR, a relatively potent agonist derived from systematic structure-activity based on the human receptors (Kawatsu *et al*., 1996; Finch *et al*., 1997).

The results of the competition binding assays with the cloned C5a rabbit receptor correlated well with the vasorelaxant effects of the C5a analogues on the rabbit pulmonary artery and portal vein, as the order of agonist potency was retained (C5a > Ac-YSFKPMPLaR; Figure 4). The relaxant effect of C5a on the portal vein and pulmonary artery isolated from normal rabbits has been previously characterized, and is mainly prostaglandin-dependent (Petitclerc & Marceau, 1991). The precise cell type responsive to the anaphylatoxin has not been determined, but, by analogy with muscular arteries of the rabbit, a low density subendothelial population of resident macrophages may be involved (Malinauskas *et al*., 1995). In the acute neutropenia assay, the molar potency of the peptide analogue was only slightly inferior to that of recombinant C5a (Figure 5; data on C5a from Drapeau *et al*., 1993). The apparent gain of potency of Ac-YSFKPMPLaR relative to C5a in assays of increasing complexity (from the binding assay, to the vascular system, to the *in vivo* neutropenia assay) is an advantage likely to be conferred by metabolic resistance, and has been observed with some other C5a analogues in the past (Drapeau *et al*., 1993). Indeed, the D-Ala-Arg C-terminal sequence of these synthetic peptides, as compared to the Gly-Arg natural sequence, is sufficient to abolish the hydrolysis by a major C5a inactivating enzyme, plasma carboxypeptidase N, and by other peptidases (Drapeau *et al*., 1993). Thus, in a previous study, the C-terminal analogue Ac-Phe-Lys-Asp-Cha-Cha-Val-D-Ala-Arg-OH was 40,000 times less potent than C5a as a competitor of ^{125}I-C5a binding to human polymorphonuclear leukocytes, but only 400 times less potent than C5a to relax isolated rabbit blood vessels and 50 times less potent to produce the neutropenia *in vivo* in rabbits (Drapeau *et al*., 1993). The analogue studied in the present paper, Ac-YSFKPMPLaR, similarly exhibited a decreasing potency gap relative to C5a in bioassays of increasing complexity, but possessed a higher receptor affinity than previously studied oligopeptide analogues, thus reaching a larger relative potency in the complex bioassays. The more complex pharmacological actions of Ac-YSFKPMPLaR, such as acute leukopenia following intravenous injection, are likely to be mediated by C5a receptors, as they are limited to the neutrophils (the lymphocytes are largely refractory, Figure 5b) and as the cloned rabbit C5a receptor has affinity for this peptide.

In summary, the present study defines a set of molecular tools (receptor sequence, agonist ligands) for the study of the rabbit C5a receptor, allowing future physiopathological investigations based on this animal model. Precise sequence information will notably allow regulatory studies based on reverse transcriptase-PCR approach and the production of more specific anti-receptor antibodies. Whether the tissue response to C5a receptor is restricted to cells of the hematopoietic lineages, and therefore amplified *via* pathological leukocyte infiltration (Petitclerc *et al*., 1996), or whether this receptor can escape such a tissue specific expression pattern and become widely expressed under certain stimulatory condition (Sayah *et al*., 1997) are issues of interest for future molecular studies.

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