C3A binds to the seven transmembrane anaphylatoxin receptor expressed by epithelial cells and triggers the production of IL-8

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Received 3 August 2000; revised 20 October 2000; accepted 21 November 2000

First published online 1 December 2000

Edited by Masayuki Miyasaka

Abstract The complement (C) plays an important role in many acute inflammatory processes. C3a is an inflammatory polypeptide named anaphylatoxin, generated during C activation and which acts through a specific receptor C3aR. In this study, we demonstrated that the epithelial cell line ECV 304 constitutively expressed C3aR (by flow cytometry and immunofluorescence) and that binding of purified C3a to epithelial cells resulted in a time- and dose-dependent upregulation of interleukin-8 (IL-8). Pre-treatment of ECV 304 with pertussis toxin inhibited IL-8 response induced by C3a, indicating that the action of C3a was mediated by a G protein coupled pathway.

Key words: Complement; C3a receptor; Anaphylatoxin; Epithelial cell; Interleukin-8

1. Introduction

The immune system is essential for protection of the host organism and the complement (C) system is an important component of this immune system.

The early stage of inflammatory process leads to the activation of the C system. One of the biological consequences of this activation is the release of potent proinflammatory molecules, the C3a and C5a anaphylatoxins, which are powerful chemoattractants and which recruit neutrophils, macrophages and mast cells at the inflammatory site. Activation of the immunocompetent cells leads to the phagocytose of invading pathogens [1].

C3a and C5a anaphylatoxins are generated by proteolytic cleavage from C3 and C5 components, respectively. C5a, the best characterised anaphylatoxin is a potent proinflammatory mediator that induces contraction of smooth muscles, enhances the vascular permeability and promotes leukocyte functions such as directed chemotaxis, degranulation, mediator release (including histamine, interleukin-1 (IL-1), IL-6, IL-8 and tumour necrosis factor (TNF) α), increased expression of adhesion molecules and production of superoxide anions [2–5]. C3a can also drive its own proinflammatory activities such as degranulation and chemotaxis of eosinophils and human mast cells and induces platelet aggregation [6–8]. Cells respond to anaphylatoxins via interaction with specific receptors, which belong to the seven transmembrane receptor superfamily and that are functionally coupled to G proteins [9,10].

The receptor for C5a (C5aR) was cloned in 1990 [11]. The expression of this receptor was traditionally thought to be restricted to granulocytes, monocytes and macrophages and cell lines of myeloid origin [12,13]. However, recent reports indicate that a variety of non-myeloid cells (including human hepatocytes, epithelial cells, endothelial cells, vascular smooth muscle and glial cells) also express C5aR [14–20]. The broad distribution of C5aR suggests it plays an important role in modulating inflammatory and immune responses.

The cloning of C3a receptor (C3aR) has recently been reported but little is known about the cellular and tissue distribution of the C3aR [21–23]. Binding and functional experiments have indicated that C3aR was present on monocyte/macrophage cells, platelets, polymorphonuclear leukocytes, rat mast cells and basophils [13,24–28]. Furthermore, the myelomonocyte U937 and myeloblastic HL60 cell line express a C3aR when differentiated to a more mature phenotype by treatment with dibutyryl cyclic adenosine monophosphate [29].

However, little is known about the expression of C3aR at the protein level and on non-myeloid cells. If C5aR was described on epithelial cells, nothing is known about it's expression and role.

The immune system is essential to eliminate a large variety of pathogens and for the protection of the host organism. This task requires concerted actions of cellular responses that are regulated by a network of cytokines. The effects of C5a on cytokine production are well characterised. C5a is a potent inducer of gene expression and protein synthesis for TNF α, IL-1, IL-6 and IL-8 [30–33].

On the contrary the effects of C3a as an inducer of cytokine production remain ill characterised and nothing is known about the effects of C3a on epithelial cells.

Thus, the purpose of the present study was to identify the expression of the C3aR on epithelial cells and to explore the

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Abbreviations: Ab, antibody; BSA, bovine serum albumin; C, complement; C3aR, C3a receptor; C5aR, C5a receptor; FACS, fluorescence analysis cell sorter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IF, immunofluorescence; IL, interleukin; MMLV, Moloney murine leukaemia virus; PBS, phosphate-buffered saline; PE, phycoerythrine; PMA, phorbol myristate acetate; PTX, pertussis toxin; RT-PCR, reverse transcription-polymerase chain reaction

0014-5793/01/$20.00
PII: S0014-5793(00)02320-6

mechanism by which C3a drives inflammatory epithelial cell responses. C3a was assessed for it's ability to modulate IL-8 production on epithelial cells.

Here we described for the first time the expression of C3aR on epithelial cell line ECV 304. C3aR expression was demonstrated at the protein and mRNA levels. C3a in addition to being direct mediator of inflammation can induce an increased level of IL-8 from epithelial cells. These results suggest that anaphylatoxin contributes to both inflammation and regulation of the immune function by modulating IL-8.

2. Materials and methods

2.1. Chemicals, cytokines and antibodies

The following materials were purchased from Sigma, St. Quentin Fallavier, France: pertussis toxin (PTX), zymosan A, phorbol myristate acetate (PMA) and S100.

Monoclonal anti-C3aR: clone BIIG1 IgG2a was from Dr. Philippe Gasque, Cardiff, UK.

Rabbit anti-human C3aR: peptide cut was from Dr. Philippe Gasque, Cardiff, UK and was characterised as described [24].

Antibodies against human CD59 (clone BRIC 229) were from Paul Morgan, Cardiff, UK.

2.2. Peptides

Multiple-assay-peptide (MAP) C3a peptides were synthesised by solid phase synthesis (Applied Biosystem). Peptides were purified by reverse phase high performance liquid chromatography (HPLC) and sequences were ascertained by amino-acid sequence. MAP C3a peptides correspond to the C-terminal of the anaphylatoxins (amino acids 64–77 for the C3a) attached to a poly-lysine core (eight peptidic monomers).

Human C3a was generated by activation of C using zymosan A (Sigma). 1 l of fresh human serum from healthy volunteers (CRTS, Bois guillaume, France) was supplemented with EGTA (10 mM), $MgCl_2$ (10 mM), ε-aminocaproic acid (50 mM) and DL-2 (mercaptoethyl) guanidinopentanoic acid (1.5 mM). C was activated by 20 g of washed zymosan A during 1 h at 37°C with gentle stirring. At the end of incubation, the pH was lowered to 5.2 by addition of HCl. The mixture was diluted with an equal volume of cold distilled water and centrifuged at 4°C, 1 h, $18\,000 \times g$. The supernatant was adjusted to 1 M NaCl and subjected to immunoaffinity chromatography on a 150 ml Sepharose anti-C3a column.

After intensive washing of the column with 1 M NaCl, phosphate-buffered saline (PBS) buffer, C3a was eluted with 0.1 M glycine–HCl, 1 M NaCl buffer pH 2.2. Solid urea was added to C3a containing fractions (8 M final concentration) which were concentrated by ultrafiltration on YM-5 membrane (Amicon, Paris, France). Purification was achieved by gel filtration on a TSK G2000 SW HPLC column (Beckman, Gagny, France) equilibrated in 20 mM sodium phosphate, 0.2 M NaCl pH 7.4.

The purity of C3a after gel filtration was assessed by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis and Coomassie blue staining. The immunoreactivity was checked by Western blotting using monoclonal anti-C3a antibody (Ab) and rabbit anti-C3a.

2.3. Cell culture

ECV 304 epithelial cell line as well as K562, U937, HL60, Jurkat cell lines was obtained from American type culture collection (Rockville, MD, USA) and cultured at 37°C in RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated foetal calf serum (Bioproducts, Gagny, France). Cell culture reagents were from Biowhittaker unless otherwise stated.

For some experiments, cells were stimulated with PMA (10 ng/ml) for 48 h.

All cell lines were tested for mycoplasma according to the manufacturer's instructions (Boehringer Mannheim, Germany).

2.4. Flow cytometry

Adherent epithelial cells were harvested by incubation in fluorescence analysis cell sorter (FACS) buffer (saline buffer containing 2% bovine serum albumin (BSA) supplemented with 10 mM EDTA), and resuspended at 10^6 cells/ml, washed twice and incubated with the appropriate primary Ab (10 μg/ml) for 30 min on ice, washed twice in ice cold FACS buffer, incubated with the appropriate phycoerythrine (PE)-labelled secondary Ab (goat anti-rabbit Ig diluted 1/200, Dako) for 30 min on ice and washed two additional times before analysis on a flow cytometer FASCscalibur (Becton Dickinson, Pont de Claix, France). The negative control consisted of cells incubated with non-immune rabbit IgG only. Data from several experiments were normalised to the staining with non-specific mouse IgG as a negative control and are expressed as arbitrary fluorescence units.

2.5. RNA preparation

Total RNAs were isolated from cultured cells using isothiocyanate guanidium and an ultracentrifugation on a cesium chloride cushion followed by phenol/chloroform extraction described previously by Shambrook et al. [34]. The quality of RNAs was controlled by electrophoresis on a 1% agarose gel and concentration was determined by absorbance at 260 nm.

50 μg of the total RNAs were treated for 20 min at 37°C with 90 U of RQ-1 RNAse-free DNAse (Promega, Charbonnières, France) in 100 μl of buffer (40 mM Tris–HCl pH 8, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$) and 200 U of RNAsin ribonuclease inhibitor (Promega) to remove all traces of contaminating genomic DNA.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

The RT reaction was carried out with 2 μg of total RNA (DNAse treated) for 1 h at 37°C in a final volume of 30 μl with 250 pmol random hexamer primer Pd(N)6 (Pharmacia, Orsay, France), 200 U Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega) and 40 U RNAse inhibitor (Promega). The absence of contaminants was routinely checked on negative control samples in which either the RNA template was replaced with diethyl pyrocarbonate water or the MMLV reverse transcriptase was omitted.

PCR was performed in a Hybaid Omnigene thermocycler (Schleicher and Schuell, Céra labo, France) with 5 μl of cDNA in a 50 μl final volume with 2.5 mM $MgCl_2$ (Promega), 200 μM deoxynucleotide triphosphates (Pharmacia), 100 pmol of each C3aR primer (Eurogentec, Belgium) and 2.5 U of Taq polymerase (Promega) in the PCR buffer. The different PCR steps were: denaturation for 3 min at 94°C; five cycles (−94°C 40 s, annealing at 56°C for 50 s and elongation at 72°C for 90 s, ramping 6 s/°C up from 56 to 72°C); 25 cycles under the same conditions as above but without ramping; end of elongation at 72°C for 10 min, and 5 min to return to room temperature.

RT-PCR products were loaded onto 1% agarose gel and separated by electrophoresis.

Different controls were performed (no RT or Southern blot) to ensure the validity of the RT-PCR. All products were also sequenced.

The sequences and the position of the primers used in this study are given as follows: C3aR sense-1: GGG TGG TGG CTT TTG TGA TG (521); C3aR sense-2: GTC CCC ACT GTC TTC CAA CC (754); C3aR antisense-1′: CAG CAG GAA ACC CAC CAC TA (1122); C3aR antisense-2′: AGG GCA TAA AGG AAG GGA TT (1391); IL-8 sense: TCT TGG CAG CCT TCC TGA TT; IL-8 antisense: AAC TTC TCC ACA ACC CTC TG; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense: TGC CAT CAA CGA CCC CTT CA; GAPDH antisense: TGA CCT TGC CCA CAG CCT TG.

For semi-quantitative RT-PCR, PCR was carried out with 25 pmol of each primer, 1.25 pmol of each GAPDH primer and 1 μCi [^{33}P]dATP (Redivue, Amersham, les Ulis, France). Primers were used with a GAPDH:cytokine molar ratio of 1:10. The number of cycles was chosen in order not to reach the plateau (25 cycles). PCR products were loaded onto a 6% acrylamide gel and separated by electrophoresis migration. Gels were dried and exposed onto Biomax Films (Kodak Films, Sigma, St. Louis, MO, USA). Autoradiograms were analysed by densitometry using Lecphor imaging system software (Biocom, Les Ulis, France).

The relative amount of each cytokine mRNA was estimated by dividing the peak densitometry area of the cytokine amplicon by this of the GAPDH amplicon.

The level of cytokine mRNA expression from unstimulated cells was set to one arbitrary unit and values for the stimulation time were calculated accordingly.

2.7. Southern blot

The human C3aR DNA was constructed by RT-PCR of PMA

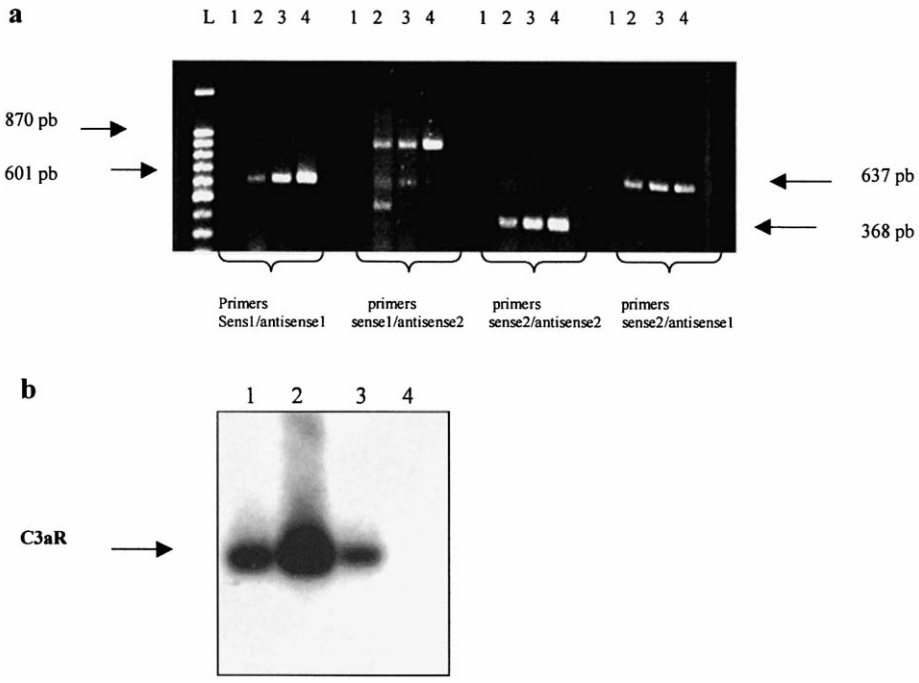

Fig. 1. a: Expression of C3aR mRNA by epithelial cell line ECV 304 and control cell line THP1 and U937: RT-PCR analysis. The expression of C3aR was assessed by RT-PCR using four different combinations of C3aR primers. L represents the size marker (100 bp ladder, Promega). Lane 1 represents a negative control in which the RT reaction product was omitted. Lane 2, epithelial cell line ECV 304. Lane 3, 48 h phorbol ester (PMA) treated U937. Lane 4, THP1. b: RT-PCR analysis to test C3aR expression followed by Southern blotting using a specific human C3aR cDNA probe. Lane 1, U937/PMA. Lane 2, THP1/PMA. Lane 3, ECV 304. Lane 4, ECV 304 without MMLV without treatment.

stimulated U937 RNA using C3aR sense 1 and C3aR antisense 1 primers.

Agarose gel electrophoresis was performed with 10 μl of RT-PCR products.

The gel was treated for 10 min in 0.15 N HCl and neutralised for 30 min in 0.4 N NaOH. Southern blotting was performed by capillary transfer during 16 h in 0.4 N NaOH on nylon plus membranes (Amersham, France).

The blot was neutralised for 10 min in saline–sodium phosphate–EDTA buffer (SSPE) 2×. The human C3aR probe was labelled by random priming with [α-^{32}P]dCTP (Redivue, Amersham, Les Ulis, France) using the kit Rediprime II (Amersham). The membrane was prehybridised at 42°C for 4 h in a hybridisation solution (50% formamide, SSPE 5×, SDS 0.1%, Denhardt solution 5×, dextran sulphate 5% and 100 μg/ml herring sperm DNA).

Hybridisation was performed at 42°C overnight in the hybridisation solution as above supplemented with the labelled probe.

The membrane was washed twice at room temperature in SSPE 2×/SDS 0.1%, 1 h in SSPE 2×/SDS 0.1% at 65°C and 1 h at 65°C in SSPE 1×/SDS 0.1% and exposed 20 min at room temperature onto Kodak Films (Sigma).

2.8. Immunocytochemistry

Epithelial cells were cultured on sterile glass coverslips for 4 days. After washing with PBS, the cells were fixed with 1% formaldehyde (Sigma) for 30 min and then cells were washed intensively with 0.2 M glycine/PBS. Abs were used at an optimal dilution of 1 μg/ml in PBS/BSA 1% and incubated at 37°C for 30 min. After washing, coverslips were incubated for 30 min at 37°C with anti-mouse IgG $(Fab')_2$ fluorescein isothiocyanate (FITC) conjugate or goat anti-rabbit-FITC conjugate (1/200) (Harlan seralab, Crowley Down, UK). After washing, coverslips were mounted in citifluor (Citifluor, London, UK) and sealed. Fluorescence was imaged by confocal laser scanning microscopy on a Leica TCS microscope.

2.9. IL-8 enzyme-linked immunosorbent assay (ELISA)

96-well plates (Costar, Corning, NY, USA) were incubated with monoclonal anti-human IL-8 Ab (T9g10, 1 μg/ml) in 0.05 M borate buffer, pH 8 and subsequently blocked with PBS containing 1% BSA. After washing, appropriate dilutions of samples were added, incubated for 1 h at room temperature, washed and then incubated with a monoclonal biotinylated anti-human IL-8 (diluted 1:2000) for 1 h at room temperature. After washing, the wells were incubated with streptavidin-peroxidase (Sigma, 1/3000). The substrate of the peroxi-

Table 1
Immunodetection of C3aR on epithelial cells by FACS analysis

	Mean of fluorescence (FL2-H channel) (PE staining)			
	rabbit anti-C3aR peptide cut	rabbit anti-C3aR peptide cut biotinylated	BIIG1	Bric 229
THP1 (*n* = *10*)	28.13 ± 9.75	96.05 ± 36.53	118.05 ± 38.73	1.11 ± 1.02
Jurkat (*n* = *6*)	1.47 ± 1.02	1.70 ± 1.18	2.83 ± 0.57	ND
ECV 304 (*n* = *7*)	40.82 ± 17.34	91.82 ± 61.80	222.36 ± 165.30	1697.57 ± 711.82
C3aR-transfected K562 (*n* = *4*)	1098 ± 21	1673.50 ± 28.50	1810 ± 41.30	ND
Vector only K562 (*n* = *3*)	2.99 ± 0.21	4.80 ± 0.07	6.20 ± 0.3	ND

All the results are represented as the mean ± S.E.M.
Cells were harvested from culture by incubation in FACS buffer supplemented with 10 mM EDTA. Cells were washed and resuspended at 10^6 cells/ml and incubated with the appropriate Ab: rabbit IgG or rabbit anti-human C3aR peptide cut, rabbit anti-human C3aR peptide cut biotinylated, monoclonal anti-human C3aR (BIIG1) or monoclonal anti-CD59 (clone BRIC229); ND: not determined.

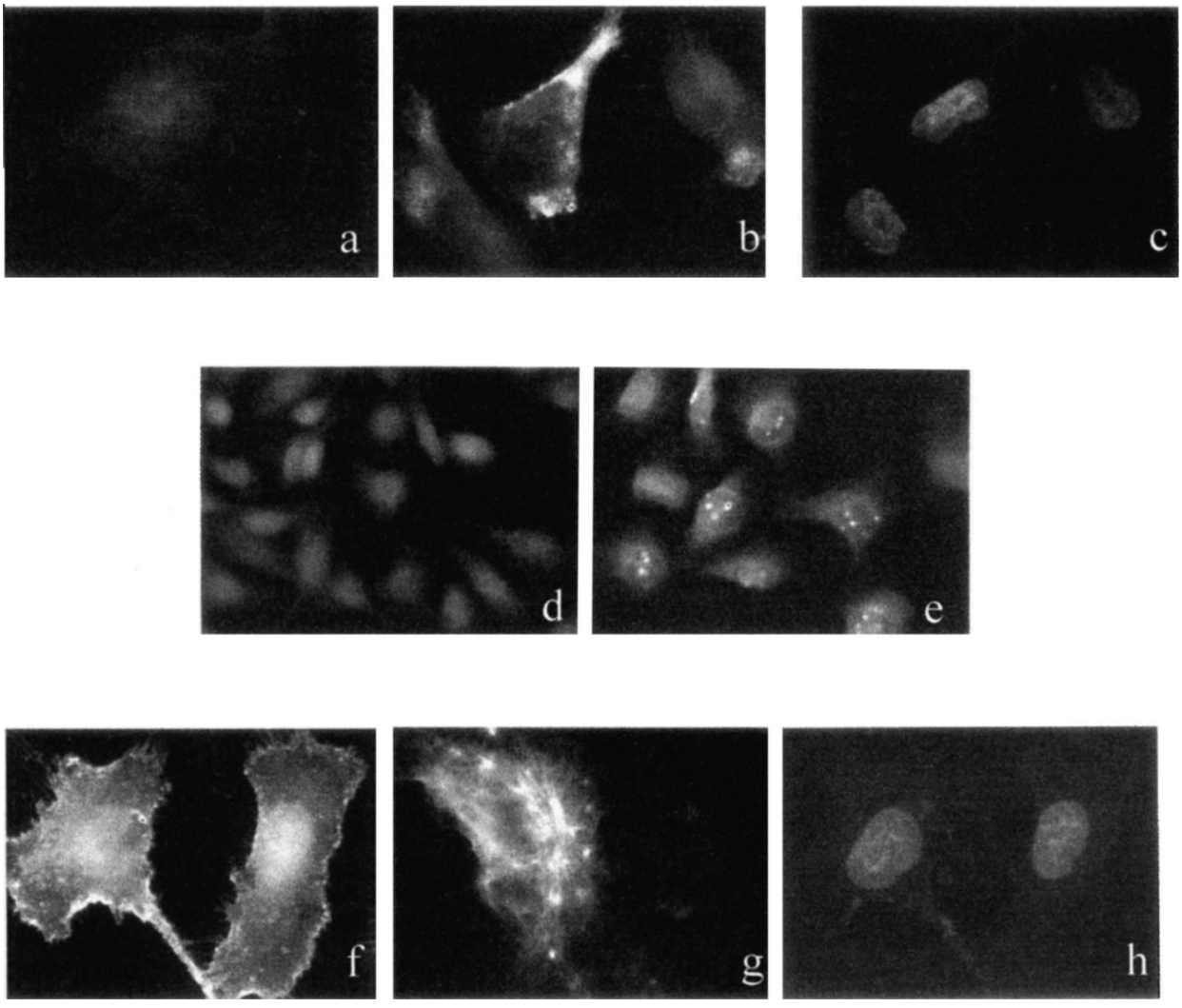

Fig. 2. Immunolocalisation of C3aR on ECV 304 endothelial cell line by indirect IF and analysis by confocal laser microscopy. Cells were incubated with either (b) rabbit anti-C3aR (1/200) followed by an FITC-conjugated goat anti-rabbit Ig Ab or with (d) monoclonal anti-C3aR BIIG1 (1/500) followed by an FITC-conjugated rabbit anti-mouse IgG Ab. Cells were also incubated with (f) BRIC 229 (mouse anti-human CD59) and (g) S100 as positive control. Extended focus views demonstrated that fluorescence was localised on the membrane. The nucleus of the cells was visualised by DAPI staining (1/2000) (c and h). C3aR staining was patchy and localised in restricted areas of the membrane. Controls consisted of cells incubated with the second Ab alone, which means goat anti-mouse IgG–FITC conjugate (a) or goat anti-rabbit–FITC conjugate (d).

dase used was the *O*-phenylenediamine and the cytokine concentration was calculated relatively to IL-8 standards.

ECV 304 was stimulated with/without anaphylatoxin, supernatants were harvested and stored at −80°C and cells were counted in a Mallasez cell. The levels of IL-8 released into the culture supernatants were measured and expressed as pg/ml of IL-8 per 10^5 cells.

3. Results

3.1. Human epithelial cell line (ECV 304) constitutively expresses a C3aR mRNA

C3aR mRNA expression by ECV 304 epithelial cell line was analysed using RT-PCR analysis (Fig. 1a).

Four oligonucleotide combinations (oligo 1/1′, 1/2′, 2/2′ and 2/1′) were tested for RT-PCR analysis. After 30 cycles the predicted amplicon sizes of 601, 870, 368 and 637 bp were detected from the epithelial cell line ECV 304 (lane 2) and the PMA differentiated U937 (lane 3) and THP1 (lane 4) cell lines.

All RNA samples were pre-treated with DNAse to eliminate contamination with genomic DNA and this was confirmed by the absence of a PCR fragment when RT product was omitted (lane 1).

The identity of the different PCR products from ECV 304 was confirmed by Southern blot analysis using a specific C3aR cDNA probe cloned from U937 (Fig. 1b). U397 (lane 1) and THP1 (lane 2) stimulated with PMA were used as positive control. The absence of contamination with genomic DNA was confirmed by the absence of a PCR fragment when RT product was omitted (lane 4)

Furthermore, all PCR products from ECV 304 were sequenced and were identical with the published sequence (data not shown).

3.2. Human epithelial cell (ECV 304) constitutively expresses membrane C3aR at the cell membrane (Table 1)

Two different Abs against the extracellular loop of the

C3aR were used for FACS analysis to detect the expression of human C3aR on epithelial cell line ECV 304. ECV 304 exhibited a mean of fluorescence of 222.36 ± 165.30 when labelled with the monoclonal Ab BIIG1. The same experiment was carried out with the polyclonal Ab biotinylated or not. ECV 304 exhibited a mean of fluorescence of 91.8 ± 61.8.

The specificity of these Abs was confirmed by staining of THP1, U937 and K562 cells, an erythroleukaemia cell line, transfected with vector Pef bos containing C3aR cDNA. Negative controls consisted of mocked cells (vector only K562). K562 transfected with Pef bos express a mean of fluorescence of 1810 ± 41.30 with the monoclonal Ab BIIG1 and a mean of fluorescence of 1673.50 ± 28.50 with the polyclonal Ab. The negative control K562 containing the vector only showed a weak mean of fluorescence (6.20 ± 0.30) with the monoclonal Ab BIIG1 as well as with the polyclonal Ab (4.8 ± 0.07).

A staining was also performed against CD59 antigen to check the quality of the cells.

Indirect immunofluorescence (IF; Fig. 2) confirmed that C3aR was expressed abundantly on the membrane of ECV 304 epithelial cell line.

The IF staining of epithelial cell line ECV 304 was shown with BIIG1 (the monoclonal Ab directed against the extracellular loop) (Fig. 2e) and confirmed by using the polyclonal Ab. Cells were strongly stained for C3aR with polyclonal Ab (Fig. 2b). Positive controls were used as staining for CD59 (Fig. 2f) and S100 protein (Fig. 2g). The distribution of C3aR over the epithelial cell was not homogeneous but localised in discrete patches on the cell body (Fig. 2b,e). C3aR was not observed in the nucleus or the cytoplasm. No staining was observed when the anti-C3aR was replaced with the second Ab alone, either goat anti-mouse IgG–FITC conjugate (Fig. 2a) or goat anti-rabbit–FITC conjugate (Fig. 2d). The nucleus of the cell was visualised by 4′,6-diamidino-2-phenylindole (DAPI) staining (Fig. 2c,h).

IL-8 is a central cytokine, which plays a central role in the inflammatory process by recruiting inflammatory cells. We decided to study the expression of this cytokine by stimulating epithelial cells with a range of concentration of anaphylatoxins and during different times. Epithelial cells at around 80% confluent were incubated with different concentrations of anaphylatoxin C3a and then the RNAs were extracted and RT-PCR was performed.

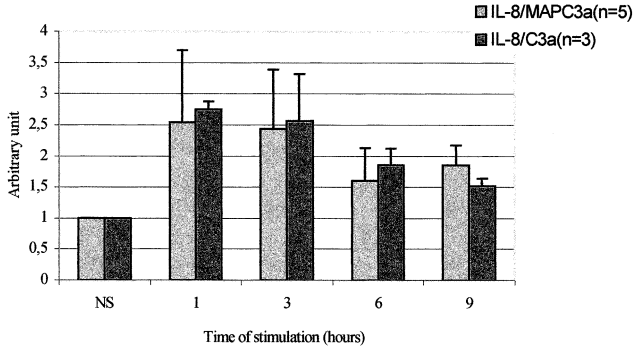

Fig. 3. Semi-quantitative RT-PCR analysis of IL-8 mRNA expression following stimulation of ECV 304 cells with MAP C3a (10^{-9} M) or C3a (10^{-8} M). RNAs were extracted at 1, 3, 6 and 9 h of stimulation and analysed by semi-quantitative RT-PCR using GAPDH as internal standard. Results are represented as histograms and expressed as relative fold increase over control (NS: non-stimulated cells). All results are represented as mean ± S.D.

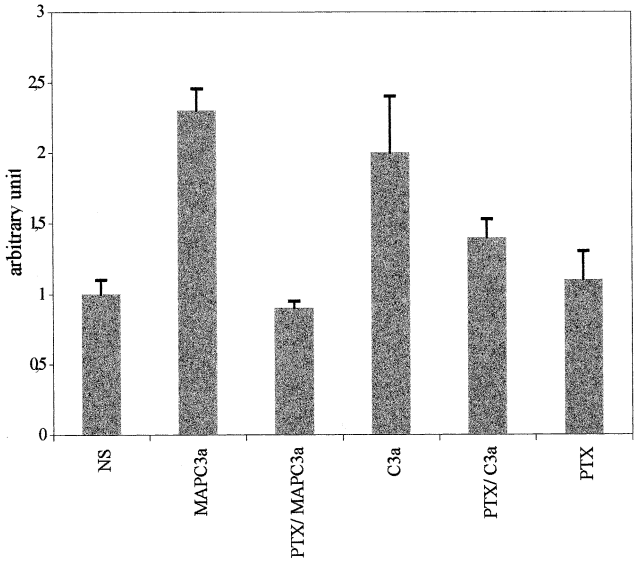

Fig. 4. Influence of PTX on the IL-8 mRNA production by epithelial cells following stimulation with C3a and MAP C3a. ECV 304 were incubated during 4 h with PTX (500 ng/ml) and then stimulated with 10^{-8} M C3a, or with 10^{-9} M of MAP C3a during 1 h. Epithelial cells were also incubated with PTX alone. Total RNAs were extracted and RT-PCR was performed. Control consists of non-stimulated cells (NS). All results are represented as the mean ± S.D. ($n = 4$).

3.3. Stimulation of epithelial cell line with 10^{-9} M MAP C3a and 10^{-8} M C3a increases IL-8 mRNA

The IL-8 mRNA level was increased at 1 h of poststimulation with C3a and MAP C3a (Fig. 3). The IL-8 mRNA level was markedly increased by 2.5-fold for MAP C3a and 2.7-fold for C3a after 1 h of stimulation. The level of IL-8 was increased 2.4-fold for MAP C3a and 2.5-fold for C3a and remained high at 3 h. The level of IL-8 mRNA remained at a higher level than that of non-stimulated cells even at 6 and 9 h of stimulation for MAP C3a and C3a.

3.4. Anaphylatoxin-induced IL-8 mRNA response was dose-dependent

Epithelial cell line ECV 304 was stimulated by a range of concentration of MAP peptides and anaphylatoxins from 10^{-7} to 10^{-11} M.

Total RNAs were extracted at 1 h of stimulation for C3a, which corresponded to the peak of IL-8 response for MAP C3a and C3a. The optimal response corresponded with 10^{-9} M of stimulation for MAP C3a and 10^{-8} M for C3a (data not shown). These results are in good agreement with previous responses that show a stronger agonist effect of MAP C3a over C3a [35,36].

3.5. C3a-mediated increases in IL-8 production in ECV 304 are PTX sensitive (Fig. 4)

C3a is known to bind to a receptor that is functionally coupled to a G protein [9,10]. In addition, cloning of human C3aR confirmed that it possesses the structure of a G protein coupled receptor. Therefore, we wondered if the observed effects of C3a on the IL-8 level were subject to inhibition by

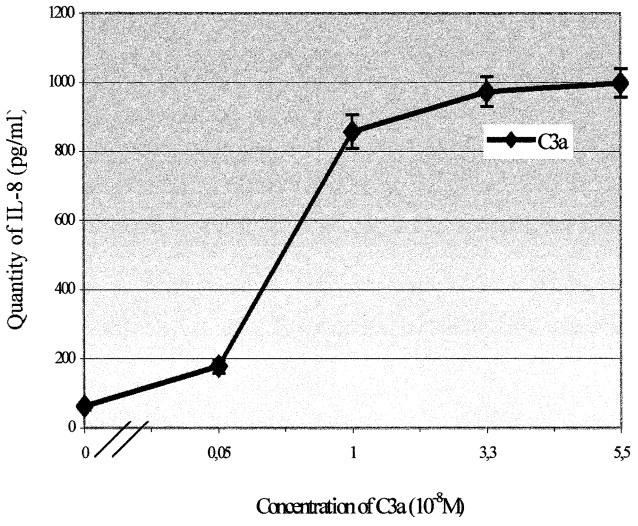

Fig. 5. Dose-dependent production of IL-8 by epithelial cells after incubation with increasing concentrations of C3a. Confluent layers of epithelial cells were rendered quiescent by overnight incubation with ultradoma medium and were then incubated for 72 h with medium containing different concentrations of C3a (from 0.05×10^{-8} M to 5.5×10^{-8} M). The mean production ± S.D. is shown for experiments performed in triplicate.

PTX. The pre-treatment of cells with PTX is known to interfere with signals mediated through Gi-protein as demonstrated in neutrophils [9]. Cells were preincubated with PTX 4 h prior to stimulation and then stimulated for 1 h with C3a. To confirm that PTX had no effect on cells, epithelial cells were incubated with PTX alone. The level of IL-8 mRNA after incubation with PTX alone is comparable with this of non-stimulated cells. Controls of the blocking effect of PTX consisted of cells stimulated with peptides alone (during 1 h for MAP C3a and C3a). Pre-treatment of ECV 304 with PTX reduced the IL-8 mRNA level. In the same way, pre-treatment of ECV 304 with PTX prior to stimulation with purified C3a blocked the IL-8 mRNA response.

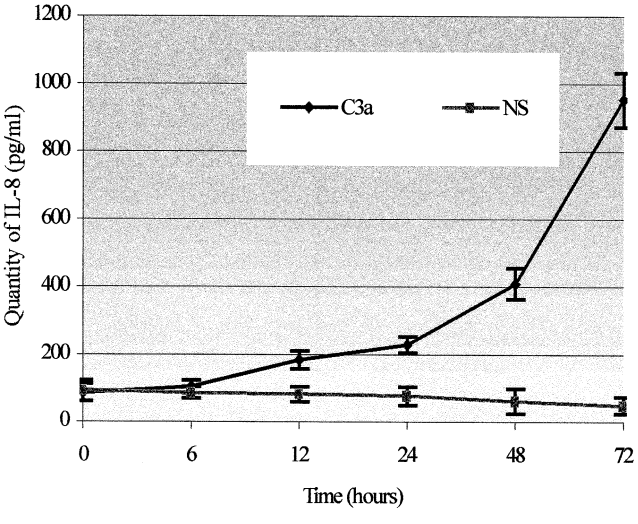

Fig. 6. Time-dependent production of IL-8 by epithelial cells after incubation with C3a (10^{-8} M). Layers of quiescent epithelial cells were incubated with 10^{-8} M of C3a or with medium alone for different lengths of time. Using a specific ELISA, the concentration of IL-8 was measured in the supernatants. The results are represented as the mean ± S.D. of three different experiments.

3.6. Epithelial cell line ECV 304 stimulated by anaphylatoxins induce a IL-8 protein production

To determine the effect of C3a on the ability of epithelial cells to produce IL-8, layers of quiescent epithelial cells were incubated at 37°C and for 72 h with different concentrations of C3a (Fig. 5). The supernatants of ECV 304 cells stimulated with anaphylatoxin were harvested after 72 h of stimulation and the concentration of IL-8 in appropriate dilutions of supernatants was assessed by specific sensitive ELISA assay (20 pg/ml). A basal level of IL-8 produced by epithelial cells was detected (92 ± 18 pg/ml).

Furthermore, epithelial cells stimulated with increasing concentrations of C3a showed a dose-dependent production of IL-8 (maximum 998 ± 42 pg/ml for C3a).

Based on these results, an optimal stimulation concentration of 10^{-8} M for C3a (Fig. 6) was chosen.

We then investigated the kinetics of IL-8 production. Quiescent epithelial cells were stimulated with 10^{-8} M of C3a for different incubation times (from 6 to 72 h).

These kinetics revealed a time-dependent production of IL-8 with a maximum production at 72 h for C3a (953 ± 49 pg/ml). No significant variation of IL-8 was detected when epithelial cells were cultured over 72 h.

Since C3a and C5a are known to interact via a specific receptor coupled to a G protein, we assessed the effect of PTX on epithelial cells preincubated with PTX 4 h prior to the stimulation with anaphylatoxins C3a. Addition of PTX prior to stimulation with C3a decreased the IL-8 production to the level observed with non-stimulated cells. Preincubation of epithelial cells with PTX alone did not affect the IL-8 production (data not shown).

4. Discussion

C is an integral buttress of the inflammatory reaction. Some functions of C include opsonisation, clearance of circulating immune complexes, lysis of nucleated and non-nucleated cells and lysis of bacteria. In addition to these activities, the C generated anaphylatoxins C3a and C5a [37]. The importance of anaphylatoxins as major participants in inflammatory processes is suggested by elevated plasma levels of C3a and C5a in local and systemic inflammatory conditions and as much as 60 μg/ml of C3a can be generated in activated human serum.

In the present study, we investigated the effects of anaphylatoxins on epithelial cells. We were interested to assess whether epithelial cells could express C3aR and to obtain further insight on the potential roles of C3a on epithelial cells in the general context of inflammation. For that purpose, we decided to examine the effects of anaphylatoxin on α chemokine IL-8 production by human epithelial cells. Indeed, IL-8 is of importance because it is involved in the attraction of neutrophils to the site of inflammation.

Until now, little work has been conducted on the effect of anaphylatoxins on epithelial cells.

Previous studies have demonstrated that C5aR is present on bronchial and alveolar epithelial cells [14,38] but nothing is known concerning C3aR.

First of all, the use of highly specific Abs reactive with the predictive large extracellular loop of the C3aR and molecular

probes allowed us to characterise expression of the C3aR protein in an epithelial cell line. The FACS analysis has revealed expression of C3aR by epithelial cells.

We then observed the distribution of C3aR on individual cells; this distribution was not homogeneous and C3aR was present in dense patches on the membrane; the distribution observed on epithelial cells was similar to that observed for C5aR on astrocytes [15]. Other proteins stained using the same protocol gave a homogeneous distribution pattern on epithelial cells, eliminating the possibility that this patched distribution of C3aR was an artefact. As this was suggested before [24], the enrichment of C3aR at localised areas on the membrane surface could enhance the ability of epithelial cells to respond to the anaphylatoxins.

To better understand the involvement of C3a anaphylatoxin in inflammation in general, we investigated the effects of C3a on epithelial cells. We demonstrated that interaction of C3a with epithelial cells enhanced the production of IL-8 in a dose- and time-dependent manner.

Involvement of anaphylatoxins in the processes of inflammation is well established [4,39].

Previous works have shown a role of C3a in the regulation of cytokine synthesis [40]. In the same way, C5a has been previously shown to induce the synthesis and secretion of a number of proinflammatory cytokines including IL-1 β and IL-8 [30,31]. We found that C3a induced IL-8 mRNA and that IL-8 is released from epithelial cells after 12 h of poststimulation by C3a and the peak of IL-8 mRNA is reached between 1 and 3 h of poststimulation by C3a. MAP C3a, an analogue of the C3a peptide, also increases IL-8 mRNA suggesting that the observed effects of C3a on IL-8 production are specific and not due to contaminants in the C3a preparation. In fact the study was first conducted with peptidic anaphylatoxin analogue MAP C3a that bears strong agonist properties [33].

Anaphylatoxins induced an IL-8 mRNA increase in a dose-dependent manner, with an optimal concentration of 10^{-9} M for MAP peptide and 10^{-8} M for anaphylatoxin.

The difference in reactivity between C3a and MAP peptide analogue is due to the superagonist properties of this analogue as demonstrated previously [35,36]. The concentration range in which C3a is active is in good agreement with the C3aR affinity constant for C3a (K_d, 2–5 nM) A dose-dependent curve with an optimal concentration for anaphylatoxin of 10^{-8} M has also been observed for chemotactic action on monocytes [41]. The results observed with MAP C3a and C3a were similar. The IL-8 mRNA increase observed with MAP peptide was confirmed with purified C3a.

Recently, the C3aR cDNA was cloned [21–23] and the C3aR was found to belong to the family of the seven transmembrane receptor. Moreover, several biological responses of C3a can be blocked by PTX, suggesting that the C3aR acts as a Gi coupled receptor.

In the present study, we showed that pre-treatment of epithelial cells with PTX for 4 h blocked the C3a-mediated enhancement of IL-8 mRNA, indicating that this molecule acts through a G protein coupled pathway, possibly involving the C3aR. This could be confirmed later by using a blocking Ab. IL-8 was also investigated at the protein level. Supernatants were tested with a specific ELISA assay and showed an upregulation of IL-8 following stimulation by C3a anaphylatoxin. The upregulation of IL-8 released after stimulation with anaphylatoxin was previously described with monocytes [41,42].

IL-8 is an important cytokine, which is released during inflammation. Attraction of inflammatory cells is predominantly mediated by IL-8, which belongs to the superfamily of chemokines [43].

It is now well established that anaphylatoxin receptors are present on non-myeloid cells [14–23]. Previously the presence of C5aR on epithelial cells was thought to be particularly relevant of the lung since C5a has been implicated as a mediator of acute lung injury [38]. However, C5aR has recently been demonstrated to be expressed in normal renal proximal tubular but not in normal pulmonary or hepatic epithelial cells [44] suggesting that C5aR may have other roles than a modulatory role for C5a/C5aR in the airway inflammatory system. As it has been studied for C5aR, it is difficult to find out a general function for C3aR on epithelial cells as it depends on the origin of the epithelial cells. Indeed epithelial cells are widely spread through the body and exhibited different characteristics according to the origin. By influencing IL-8 production in epithelial cells, C3a and its receptor may contribute to the regulation of both immune responses and inflammation.

This idea has already been suggested by the fact that the deletion of C5aR in mice has revealed that C5aR is important in mice in the protection against infection by bacterium *Pseudomonas aeruginosa* [45]. The expression of C3aR on epithelial cells in inflammation conditions could be important in perpetuation of inflammatory response.

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