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Induction of Functional Anaphylatoxin C₅a Receptors on Hepatocytes by In Vivo Treatment of Rats with IL-6

Henrik L. Schieferdecker,‡* Gerald Schlaf, † Milena Koleva,* Otto Götte, † and Kurt Jungermann*§

In normal rat liver, anaphylatoxin C₅a receptors (C₅aR) are only expressed by nonparenchymal cells, mainly Kupffer cells and hepatic stellate cells, but not by parenchymal cells, i.e., hepatocytes (HC). Nevertheless, C₅a stimulates glucose output by HC. This HC-specific defense reaction is induced indirectly via prostanoids secreted by the C₅aR-expressing Kupffer cells and hepatic stellate cells. It is shown here that under inflammatory conditions simulated by in vivo treatment of rats with IL-6 C₅aR mRNA and protein were induced in HC in a time-dependent manner. Maximal mRNA and protein expression were observed at 4–8 h and 8–10 h, respectively, after IL-6 injection. The newly expressed receptors were functional, because recombinant rat C₅a significantly activated glycogen phosphatase in HC isolated from IL-6-treated but not in HC from control rats. In perfused livers of IL-6-treated animals in contrast to control animals, recombinant rat C₅a-induced glucose output was not impaired by inhibition of prostanoid synthesis and function with the cyclooxygenase inhibitor indomethacin and the thromboxane receptor antagonist daltroban. These results indicate that HC-specific defense reactions might be differently regulated under normal and inflammatory conditions as shown here for the indirect prostanoid-dependent or direct C₅a-induced activation of hepatocellular glycogen phosphatase and glucose output in control or IL-6-treated rats, respectively. The Journal of Immunology, 2000, 164: 5453–5458.

Activation of the complement system via the classical, the alternative, or the mannann-binding lectin pathway leads to the generation of anaphylatoxins C₃a and C₅a. C₅a, a glycopeptide of 77 and 74 aa in rat and human, respectively (1–4), causes the degranulation of mast cells, the contraction of smooth muscle cells, an increase in vascular permeability, and the chemotaxis and activation of neutrophils with local release of reactive oxygen species, eicosanoids, and cytokines (5–7). C₅a receptors (C₅aR)² have been cloned in various species including human (8, 9), mouse (10), dog (11), guinea pig (12), and rat (13, 14). They belong to the seven transmembrane domain receptor family, are coupled to the α-subunit of a pertussis toxin-sensitive G-protein (15, 16), and act via an increase in inositol-1,4,5-trisphosphate and intracellular Ca²⁺.

C₅aR are known to be expressed on cells of the myeloid lineage including neutrophils, eosinophils, monocytes, and macrophages (17) but recently have also been found in nonmyeloid cells of human lung and liver (18) as well as in astrocyte-derived (19) and hepatocyte (HC)-derived (20, 21) cell lines. In contrast to these findings, C₅aR mRNA (22) and protein (23) were expressed by Kupffer cells, hepatic stellate cells, and (weakly) sinusoidal endothelial cells but not by HC isolated from normal rat liver. This expression pattern was in line with functional studies demonstrating that recombinant C₅a (rrC₅a) activated glycogen phosphatase (GPH) (24, 25) and induced glucose output (26, 27) in HC indirectly by stimulating PG and thromboxane release from Kupffer cells (24) and hepatic stellate cells (25). Analogously, C₅a induced the synthesis of acute phase proteins in HC also indirectly by initiating proinflammatory cytokine formation by Kupffer cells (28). Thus, the lack of expression of C₅aR on rat HC under normal conditions has been proven by molecular as well as functional evidence.

An up-regulation of C₅aR expression in human skin and brain has been reported under various inflammatory conditions such as pyrogenic granuloma and lichen planus (29) or allergic encephalomyelitis (30) as well as in the inflamed CNS of different pathologies (31, 32). C₅aR were induced on neurons and glia of transgenic mice with astrocytes constitutively expressing IL-3 (33), in rats with diffuse axonal injury (34), in mice with experimental Listeria meningococcal infection (35), and in patients with Huntington’s disease (36).

Also, the i.p. injection of LPS, a major trigger of inflammation, increased C₅aR mRNA expression in various tissues including the liver of mouse (18) and rat (14, 37). It was postulated that this enhanced expression, at least in part, was due to an induction of C₅aR on HC. Because LPS does not act directly on HC (28), it was therefore investigated whether the proinflammatory cytokine IL-6, which is the major mediator of inflammation in the liver, as has been shown by its ability to induce the synthesis of acute phase proteins in HC (38–41), might elicit the expression of C₅aR mRNA and protein in rat HC. It was found that in vivo treatment of rats with IL-6 caused the expression of functional C₅aR in HC as shown by RT-PCR, immunocytochemistry, flow cytometry, and C₅a-induced direct activation of GPH in isolated HC as well as prostanoid-independent glucose output in the perfused liver.

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2 Abbreviations used in this paper: C₅aR, C₅a receptor; GPH, glycogen phosphorylase; HC, hepatocytes; NA, noradrenaline; NCS, newborn calf serum; rrIL-6, recombinant human IL-6; rrC₅a, recombinant rat C₅a; rsa, rat serum albumin.

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Materials and Methods

Chemicals
All materials were of analytical grade and from commercial sources. Recombinant human IL-6 (rhIL-6) was purchased from Pharma Biotechnologie (Hannover, Germany), and recombinant rat IL-6 (rrIL-6) was obtained from R&D Systems (Wiesbaden, Germany). Percoll was obtained from Pharmacia (Freiburg, Germany); M199 was obtained from AppliChem (Darmstadt, Germany); newborn calf serum (NCS) was obtained from PAA Laboratories (Coibi, Germany); dexamethasone and indomethacin was obtained from Sigma (Deisenhofen, Germany); and insulin, penicillin, streptomycin sulfate, and noradrenaline (NA) was obtained from Serva (Heidelberg, Germany). Tissue culture dishes were obtained from Nunc (Wiesbaden, Germany). Daltroban (BM 13.505, 4-[2-(4-chlorobenzenesulfonylamo)-ethyl] benzene-acetic acid) was kindly provided by Roche (Mannheim, Germany).

Preparation of rrC5a
RrC5a was prepared by synthesis of a cDNA from rat liver RNA and a subsequent PCR using degenerate 5' and 3' primers that were designed according to sequence data published in the EMBL GenBank (accession no. X91892, ID: RNC5AARPT) (4) as described previously (4, 24). RrC5a contained in addition to the original sequence of amino acids 1–77 the N-terminal sequence MRGSHHHHHHGHGS used for its purification from bacterial lysates by Ni2+–chelate chromatography and was depleted of endotoxins by affinity chromatography on polymyxin B agarose. Endotoxin depletion of the C5a stock solution (100 μg/ml) was demonstrated by a negative Limulus amebocyte lysate assay (Sigma) that had a detection limit of 0.01 EU/ml or 1 pg/ml.

Animal treatment
Male Wistar rats (Winkelmann, Borchen, Germany), weighing 170–230 g for perfusion experiments and 130–200 g for the isolation of HC, were kept on a 12-h day/night rhythm (light 7 a.m. to 7 p.m.) with free access to water and a standard rat diet (Sinnfu, Soest, Germany) for at least 2 wk before the experiments. The animals were injected with 0.02 μg rhIL-6 or rrIL-6/g body weight in 0.9% NaCl containing 0.1% albumin (RSA) (“IL-6-treated” animals) or only with 0.9% NaCl/RSA (“NaCl/RSA-treated” or “control” animals) directly or 2, 4, 8, 10, or 30 h before isolation of HC or start of the perfusion experiments as indicated. Due to the high costs of rrIL-6, experiments were performed with rhIL-6. The efficiency of rhIL-6 in the rodent system (42) was confirmed for each experimental series by one control experiment with rrIL-6 (see Results). Treatment of the animals followed the German Law on the Protection of Animals. The experimental protocol was approved by the local ethical committee for animal research.

HC preparation
HC were prepared according to Meredith by Ca2+-free liver perfusion without the use of collagenase (43). Briefly, the liver was perfused via the portal vein in a nonrecirculating manner with a Ca2+-free Krebs-Henseleit buffer containing 15 mM glucose, 2 mM lactate, 0.2 mM pyruvate, and 2 mM EDTA at 37°C and a flow rate of 10 ml/min. After 45 min, the liver was excised, the liver capsule was opened, and HC were suspended in Krebs-Henseleit buffer containing 1 mM CaCl2 and filtered through nylon gauze (mesh diameter, 60 μm). Viable HC were obtained by removing detritus in two subsequent washing steps with centrifugation at 50 x g and further purified through a gradient with 58% Percoll. Purity of HC as identified on the basis of their typical light microscopic appearance was >98%.

RNA isolation and RT-PCR
Total RNA from freshly isolated HC was isolated by the RNeasy Kit provided by Qiagen (Hilden, Germany), preincubated for 10 min at 68°C with 500 ng oligo-d(T)12–18, and transcribed into cDNA with reverse transcriptase (SuperScript II; Life Technologies, Eggenstein, Germany). The cDNA thus generated was amplified with C5aR- or β-actin-specific primers as described in detail elsewhere (22). The C5aR product yielded in HC from IL-6-treated rats was cloned with a SureClone ligation kit (Pharmacia) into pUC 18 and sequenced for identification using dye-deoxy terminator NTPs (Perkin-Elmer, Weiterstadt, Germany).

Cytosin immunofluorescence
A total of 5 x 104 HC in a volume of 150 μl PBS were centrifuged onto a glass slide using a Cytopro cytosin centrifuge (Berthold Schlag, Bergisch Gladbach, Germany) for 6 min at 1300 rpm. Cells on the slides were washed with PBS, and surface Ags were fixed with 0.75% paraformaldehyde at 4°C for 10 min. HC were then incubated with the primary mAbs R63 (anti-C5aR) or MOPC-21 (IgG1-isotype control) at concentrations of 5 μg/ml (R63) or 10 μg/ml (MOPC-21) in PBS for 50 min at 4°C, washed three times with PBS, and incubated with FITC-conjugated goat anti-mouse IgG at a dilution of 1:100 in PBS for another 50 min at 4°C. After three additional washing steps, immunofluorescence patterns were analyzed using a Zeiss Photomicroscope III (Zeiss, Oberkochen, Germany).

FACS analysis
For FACS analysis, HC were resuspended at 2 x 104 cells/ml in “FACS buffer” (PBS/1.5% FCS/0.1% NaN3). The subsequent incubation steps were performed on ice to prevent internalization of surface receptors. For each analysis, 2 x 104 HC were incubated for 45 min in 100 μl FACS buffer supplemented with 4 μg/ml R63 (anti-C5aR) or 10 μg/ml MOPC-21. After three washing steps, cells were incubated with a FITC-conjugated secondary rabbit anti-mouse IgG Ab (1:100) as above but in the dark. After three additional washing steps, cells were fixed by resuspension in 200 μl FACS buffer containing 1% formaldehyde, stored in the dark, and analyzed by flow cytometry with a Coulter EPICS XL-MCL (Beckman-Coulter, Krefeld, Germany).

Determination of GPH activity
For the determination of GPH activity, HC isolated from IL-6-treated or control rats were suspended at 1 g wet weight/100 ml HEPES-buffered

FIGURE 1. C5aR mRNA expression in HC isolated from IL-6-treated but not in HC from control rats. Animals were injected with 0.02 μg/g body weight rhIL-6 (IL-6) or NaCl/RSA (control animals). cDNA was generated by reverse transcription of equal amounts of total RNA from HC isolated directly or 2, 4, 8, 10, or 30 h after injection and then was amplified in the presence of sequence-specific primers for the rat C5aR (511 bp) or rat β-actin (769 bp). PCR products were visualized after electrophoresis in agarose gels by ethidium bromide staining (B) and semiquantified by video densitometry (A). A, Data represent means ± SEM of three (0, 2, 4, 8, and 10 h) or two (30 h) independent experiments. B, Data represent one exemplary result of the experiments shown in A.
C5aR protein expression in HC isolated from IL-6-treated but not in HC from control rats (immunocytochemistry). Cytosin preparations of HC isolated 4, 8, and 10 h after injection of rats with IL-6 or NaCl/RSA (controls), respectively, were fixed with parafomaldehyde and stained with mAb R63 (anti-C5aR) followed by FITC-conjugated goat anti-mouse IgG and photographed. C5aR protein expression was neither detectable in HC isolated 4, 8, and 10 h after NaCl/RSA treatment nor in HC isolated 4 h after IL-6 treatment. HC isolated 8 h after IL-6 treatment expressed C5aR protein; this expression was slightly stronger in HC isolated 10 h after IL-6 treatment. The figure shows one representative result from four independent experiments. Control staining with the IgG isotype control Ab MOPC-21 in HC from control and IL-6-treated rats was always negative (not shown).

FIGURE 2.

Determination of glucose release and flow reduction in the perfused liver

Rat livers were perfused in situ via the portal vein in a nonrecirculating fashion with Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 5 mM glucose, 2 mM lactate, and 0.2 mM pyruvate at a rate of 4 ml/min per gram of liver. The buffer was equilibrated with 95% O2 and 5% CO2 at 37°C. Livers were preperfused for 15 min with buffer and for an additional 15 min with buffer containing 0.1% DMSO and indomethacin and daltroban (20 µM each) where indicated. RrC5a was then infused to a final concentration of 100 nM for 30 s. After an additional 10 min, NA was infused for a second stimulation to a final concentration of 1 µM as a control. The effluvate was fractionated in 1-min intervals to measure flow. Glucose was determined in these fractions using a commercial test kit from Merck (Darmstadt, Germany) (45).

Statistics

Values are given as means ± SEM of the number of experiments indicated. Two groups were compared by Student's t test for unpaired samples. Values of p < 0.05 were regarded as significant differences.

Results

Induction of C5aR mRNA expression in HC by in vivo treatment of rats with IL-6

HC from normal rats do not express C5aR mRNA (22). Treatment of rats with 0.02 µg rhIL-6/g body weight caused a time-dependent expression of C5aR mRNA in HC (Fig. 1). HC isolated directly after IL-6 injection did not express C5aR mRNA. C5aR mRNA was first detected at 2 h and was maximally expressed between 4 and 8 h after IL-6 injection, depending on the experiment. Thereafter, C5aR mRNA expression declined until it was almost undetectable at 30 h after injection. Analogous results were obtained by treatment of the animals with 0.02 µg rIL-6/g body weight (data not shown). RT-PCR amplification of total RNA with C5aR-specific primers yielded a single cDNA product corresponding to the expected 511 bp (Fig. 1B); its identity was verified by sequencing (data not shown). In HC from control animals, a very faint or no expression of C5aR mRNA could be detected at 4 and 8 h after NaCl/RSA injection, but the expression was always significantly lower than that induced by IL-6 treatment (Fig. 1A).

Induction of C5aR protein expression in HC by in vivo treatment of rats with IL-6

HC from normal rats do not express C5aR protein (23). HC isolated after treatment of rats for 4 h with rIL-6 did not express C5aR protein as revealed by immunofluorescence cytochemistry of cytospins (Fig. 2). In HC obtained 8 h after IL-6 injection, substantial C5aR protein expression was demonstrated; C5aR expression was similar or even slightly stronger in HC isolated 10 h after IL-6 treatment. Analogous results were obtained by treatment of the animals with 0.02 µg rrIL-6/g body weight (data not shown). In HC isolated from control animals, significant C5aR expression was still detectable at 30 h. In HC isolated from IL-6-treated rats, C5aR expression was similar or even slightly stronger in HC isolated 10 h after IL-6 treatment. Analogous results were obtained by treatment of the animals with 0.02 µg rrIL-6/g body weight (data not shown). In HC isolated from control animals, significant C5aR expression was still detectable at 30 h.
protein expression was neither detected 4, 8, nor 10 h after NaCl/RSA injection.

For semiquantification, C5aR protein expression was also investigated by flow cytometry (Fig. 3). In HC isolated 4 h after IL-6 treatment of rats, no C5aR protein expression could be detected. In contrast, a substantial C5aR expression was observed in HC isolated 8 h after IL-6 injection. The expression remained stable or in some experiments slightly increased up to 10 h after IL-6 injection. Analogous results were obtained by treatment of the animals with 0.02 μg rrIL-6/g body weight (data not shown). In HC isolated from control animals, C5aR protein could not be detected 4, 8, or 10 h after NaCl/RSA injection.

Induction of C5a reactivity in HC by in vivo treatment of rats with IL-6

Direct activation of hepatocellular GPH by rrC5a. To examine the functioning of the newly expressed C5aR, it was investigated whether rrC5a directly activated GPH in HC from IL-6-treated rats. RrC5a enhanced GPH activity in isolated HC slightly 4 h and strongly 8 and 10 h after IL-6 injection (Table 1). These findings were in line with the expression of C5aR protein (cf. Fig. 2 and 3). After 10 h, rrC5a activated hepatocellular GPH to a similar extent as NA, which is known to directly increase GPH activity in HC

<table>
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Table 1. Direct activation by rrC5a of GPH in HC isolated from IL-6-treated but not in HC from control rats

*Isolated HC from rats that had been treated with NaCl/RSA (control) or with IL-6 for the time period indicated were stimulated with rrC5a (final concentration, 100 nM) or, for comparison, with NA (1 μM) in the presence of indomethacin (final concentration, 20 μM) as described in Materials and Methods. The reactions were stopped after 2 min, and GPH activity was determined in cell homogenates. Values represent increases in GPH activity compared to basal unstimulated values (100%) and are means ± SEM of the number of experiments indicated.

* p ≤ 0.05, significant differences compared with basal unstimulated values (Student’s t test for unpaired samples).

### Discussion

It is shown in this study that C5aR that are not normally expressed by HC (22, 23) were up-regulated in these cells both on the mRNA (Fig. 1) and the protein level (Fig. 2 and 3) under inflammatory conditions as simulated in vivo treatment of rats with the proinflammatory cytokine IL-6. It is demonstrated further that the up-regulated C5aR were functional both in isolated HC (Table 1) and in perfused livers (Fig. 4).

**C5aR expression in HC of IL-6-treated rats**

In the early phase of inflammation, when the liver is still in its normal state and HC do not express C5aR (22, 23), the anaphylatoxin C5a generated systemically cannot directly elicit defense
reactions in HC such as glucose release (26, 27) or acute phase protein synthesis (28); it can only act indirectly via the release of prostanooids or cytokines, respectively, from Kupffer cells (26, 28) and hepatic stellate cells (27), which bear the C5aR (22, 23). During prolonged systemic or local hepatic inflammation the pattern of receptor expression on liver cells may change. HC might express C5aR, so that they can be stimulated directly by C5a.

In this study, experimental evidence is provided that in vivo treatment of rats with the proinflammatory cytokine IL-6 indeed induced C5aR expression in HC (Figs. 1–3). This induction was independent of whether rhIL-6 or rIL-6 was used, confirming the activity of human IL-6 in the rodent system (42). In some cases, a slight increase in C5aR mRNA was also observed in HC isolated from NaCl/RSA-treated rats (Fig. 1A). Nevertheless this slight, presumably “stress-induced,” C5aR mRNA expression was much weaker than that induced by IL-6 and did not lead to the surface expression of C5aR protein (Figs. 2 and 3).

The finding of an inducible C5aR expression by the proinflammatory cytokine IL-6 is in line with previous observations in other tissues or cell systems having demonstrated an up-regulated C5aR expression during inflammatory processes. Keratinocytes expressed detectable levels of C5aR mRNA only in inflamed but not in normal skin (29), and reactive astrocytes and microglia and, to a lesser extent, also endothelial cells up-regulated C5aR expression in the inflamed human CNS (30).

Inducible C5aR expression under inflammatory conditions might explain contradictory results concerning C5aR expression by HC. While HC isolated from normal rats have been shown not to express C5aR mRNA and protein (22, 23), human HC (18, 20, 21) were reported to express C5aR. In these studies C5aR were detected either by in situ hybridization of human liver tissue (18), the source of which was not indicated, or on HepG2 cells (20, 21). The present investigation provides evidence that the findings with human liver tissue might not represent results obtained with HC in a normal state but most likely indicate a disease-induced up-regulation of C5aR mRNA in the livers of patients from whom the tissues were obtained. Similarly, the findings with HepG2 cells presumably are due to an up-regulation of C5aR during transformation of normal HC into hepatoma cells. These assumptions are confirmed by 1) a recent study demonstrating the absence of C5aR in human HC using normal liver tissue (46) and 2) the detection of C5aR mRNA in the rat hepatoma cell lines FAO and H4IIE but not in primary rat HC (23), presumably indicating a frequent or even general activation of the C5aR gene during malignant transformation.

An up-regulation of C5aR mRNA expression in the liver was observed previously after in vivo treatment of rats with LPS (14, 37). However, it was not investigated whether this enhanced expression occurred in nonparenchymal liver cells or in HC. Because LPS did not up-regulate C5aR mRNA expression directly in cultures neither of Kupffer cells, hepatic stellate cells, sinusoidal endothelial cells, nor of HC (C. Mäck, M. Koleva, H. L. Schiefer-decker, and K. Junkermann, unpublished observations), it is likely that the enhanced C5aR mRNA expression in the liver following LPS treatment in vivo (14, 37) was induced by mediators released from responder cells in the periphery or locally within the liver. Among the most important mediators of inflammation is the proinflammatory cytokine IL-6, which can be released from Kupffer cells after stimulation with LPS (47). It has to be elucidated in further studies 1) whether the enhanced C5aR expression in the liver after LPS treatment in vivo occurs mainly in HC and 2) whether LPS acts indirectly via IL-6.

**Functioning of C5aR in HC of IL-6-treated rats**

Among the most important functions of HC is the release of glucose from glycogen stores. In normal livers in which the HC are devoid of C5aR, the anaphylatoxin cannot enhance glucose output from HC directly but only indirectly by stimulating the release of prostanooids from Kupffer cells and hepatic stellate cells (24–28). Thus, the C5a-dependent increase in glucose output from perfused livers of normal rats can be inhibited by the cyclooxygenase inhibitor indomethacin and the thromboxane receptor antagonist daltroban (27). Similarly, the C5a-dependent activation of GPH cannot be demonstrated in HC monocytes but only in cocultures of HC with prostanooid-secreting Kupffer cells (24) or hepatic stellate cells (25). Therefore, the findings of this study that C5a enhanced glucose output from perfused livers of IL-6-treated rats without impairment by indomethacin and daltroban (Fig. 4) and that it activated GPH in monocytes of HC from these animals (Table I) indicate that the newly expressed C5aR on HC were functional.

The data presented in this study demonstrate that hepatic defense reactions, as shown here for the short-term glucose output from HC, can be regulated by C5a via different mechanisms during the early and the later stages of inflammation. In the early stages, when HC are still devoid of C5aR, glucose output is stimulated by C5a indirectly via the release of prostanooids from Kupffer cells and hepatic stellate cells. In the later stages, when HC have been induced to express C5aR themselves, glucose output can be elicited by C5a directly without the intervention of nonparenchymal cells.

**References**


