N-Formylpeptide and Complement C5a Receptors Are Expressed in Liver Cells and Mediate Hepatic Acute Phase Gene Regulation

By Roderick McCoy,*‖ David L. Haviland,*¶ Ernesto P. Molmenti,*‖ Theodoros Ziambaras,*‖ Rick A. Wetsel,*§¶ and David H. Perlmutter*‡‖

*From the Departments of *Pediatrics, ‡Cell Biology and Physiology, §Microbiology and Immunology, Washington University School of Medicine; and Divisions of ‖Gastroenterology/Nutrition and ¶Pulmonary Medicine, St. Louis Children's Hospital, St. Louis, Missouri 63110*

Summary

Although the classical chemotactic receptor for complement anaphylatoxin C5a has been associated with polymorphonuclear and mononuclear phagocytes, several recent studies have indicated that this receptor is expressed on nonmyeloid cells including human endothelial cells, vascular smooth muscle cells, bronchial and alveolar epithelial cells, hepatocytes, and in the human hepatoma cell line HepG2. In this study, we examined the possibility that other members of the chemotactic receptor family are expressed in HepG2 cells and human liver, and the possibility that such receptors mediate changes in acute phase gene expression in HepG2 cells. Using polymerase chain reaction (PCR) amplification of HepG2 mRNA with primers based on highly conserved regions of the chemotactic subgroup of the G protein–coupled receptor family, we identified a PCR fragment from the formyl-methionyl-leucyl-phenylalanine (FMLP) receptor, as well as one from the C5a receptor. Immunostaining with antipeptide antisera to FMLPR confirmed the presence of this receptor in HepG2 cells. Receptor binding studies showed specific saturable binding of a radioiodinated FMLP analogue to HepG2 cells ($K_d \sim 2.47$ nM; $R \sim 6 \times 10^3$ plasma membrane receptors per cell). In situ hybridization analysis showed the presence of FMLPR mRNA in parenchymal cells of the human liver in vivo. Both C5a and FMLP mediated concentration- and time-dependent changes in synthesis of acute phase proteins in HepG2 cells including increases in complement C3, factor B, and α1-antichymotrypsin, as well as concomitant decreases in albumin and transferrin synthesis. The effects of C5a and FMLP on the synthesis of these acute phase proteins was evident at concentrations as low as 1 nM, and they were specifically blocked by antipeptide antisera for the corresponding receptor. In contrast to the effect of other mediators of hepatic acute phase gene regulation, such as interleukin 6, the effects of C5a and FMLP were reversed by increased concentrations well above the saturation point of the respective receptor. These results suggest that acute phase gene regulation by C5a and FMLP is desensitized at high concentrations, a property that is unique among the several known mechanisms for hepatic acute phase gene regulation.

The complement C5a fragment and the synthetic *N*-formyl peptide FMLP are best known as prototype activators of neutrophils and neutrophil chemotactic activity. C5a is a 11,200-D cationic glycopeptide that is derived from the first 74 amino acid residues of the amino terminus of the C5 α chain (1, 2). It is generated by the action of the classical complement pathway (C4b, 2a,3b) or alternative complement pathway (C3b, Bb,P) convertases during complement activation (3–5). C5a has been shown to mediate chemotactic activity, phagocytic activity, release of active oxygen intermediates, release of granular enzymes, and increase in adhesion/aggregation properties in neutrophils (reviewed in 6–8). C5a is also thought to have vasoactive properties, mediating vasodilatation (9), increased vascular permeability (10), smooth muscle contraction (11), and release of histamine from mast cells (12). FMLP is one of several *N*-formyl tripeptides originally discovered by Schiffmann et al. (13) as the active principle in supernatants of bacterial cultures that were chemotactic for mammalian phagocytes in vitro. FMLP is now known to activate a complex program in neutrophils

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/07/0207/11 $2.00
Volume 182 July 1995 207–217

that results in directed cell migration, release of active oxygen intermediates, and release of proteolytic and phlogistic granular contents (reviewed in 14).

It has been known for some time that the effects of C5a and FMLP are mediated by distinct specific receptor-activated signal transduction pathways. In each case, signal transduction is associated with cellular calcium mobilization, is regulated by homologous desensitization, and is inhibited by pertussis toxin, providing evidence that members of the G protein–coupled receptor family are involved. Sequence analyses of cDNAs for high affinity C5a (15, 16) and FMLP (17) receptors have shown by structural criteria that these receptors do indeed belong in the G protein–coupled receptor family. In fact, the structures of the C5a and FMLP receptors have been shown to be highly conserved in a subfamily of G protein–coupled receptors that include the IL-8A, IL-8B, C-C chemokine, and MIP-1α receptors (18, 19). In addition to seven transmembrane-spanning domains that are relatively conserved among the entire G protein–coupled receptor family, the so-called "chemokine" receptor subfamily is highly conserved in the sequence of the first, second, third, sixth, and seventh membrane-spanning domains and has a highly conserved stretch of nine amino acids on the cytoplasmic loop just after the third transmembrane domain. This receptor subfamily is also related by pharmacological characteristics, as well as functional involvement in chemotaxis and inflammatory activation.

Although C5aR and FMLPR expression has been associated with cells of myeloid origin, such as neutrophils, eosinophils, monocytes, macrophages, and myeloid cell lines U937 and HL-60, several recent studies have suggested that C5aR is expressed in nonmyeloid cells. Foreman et al. (20) have shown that C5a binds to human endothelial cells. We have shown that C5aR is expressed in vascular smooth muscle cells, endothelial cells, bronchial and alveolar epithelial cells of the lung, as well as in parenchymal cells of the liver and human hepatoma HepG2 cells (21). C5aR expression in vascular smooth muscle cells and endothelial cells could be responsible for the vasoactive properties of C5a, but there are no previous data that would provide an explanation for the function of C5aR in bronchoalveolar epithelial cells and hepatocytes. In this study, we used HepG2 mRNA in PCR with primers based on highly conserved regions of the chemotactic G protein–coupled receptor subfamily to examine the possibility that other members of the subfamily are expressed in liver cells. We also examined the possibility that expression of C5aR and/or other chemotactic receptors in liver cells was functionally significant by determining whether ligands for these receptors could elicit acute phase gene expression in HepG2 cells, a model system reflecting the acute inflammatory response of liver.

Materials and Methods

PCR of Chemotactic Receptors, Subcloning, and Sequencing. Total cellular RNA was isolated from HepG2 cells by guanidine isothiocyanate extraction/cesium chloride centrifugation/ethanol precipitation (22) and poly(A) RNA was prepared by oligo (dT)-cellulose column chromatography. Double-stranded cDNA was synthesized using a kit from Amersham Corp. (Arlington Heights, IL). Addition of BstXI nonpalindromic linkers to the blunt-end cDNA and size fractionation of cDNA on a 1% agarose gel were performed using a kit from InVitrogen (San Diego, CA). cDNA larger than 1.0 kb was recovered from the gel and ligated to BstXI-digested CDM8 vector (23). *Escherichia coli* MC1061/p3 cells were transformed with the ligated DNA using the InVitrogen kit. The completed library represented $\sim 4 \times 10^5$ recombinants. Plasmid DNA was isolated from an amplified aliquot of the transformed library using a kit from QIAGEN Inc. (Chatsworth, CA). This plasmid DNA was used as template for PCR. Several sets of degenerate oligonucleotides were used in the PCR: one primer pair was based on highly conserved regions in the second and sixth transmembrane domains of the tachykinin G protein–coupled receptors (24, 25) (MII sense: CTIGCI$^{T}/_{C}$TIGCIGA$^{T}/_{C}{}^{T}/_{C}$T; MVI antisense: GGGIAGCCAGCAGAI$^{G}/_{C}{}^{G}/_{A}{}^{T}/_{C}{}^{G}/_{A}$AA); a second primer pair was based on highly conserved regions in the third and sixth transmembrane domains of the tachykinin G protein–coupled receptors (24, 25) (MIII sense: CTGTG$^{C}/_{T}$G$^{C}/_{T}{}^{C}/_{G}$AT$^{T}/_{C}$GCIIT$^{G}/_{T}$GA$^{T}/_{C}{}^{A}/_{C}$G$^{C}/_{G}$TAC; MVI antisense: exactly as delineated above); a third primer pair was based on highly conserved regions in the second and seventh transmembrane domains of the chemotactic G protein–coupled receptors, including IL-8RA, IL-8RB, C5aR, FMLPR, C-C CKR R (18) (M2CC sense: $^{T}/_{C}$TIAA$^{T}/_{C}{}^{T}/_{C}$ TIGCI$^{T}/_{G}$CTIGCIGA$^{T}/_{C}{}^{T}/_{C}$T; M7CC antisense: TGICCI$^{G}/_{A}{}^{T}/_{C}$IA$^{C}/_{A}$I$^{G}/_{A}$C$^{G}/_{A}$TAIA$^{T}/_{G}$AIATIGG$^{G}/_{A}$T); a fourth set was identical to the set used by Neote et al. (18) to clone C-C chemokine receptors from U937 cells [M2CC G sense: CT$^{G}/_{C}$AAC$^{C}/_{T}$TGGC$^{C}/_{G}{}^{T}/_{G}$ T$^{G}/_{A}$GC$^{C}/_{T}$GAC$^{C}/_{T}$T$^{A}/_{C}$CTC; M7CCG antisense: TGGCC$^{G}/_{C}{}^{A}/_{G}{}^{T}/_{C}{}^{G}/_{C}A^{A}/_{C}{}^{G}/_{C}{}^{A}/_{G}C^{G}/_{A}$TAGA$^{T}/_{G}{}^{G}/_{C}$ATGGGGTT; DRYCCG antisense: GC$^{A}/_{G}$ TGGAC$^{A}/_{G}$ATNGCNAGG TANCG$^{A}/_{G}$TC]. PCR conditions were 55 cycles at 92°C for 1 min, 50–55° for 1 min, and 70°C for 2 min. PCR products were subcloned in the pGEM3z plasmid (Promega Corp., Madison, WI) and plasmid DNA was subjected to sequencing with the Sequenase kit (U.S. Biochemicals Corp., Cleveland, OH).

In Situ Hybridization Analysis. Human liver was obtained from two different organ donors through the Mid America Transplant Association (St. Louis, MO). The studies were approved by the Human Studies Committee of Washington University School of Medicine. In a protocol that has been previously described (26), cryostat sections (5–8 μm) of liver were fixed in 4% paraformaldehyde and prehybridized after proteinase K digestion (1 μg/ml for 10 min). Hybridization with ^{35}S-UTP–labeled antisense cRNA probe was performed at 55°C for 24 h in 60% formamide, 15% dextran sulfate, 1× Denhardt's solution, 1 mM EDTA, 10 mM Tris HCl, pH 8, 400 mM NaCl. Sections were then subjected to RNAse A digestion to remove all nonhybridized single-stranded RNA, washed at stringencies up to 0.1× SSC at SS°C for 30 min, and exposed to photoemulsion for 26 d at 4°C. Cellular expression of mRNA was indicated by the presence of silver grains. To be certain that the hybridization signals were specific, sequential sections of the same tissue were hybridized under identical conditions using ^{35}S-UTP–labeled sense probe.

Flow Cytometry. Flow cytometric analysis was conducted as follows: 4×10^5 HepG2 cells were removed from flasks using PBS with 5 mM EDTA. Cells were washed twice in 0.1% BSA-PBS supplemented with 1 mM $CaCl_2$ and 0.05% NaN_3, dispersed into 15-ml conical tubes, and incubated with 5 μl of the anti-FMLPR antiserum on ice for 30–45 min. Cells were kept on ice so as to minimize downmodulation of FMLPR caused by receptor internalization. Cells were washed three times in cold buffer and incubated in the dark for 30 min with FITC–goat anti-rabbit IgG

on ice. Cells were washed three times in cold buffer (10 ml), fixed in 1% paraformaldehyde, and rapidly resuspended in 200 μl of buffer for analysis. Controls received irrelevant primary antibody (preimmune serum) or second antibody alone.

Immunofluorescence Studies. HepG2 cells were seeded onto glass coverslips, cultured for 3 d, rinsed with PBS, and then permeabilized with acetone for 1 min at room temperature. Cells were then incubated with rabbit anti-human FMLPR antiserum or preimmune rabbit serum in PBS with 3% BSA for 2 h at room temperature. Coverslips were washed three times in PBS, 1% Triton X-100, 0.2% Tween 20 and then incubated for 1 h at room temperature in the dark with FITC-anti-rabbit IgG. The coverslips were then washed three times and mounted on glass slides for microscopic viewing.

Receptor-binding Assays. The FMLP analogue formylNLe-Leu-Phe-NLe-Tyr-Lys was obtained from Sigma Immunochemicals (St. Louis, MO) and was labeled with Na ^{125}I using chloramine T exactly as described in previous studies (27). ^{125}I-FMLP analogue was separated from free ^{125}I by Sephadex G-10 (Pharmacia, Piscataway, NJ) gel filtration. Specific radioactivity was ~2,000–6,000 cpm/ng of peptide. Monolayers of HepG2 cells were washed vigorously with PBS and then incubated for 2 h at 4°C in binding medium (DMEM, 10 mM Hepes, pH 7.2, 1 mg/ml BSA, 0.05% Tween 80) with ^{125}I-FMLP analogue in several different concentrations. Duplicate samples were incubated in the absence (total binding) or presence (nonspecific binding) of a 200-fold molar excess unlabeled FMLP. After the 2-h incubation at 4°C, the cells were washed with PBS and then solubilized in 1 N NaOH for gamma counting. Specific binding was determined by the difference between total and nonspecific binding.

Regulation of Acute Phase Protein Biosynthesis. Monolayers of HepG2 cells were rinsed and then incubated at 37°C for different time intervals in serum-free DMEM alone or supplemented with FMLP or recombinant human C5a (Sigma) in different concentrations. At the end of each of these incubations, the monolayers were again rinsed and incubated at 37°C for 30 min in the presence of methionine-free medium containing 250 μCi/ml [^{35}S]methionine. Cells were then solubilized and cell lysates were subjected to clarification. Cell lysates were also subjected to TCA precipitation to determine relative total protein synthesis according to previously described protocols (28). Aliquots of cell lysates were incubated overnight at 4°C in 1% Triton X-100, 1.0% SDS, 0.5% deoxycholic acid, with excess antibody. Immune complexes were then precipitated with excess formalin-fixed staphylococci-bearing protein A (Immunoprecipitin; Life Technologies, Inc., Gaithersburg, MD), washed, released by boiling in Laemmli sample buffer, and applied to SDS-PAGE under reducing conditions. Gels were dried for fluorography as previously described (28). A laser densitometer (model 2222 Ultrascan XL; LKB Instruments, Inc., Houston, TX) was used for scanning of fluorograms.

Antibodies. Synthetic peptides with sequences that corresponded to divergent regions of the amino-terminal extracellular tails of C5aR (C5aR 7-24: TTPDYGHYDDKDTLDLTN) and of FMLPR (FMLPR 4-21: NSSLPTNISGGTPAVSAG) were coupled to KLH. The KLH-coupled peptides were used as immunogens to raise antipeptide antisera in rabbits. By ELISA, anti-C5AR and anti-FMLP antisera were specific, sensitive, and were shown to specifically inhibit immunofluorescent staining of dbcAMP-stimulated U937 cells by C5a and FMLP.

Results

Identification of FMLPR Expression in HepG2 Cells. In previous studies, we discovered C5aR expression in HepG2 cells and human liver parenchymal cells in vivo (21). To determine whether other members of the chemotactic G protein–coupled receptor family are expressed in HepG2 cells, we subjected plasmid DNA from an HepG2 cDNA library to PCR with primers based on highly conserved regions of the tachykinin G protein–coupled receptor family and the subgroup of chemotactic G protein–coupled receptors (Fig. 1). A single PCR fragment of 1,400 bp was generated with positive control primers based on α_1-antitrypsin (α_1-AT)[1] sequence (lane *1*). PCR fragments were not detected in the absence of template (lane *2*). Multiple PCR fragments of ~200–1,600 bp were generated with primers based on the tachykinin G protein–coupled receptors (lanes *3–5*). A single PCR product of ~780 bp was detected with primers based on the chemotactic G protein–coupled receptors (lane *6*). This product was subjected to DNA sequence analysis and found to be identical to the human FMLPR sequence (17). We did not detect separate PCR products for any of the other chemotactic receptors. Using another pair of primers identical to the ones that were used to detect C5aR, IL-8AR, IL-8BR, and C-C chemokine R in U937 cells by Neote et al. (18), we detected a PCR fragment that was identical to C5aR. Even with this last pair of primers, however, there was no evidence for IL-8AR, IL-8BR, or C-C chemokine R in HepG2 cells (data not shown).

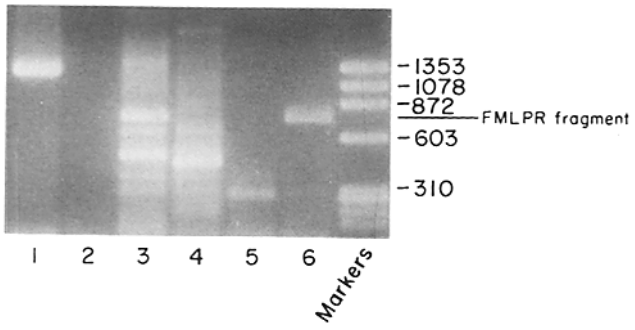


Figure 1. PCR amplification of G protein–coupled receptors from HepG2 cells. Total RNA was isolated from HepG2 cells and subjected to PCR as described in Materials and Methods. (Lane *1*) Primers for amplification of α1-antitrypsin cDNA. (Lane *2*) No template. (Lane *3*) 5′ primer for the second transmembrane domain of the chemotactic G protein-coupled receptors (*M2CC*) and 3′ primer for the sixth transmembrane domain of the tachykinin G protein–coupled receptors (*MVI*). (Lane *4*) 5′ primer for the third transmembrane domain of the tachykinin G protein–coupled receptors (*MIII*) and 3′ primer for the seventh transmembrane domain of the chemotactic G protein–coupled receptors (*M7CC*). (Lane *5*) 5′ primer for the second transmembrane domain of the tachykinin G protein–coupled receptors (*MII*) and 3′ primer of *M7CC*. (Lane *6*) 5′ primer of *M2CC* and 3′ primer of *M7CC*. The relative migration of DNA markers and of the 780-bp fragment corresponding to FMLPR are indicated at the right margin.

Next, we examined the possibility that FMLPR could be detected by immunofluorescence at the cell surface of HepG2. HepG2 cells were incubated with anti-FMLPR peptide antiserum, anti-C5aR peptide antiserum, or preimmune serum

[1] *Abbreviations used in this paper:* ACT, antichymotrypsin; AT, antitrypsin.

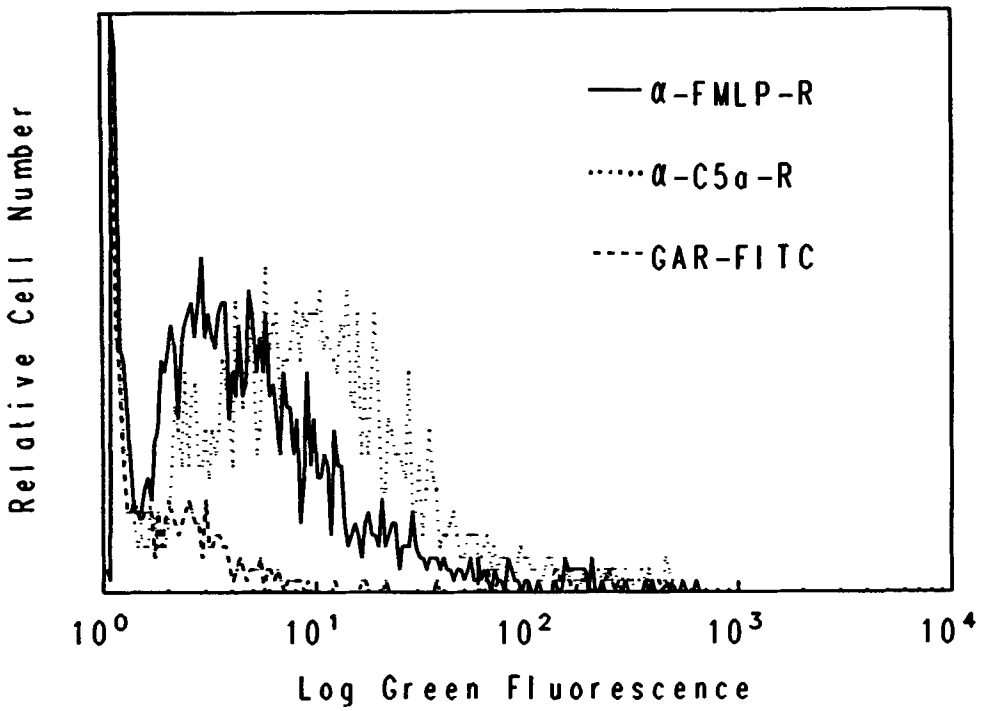


Figure 2. Flow cytometry of HepG2 cells. HepG2 cells were incubated with anti-FMLPR antiserum (*solid line*, *α-FMLPR*), anti-C5aR antiserum (*dotted line*, *α-C5a-R*), and preimmune serum (*dashed line*, *GAR-FITC*), and then analyzed by flow cytometry as described in Materials and Methods.

and then incubated with FITC-conjugated anti-Ig for flow cytometric analysis (Fig. 2). The results showed a significant shift for anti-FMLPR and by anti-C5aR staining, but not for preimmune serum or anti-IL-8AR staining (data not shown).

To determine whether FMLPR is functionally active in these cells and to provide quantitative characterization of FMLPR expression, HepG2 cells were subjected to receptor-binding assays with a radioiodinated FMLP analogue (Fig. 3). These studies demonstrated the presence of specific and saturable binding of ^{125}I FMLP analogue to HepG2 cells (Fig. 3 *A*). Scatchard plot analysis of these binding data predicts a K_d of ~2.47 nM and ~6,000 plasma membrane receptors per cell (Fig. 3 *A*). Competitive binding assays show that recognition of radioiodinated FMLP analogue by HepG2 cells is specific (Fig. 3 *B*): it is inhibited by FMLP in a concentration-dependent manner, but is not inhibited by C5a in concentrations that inhibit binding of ^{125}I-C5a to HepG2 cells (21), and is not inhibited by peptide 105Y in concentrations that inhibit binding of ^{125}I-peptide 105Y to HepG2 cells (27).

To exclude the possibility that FMLPR expression in HepG2 cells is a result of transformation of liver cells into a con-

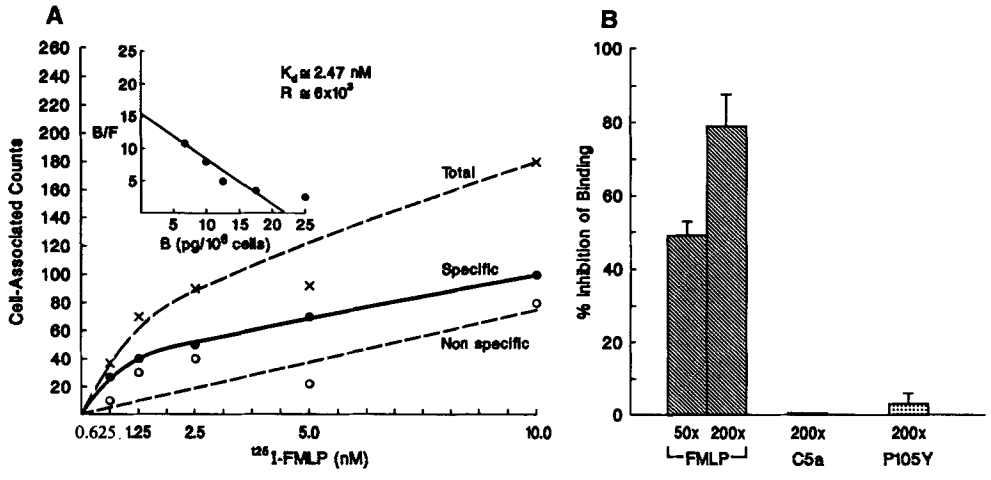


Figure 3. Specific binding of ^{125}I-FMLP analogue to HepG2 cells. (*A*) HepG2 cells were incubated for 2 h at 4°C with concentrations of ^{125}I-FMLP analogue shown on the horizontal axis in the absence (total) or presence (nonspecific) of 250-fold molar excess of unlabeled FMLP. The difference between total and nonspecific binding is designated specific binding. The results are reported as cell-associated radioactivity on the vertical axis. The inset shows the Scatchard plot of the specific ^{125}I-FMLP analogue binding. (*B*) Percent inhibition of binding of ^{125}I-FMLP analogue (5 nM) to HepG2 by FMLP, C5a, and peptide 105Y. Fold molar excess competitor is shown at the bottom of each bar. Results are reported as mean ± 1 SD.

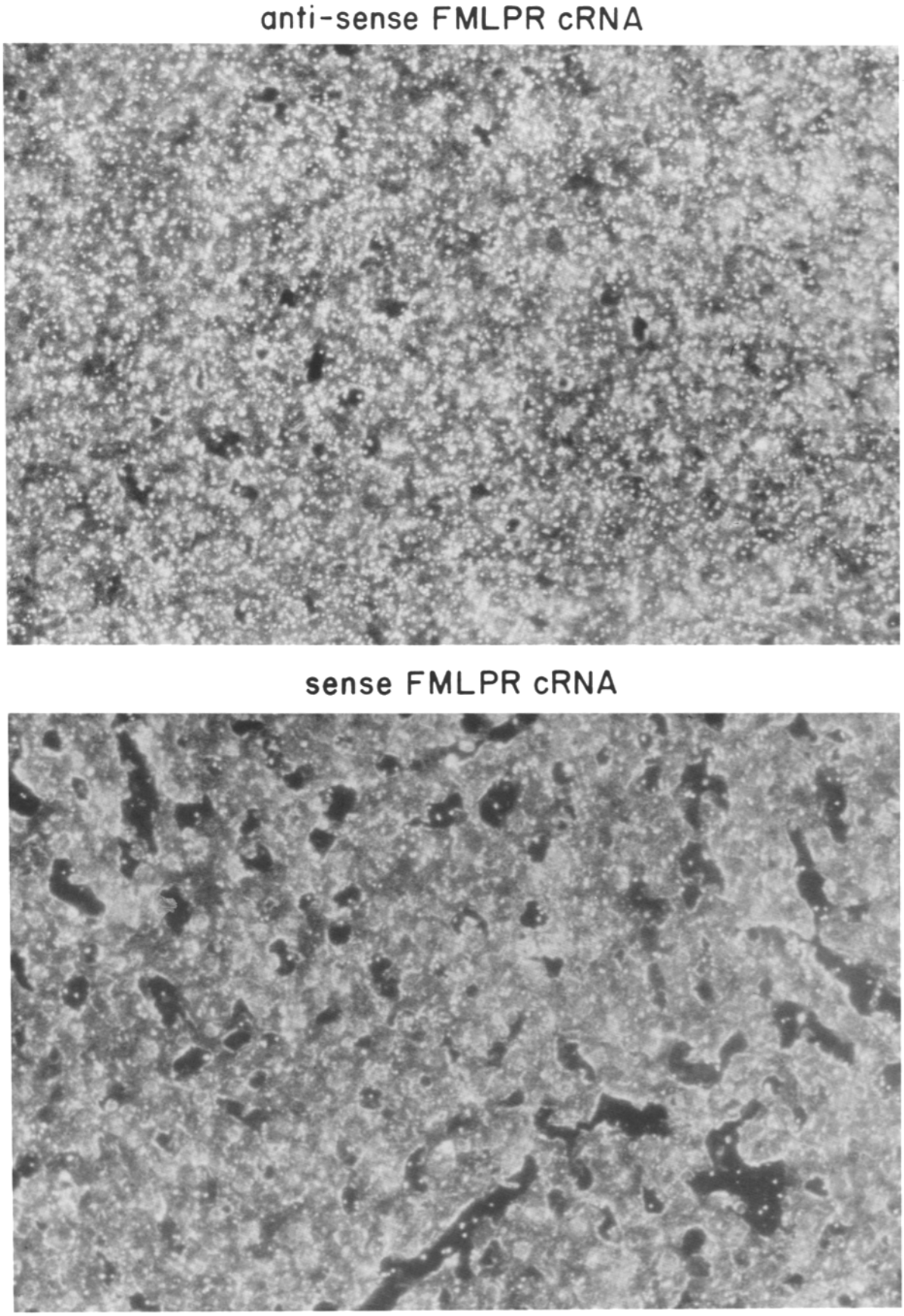


Figure 4. In situ hybridization of human liver tissue. (*Top*) A section of human liver probed with an antisense ^{35}S-UTP-labeled-FMLPR cRNA as described in Materials and Methods (×200). Background signal was determined by incubating an adjacent section of human liver with sense ^{35}S-UTP-labeled FMLPR cRNA (*bottom*).

tinuous cell line and not necessarily a reflection of the physiological function of hepatocytes in vivo, we examined by in situ hybridization analysis the possibility that FMLPR is expressed in liver cells in vivo. Sections of human liver were subjected to in situ hybridization with ^{35}S-UTP–labeled antisense FMLPR cRNA (Fig. 4). The results show localization of silver grains to parenchymal cells throughout the liver lobule (Fig. 4, *top*). This hybridization is specific for FMLPR mRNA as demonstrated by the absence of silver grains in serial sections incubated with ^{35}S-UTP–labeled sense FMLPR cRNA (Fig. 4, *bottom*). These data provide further evidence that FMLPR is expressed in liver cells.

Effect of C5a and FMLP on Acute Phase Protein Biosynthesis in HepG2 Cells. Because C5a and FMLP play a role in the inflammatory response of myeloid cells, we examined the possibility that the interaction of these peptides with their receptors on HepG2 cells induces a hepatic inflammatory response. Many previous studies have shown that a hepatic inflammatory response, or what is now called the "hepatic acute phase response," can be elicited in the HepG2 cell line (reviewed in 29, 30). First, we examined the effect of C5a and FMLP on synthesis of a prototype positive acute phase reactant, C3 (Fig. 5). C3 expression is upregulated by several different acute phase cytokines including IL-1, TNF, and IFN-γ. HepG2 cells were incubated at 37°C for 10 h in serum-free medium or supplemented with C5a, FMLP, or as positive control, a combination of IL-1β and IL-6. Cells were then subjected to labeling with [^{35}S]methionine for analysis of the synthesis of specific proteins. The results show that both C5a and FMLP mediate increases in C3 synthesis (Fig. 5, *left*). The effects of C5a and FMLP on C3 synthesis were evident at concentrations as low as 1 nM and were less than but comparable in magnitude to the combined effect of IL-1β and IL-6. The effects were specific in that both C5a and FMLP mediated concentration-dependent decreases in synthesis of albumin, a prototype negative acute phase reactant, in the same cell monolayers (Fig. 5, *right*). The effect of C5a on albumin biosynthesis was first evident at 10 nM, whereas that of FMLP was evident at 1 nM. In each case, the magnitude of the effect was less than but comparable to that of the combination of IL-1β and IL-6. There was some variation in the magnitude of the effect of C5a and FMLP on synthesis of C3 and albumin from one experiment to the next. Results of at least five different experiments are shown in Table 1 to show the magnitude variation in terms of standard deviations.

Next, we examined the time dependence of the effect of C5a and FMLP on synthesis of C3 and albumin. Separate monolayers of HepG2 cells were incubated for time intervals of up to 24 h with serum-free medium alone or supplemented with C5a or FMLP and then subjected to analysis for synthesis of C3 and albumin. Results for time intervals of up to 6 h are shown in Fig. 6. The results show that the effects of C5a and FMLP, including increase in C3 synthesis and decrease in albumin synthesis, are time dependent and become evident as early as 6 h after addition.

We also examined the effect of C5a and FMLP on a number

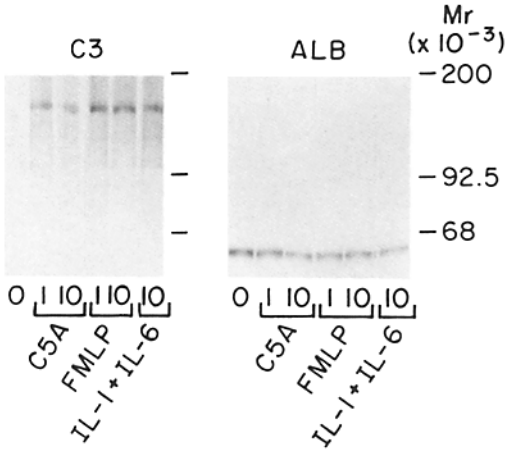


Figure 5. Effect of C5a and FMLP on synthesis of C3 and albumin in HepG2 cells. Separate monolayers of HepG2 cells were incubated at 37°C for 10 h in serum-free medium or medium supplemented with C5a (1 and 10 mM), FMLP (1 and 10 nM) or, as positive control, a combination of IL-1β (10 ng/ml) and IL-6 (10 ng/ml). Cells were then subjected to labeling with [^{35}S]methionine for 30 min. Separate aliquots of the same cell lysates were subjected to immunoprecipitation with anti-C3 and anti-albumin for analysis by SDS-PAGE next to molecular mass markers as indicated at the right margin. There were no significant differences in total TCA-precipitable radioactivity between monolayers.

of other acute phase proteins. For these experiments, HepG2 cells were incubated for 6 h with C5a or FMLP and then subjected to labeling with [^{35}S]methionine. Radiolabeled cells were lysed, and separate aliquots of the same cell lysates were subjected to immunoprecipitation with different antibodies (Fig. 7). Acute phase proteins that are regulated by several different cytokines were initially selected for analysis. The results show that C5a and FMLP have similar effects, mediating increases in synthesis of C3, complement Bf, and proteinase inhibitor α_1-antichymotrypsin (α_1-ACT). In the same experimental material, C5a and FMLP mediated decreases in synthesis of albumin and transferrin, but had no effect on α_1-AT or C5. Several other acute phase proteins were examined. Synthesis of positive acute phase proteins fibrogen and C4 was minimally and inconsistently increased by C5a and

Table 1. *Effect of C5a and FMLP on Net Synthesis of Plasma Proteins in HepG2 Cells*

		Percent change from control‡	
Plasma protein	N*	C5a§	FMLP§
C3	6	349 ± 69	370 ± 74
Albumin	5	52 ± 21	46 ± 24

* Number of separate experiments.
‡ Mean ± SD as determined by densitometric scanning.
§ 5 nM; 6-h incubation.

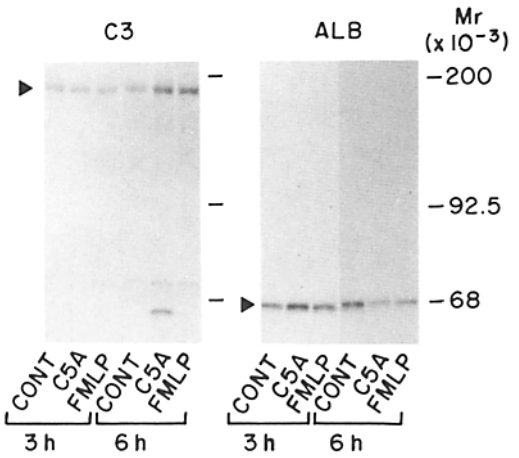


Figure 6. Time required for C5a and FMLP to mediate changes in synthesis of C3 and albumin (*ALB*) in HepG2 cells. Separate monolayers of HepG2 cells were incubated at 37°C for 3 or 6 h in serum-free medium or medium supplemented with C5a (10 nM) or FMLP (10 nM). Synthesis of C3 (*left*) and albumin (*right*) was then analyzed exactly as described in the legend for Fig. 5.

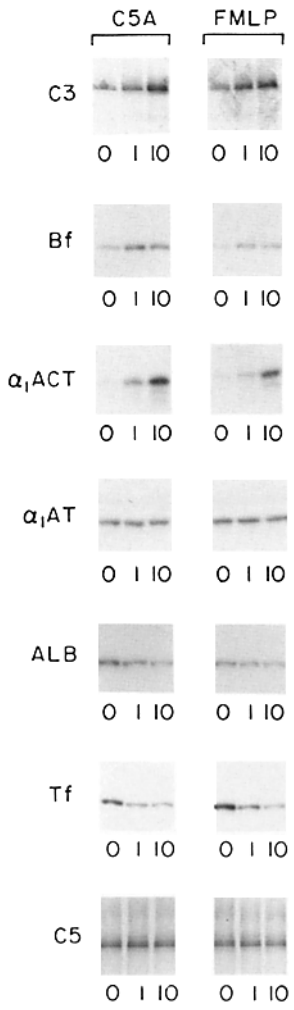
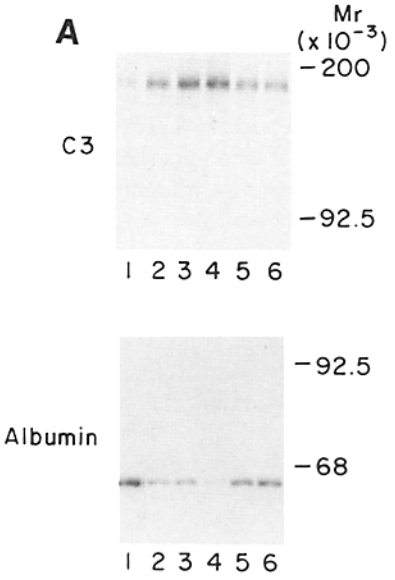


Figure 7. Effect of C5a and FMLP on synthesis of several acute phase proteins in HepG2 cells. Separate monolayers of HepG2 cells were incubated at 37°C for 6 h with serum-free medium (*0*), medium supplemented with C5a at 1 and 10 nM (*left*), or medium supplemented with FMLP at 1 and 10 nM (*right*). Synthesis of acute phase proteins was then analyzed exactly as described in the legend for Fig. 5, except that antibodies to C3, Bf, α_1-ACT, α_1-AT, albumin, transferrin, and C5 were used for immunoprecipitation of separate aliquots of the same radiolabeled cell lysates.



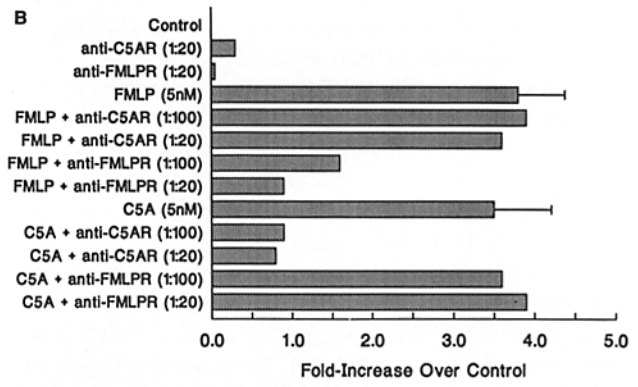


Figure 8. (*A*) Effect of FMLP on synthesis of C3 and albumin in HepG2 cells: blocking by anti-FMLPR and anti-C5aR antisera. Separate monolayers of HepG2 cells were incubated at 37°C for 6 h in serum-free control medium (lane *1*), medium supplemented with FMLP (5 nM; lane *2*), or medium supplemented with FMLP (5 nM) + either anti-C5aR antiserum (at dilutions of 1:100 [lane *3*] or 1:20 [lane *4*]) or anti-FMLPR antiserum (at dilutions of 1:100 [lane *5*] or 1:20 [lane *6*]). Synthesis of C3 and albumin was then analyzed exactly as described in the legend for Fig. 5. (*B*) Effect of FMLP and C5a on synthesis of C3 in HepG2 cells: blocking by anti-FMLPR and anti-C5aR antiserum. HepG2 cells were incubated at 37°C for 6 h in serum-free control medium, medium supplemented with FMLP (5 nM), medium supplemented with FMLP (5 nM) + either anti-C5aR antiserum (1:100 and 1:20 dilutions) or anti-FMLPR (1:100 and 1:20 dilutions), medium supplemented with C5a (5 nM), medium supplemented with C5a (5 nM) + either anti-C5aR antiserum (1:100 and 1:20 dilutions) or anti-FMLPR (1:100 and 1:20 dilutions), medium supplemented with anti-C5a antiserum (1:20 dilution) alone or medium supplemented with anti-FMLPR antiserum (1:20 dilution) alone. C3 synthesis was analyzed by the method described in the legend for Fig. 5, but here the radiolabeled polypeptide corresponding to pro-C3 on the fluorograms was subjected to densitometric scanning. Results are reported on the horizontal axis as fold-increase over control with mean ± 1.0 SD when three or more separate experiments were available.

FMLP (data not shown). There was no evidence for induction of serum amyloid A or C-reactive protein synthesis in HepG2 cells that had been incubated with C5a or FMLP (data not shown).

Next, we examined the capacity for antibodies to C5aR and FMLPR to inhibit the effects of C5a and FMLP on acute phase synthesis in HepG2 cells. Synthetic peptides corresponding to divergent regions in the amino-terminal extracellular domains of C5aR and FMLPR were coupled to KLH and used to raise anti-C5aR and anti-FMLPR antisera. Separate monolayers of HepG2 cells were incubated for 6 h in serum-free control medium, medium supplemented with FMLP, or medium supplemented with FMLP and either anti-C5aR antiserum or anti-FMLPR antiserum. These monolayers were then analyzed for synthesis of C3 and albumin (Fig. 8 *A*). The results show that FMLP mediates an increase in C3 synthesis. This increase is not blocked by anti-C5aR, but is blocked in a concentration-dependent manner by anti-FMLPR antiserum. FMLP also mediates a decrease in albumin synthesis that is not blocked by anti-C5aR, but is blocked in a concentration-dependent manner by anti-FMLPR antiserum. In other experiments summarized in Fig. 8 *B* we found that the effect of C5a on C3 synthesis was blocked by anti-C5aR but not by anti-FMLPR. Anti-C5aR and anti-FMLPR antisera did not independently affect C3 synthesis. Taken together, these data indicate that the effect of C5a and FMLP on acute phase synthesis in HepG2 cells is mediated by C5aR and FMLPR, respectively.

Finally, we examined the effect of very high concentrations of chemotactic peptides on synthesis of acute phase proteins in HepG2 cells. Previous studies of neutrophils, particularly neutrophil chemotactic activity, have shown desensitization to chemotactic peptides as the neutrophils are exposed to increasing concentrations, concentrations well above the point at which receptor is saturated (14). For this experiment (Fig. 9), HepG2 cells were incubated for 6 h in serum-free medium, medium supplemented with FMLP in several different concentrations, or medium supplemented with a prototype acute phase cytokine IL-6 in several different concentrations. Again, acute phase protein synthesis was assayed. In this case, we examined α_1-ACT synthesis. The results show that FMLP mediates a concentration-dependent increase in α_1-ACT synthesis up to an FMLP concentration of 20 nM. At higher FMLP concentrations, α_1-ACT synthesis returns to control levels (Fig. 9, *left*). In other experiments, there was also evidence for dissipation of the effect of both FMLP and C5a on C3 and albumin synthesis at the higher concentrations of the chemotactic peptides (data not shown). IL-6 also mediates an increase in α_1-ACT synthesis (Fig. 9, *right*). Because this experiment was started at very high concentrations of IL-6 (0.2 μg/ml) by design, one does not observe the concentration dependence of the increase. Previous studies have shown that the effect of IL-6 on α_1-ACT synthesis is concentration dependent at lower doses (29). Most important, however, this result shows that once the IL-6 reaches its maximal effect (0.2 μg/ml), there is a plateau, even up to 100-fold higher concentrations (20 μg/ml). Taken together, these results suggest that there is desensitization of the effect of chemotactic peptides at supersaturating concentrations in HepG2 cells and show that there is a difference in the pharmacologic characteristics of hepatic acute phase regulation by chemotactic peptides as compared to the prototype acute phase cytokines.

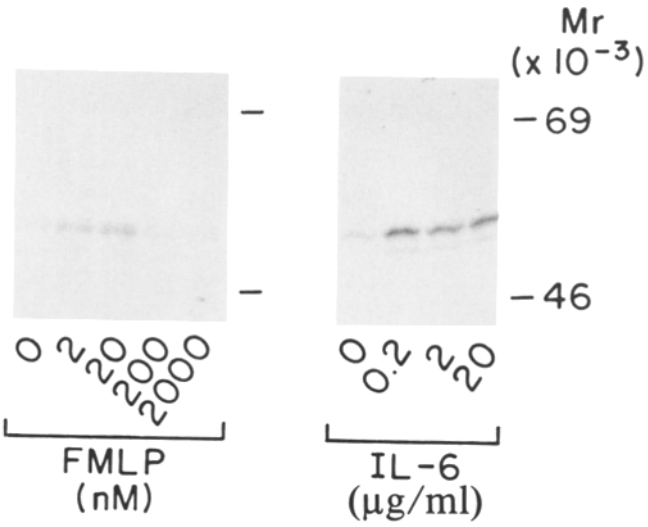


Figure 9. Effect of supersaturating concentrations of FMLP and IL-6 on α1-ACT synthesis in HepG2 cells. Separate monolayers of HepG2 cells were incubated at 37°C for 6 h in serum-free control medium (*0*), medium supplemented with FMLP in several different concentrations (*left*), or medium supplemented with IL-6 in several different concentrations (*right*). α1-ACT synthesis was analyzed exactly as described in the legend for Fig. 5.

Discussion

The results of these studies show that liver cells express two members of the chemotactic G protein–coupled receptor family, or chemokine receptor family. In a previous study, we demonstrated C5a expression in human hepatoma cells and in human liver parenchyma in situ (21). Here, we detected FMLPR expression in HepG2 and human liver parenchymal cells using extremely sensitive assay systems such as PCR and in situ hybridization analysis. Expression of these two members of the chemotactic G protein–coupled receptor family in liver cells is specific in that there is no apparent expression of other members of this family in HepG2 cells. The primer used for PCR of HepG2 mRNA in this study included a pair that have amplified, from U937 cells, IL-8RA, IL-8RB, C-C CKR, orphan receptor HUMSTR, and CMV US 28 (18). Another pair of primers would be expected to amplify several other tissue-specific orphan receptors (32–35). On the basis of this specificity, we examined the possibility that C5aR and FMLPR play a role in the function of liver cells. Immunofluorescence analysis showed that both receptors are expressed on the surface of HepG2 cells (Fig. 2), and radioligand-binding assays showed that these receptors recognize their ligand with characteristics similar to those originally described in cells of myeloid lineage (Fig. 3). Finally, each of these receptors activates a signal transduction pathway

for regulation of acute phase protein biosynthesis in HepG2 cells (Figs. 5–9).

During the host response to inflammation/tissue injury, there is a highly coordinated series of changes in intermediate metabolism with fever, muscle proteolysis, lipolysis, leukocytosis, and marked changes in concentrations of plasma glycoproteins termed acute phase plasma proteins. Concentrations of several plasma proteins increase profoundly (C-reactive protein, serum amyloid A), others increase moderately (fibrinogen, α_1-AT, α_1-ACT, complement proteins factor B and C3), and other plasma proteins decrease (albumin, transferrin). Because these acute-phase plasma proteins are predominantly derived from the liver, this part of the host response to inflammation/injury has been called the hepatic acute phase response. In recent years, it has become possible to identify several specific cytokines that are produced by local tissue and circulating mononuclear cells in response to prototype inflammatory stimuli, and that can elicit the diverse biological effects characteristic of the acute phase response. It has also become possible to demonstrate the effects of these cytokines on hepatic acute phase genes in tissue culture using several well-characterized hepatoma cell lines as well as primary cell culture systems (reviewed in 29). Each of these cytokines, including IL-6, IL-1β, IL-1α, TNF-α, leukemia inhibitory factor (LIF), oncostain M, and IL-11, mediates changes in expression of at least several if not many acute phase genes in cell lines such as HepG2 (36–41). The effect of each of these cytokines is also distinguished by mediating increases in the expression of "positive" acute phase reactants such as α_1-ACT, factor B, C3, and serum amyloid A at the same time as mediating decreases in expression of "negative" acute phase reactants such as albumin, prealbumin, and transferrin. Data reported in this study show that C5a and FMLP have effects that are similar to these acute phase cytokines. Each mediates, through its receptor, concentration- and time-dependent increases in acute phase reactants such as C3, factor B, and α_1-ACT, and decreases in negative acute phase reactants such as albumin and transferrin.

The effects of C5a and FMLP are not as extensive as those of IL-6, which affect a number of additional acute proteins, including C-reactive protein, serum amyloid A, α1-acid glycoprotein, α_1-AT, fibrinogen, and others. Moreover, targeted disruption of the IL-6 gene severely impairs the hepatic acute-phase response elicited in vivo by turpentine (42). Thus, disruption of the IL-6 gene provides evidence for the primacy of IL-6 in mediating the physiologic hepatic acute phase gene expression. In the IL-6 knockout mouse, however, some of the hepatic acute phase response to turpentine is retained and the response to endotoxin is only reduced by ~50–60, raising the possibility that other mediators such as IL-1, TNF, LIF, oncostatin M, IL-11, and C5a play a significant role in the physiologic acute phase response in vivo.

In contrast to the effect of cytokines such as IL-6, IL-1, and TNF, the effect of C5a and FMLP on acute phase protein synthesis in HepG2 cells is abrogated at supersaturating concentrations. The effects of C5a and FMLP on neutrophil chemotaxis are also abrogated at supersaturating concentrations, a phenomenon that has been attributed to desensitization of the C5aR and FMLPR. Desensitization presumably permits the neutrophil to stop migrating as it reaches its destination. It is more difficult to conceptualize an explanation for desensitization of the hepatic acute phase response. This mechanism presumably provides for the termination of the hepatic acute phase response or at least the portion of the response that is elicited by complement fragments or bacterial peptides.

Although these studies show that FMLPR is expressed by liver cells and is capable of regulating acute phase protein biosynthesis, the studies do not provide any information about what ligand FMLPR recognizes in vivo. There are many reports of bacterial translocation across the intestine and into the portal vein which raise the possibility that bacterial peptides could in fact reach FMLPR on liver parenchymal cells. A recent study by Gao et al. (43) has shown that acetylated FMLP analogues as well as nonacetylated, nonformylated FMLP analogues may be recognized by FMLPR. This last study has, therefore, provided evidence for the possible existence of other endogenous mammalian ligands.

The authors are indebted to Drs. Patricia Nelson and Anna August for construction of HepG2 cDNA libraries, Dr. Harvey Colten for advice, and Joyce L. Williams for preparation of the manuscript.

The studies were supported in part by U.S. Public Health Service HL37784 and AI25011, Research Career Development Award AI00919 to R. A. Wetsel, the Monsanto-Washington University Biomedical Program, and a Burroughs Wellcome Trust Scholar Award in Experimental Therapeutics.

Address correspondence to David H. Perlmutter, M.D., Department of Pediatrics, Washington University School of Medicine, One Children's Place, St. Louis, MO 63110.

Received for publication 7 February 1995.

References

1. Tack, B.F., S.C. Morris, and J.W. Prahl. 1979. Fifth component of human complement: purification from plasma and polypeptide chain structure. *Biochemistry.* 18:1490–1497.
2. Fernandez, H.N., and T.E. Hugli. 1978. Primary structural analysis of the polypeptide portion of human C5a anaphylatoxin. *J. Biol. Chem.* 253:6955–6964.
3. Cooper, N.R., and H.J. Muller-Eberhard. 1970. The reaction mechanism of human C5 in immune hemolysis. *J. Exp. Med.* 132:775–793.
4. Medicus, R.G., O. Gotze, and H.J. Muller-Eberhard. 1976. Alternative pathway of complement: recruitment of precursor properdin by the labile C3/C5 covertase and the potentiation of the pathway. *J. Exp. Med.* 144:1076–1093.
5. Goldlust, M.B., H.S. Shin, C.H. Hammer, and M.M. Mayer. 1974. Studies of complement complex C5b,6 eluted from -EAC-6: reaction of C5b,6 with EAC4b,3b and evidence on the role of C2a and C3b in the activation of C5. *J. Immunol.* 113:998–1007.
6. Goldstein, I.M. 1992. Complement: biologically active products. *In* Inflammation: Basic Principles and Clinical Correlates. J.I. Gallin, I.M. Goldstein, and R. Snyderman, editors. Raven Press, Ltd., New York. pp. 63–80.
7. Hugli, T.E., and E.L. Morgan. 1984. Mechanisms of leukocyte regulation by complement-derived factors. *In* Contemporary Topics in Immunobiology. Plenum Press, New York. pp. 109–117.
8. Morgan, E.L. 1986. Modulation of the immune response by anaphylatoxins. *Complement Inflammation.* 3:128–136.
9. Schumacher, W.A., J.C. Fantone, S.E. Kunkel, R.C. Webb, and B.R. Lucchesi. 1991. The anaphylatoxins C3a and C5a are vasodilators in the canine coronary vasculature in vitro and in vivo. *Agents Actions.* 34(Suppl.):345–349.
10. Shin, H.S., R. Snyderman, E. Friedman, A. Mellors, and M.M. Mayer. 1968. Chemotactic and anaphylatoxic fragment cleaved from the fifth component of guinea pig complement. *Science (Wash. DC).* 162:361–363.
11. Cochrane, C.G., and H.J. Muller-Eberhard. 1968. The derivative of two distinct anaphylatoxin activities from the third and fifth components of human complement. *J. Exp. Med.* 127: 371–386.
12. Johnson, A.R., T.E. Hugli, and H.J. Muller-Eberhard. 1975. Release of histamine from rat mast cells by the complement peptides C3a and C5a. *Immunology.* 28:1067–1074.
13. Schiffmann, E., B.A. Corcoran, and S.M. Wahl. 1975. N-formylmethionyl peptides as chemoattractants for leukocytes. *Proc. Natl. Acad. Sci. USA.* 72:1059–1062.
14. Snyderman, R., and R.J. Uhing. 1992. Chemoattractant stimulus-response coupling. *In* Inflammation: Basic Principles and Clinical Correlates. J.I. Gallin, I.M. Goldstein, and R. Snyderman, editors. Raven Press, Ltd., New York. pp. 421–439.
15. Gerard, N.P., and C. Gerard. 1991. The chemotactic receptor for human C5a anaphylatoxin. *Nature (Lond.).* 349:614–617.
16. Boulay, F., L. Mery, M. Tardif, L. Brouchon, and P. Vignais. 1991. Expression cloning of a receptor for C5a anaphylatoxin on differentiated HL-60 cells. *Biochemistry.* 30:2993–2999.
17. Boulay, F., M. Tardif, L. Brouchon, and P. Vignais. 1990. Synthesis and use of a novel N-formylpeptide derivative to isolate a human N-formylpeptide receptor cDNA. *Biochem. Biophys. Res. Commun.* 168:1103–1109.
18. Neote, K., D. DiGregorio, J.Y. Mak, R. Horuk, and T.J. Schall. 1993. Molecular cloning, functional expression, and signalling characteristics of a C-C chemokine receptor. *Cell.* 72:415–425.
19. Kelvin, D.J., D.F. Michiel, J.A. Johnston, A.R. Lloyd, H. Prenger, J.J. Oppenheam, and J.-M. Wang. 1993. Chemokines and serpintines: the molecular biology of chemokine receptors. *J. Leukocyte Biol.* 54:604–612.
20. Foreman, K.E., A.A. Vaporiciyan, B.K. Bonish, M.L. Jones, K.J. Johnson, M.M. Glovsky, S.M. Eddy, and P.A. Ward. 1994. C5a-induced expression of P-selectin in endothelial cells. *J. Clin. Invest.* 94:1147–1155.
21. Haviland, D.L., R.L. McCoy, W.T. Whitehead, H. Akama, E.P. Molmenti, A. Brown, J.C. Haviland, W.C. Parks, D.H. Perlmutter, and R.A. Wetsel. 1995. Cellular expression of the C5a anaphylatoxin receptor (C5aR): demonstration of C5a on non-myeloid cells of the liver and lung. *J. Immunol.* 154:1861–1869.
22. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.
23. Seed, B. 1987. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. *Nature (Lond.).* 329:840–842.
24. Libert, F., M. Parmentier, A. Lefort, C. Dinsart, J. Vand Sande, C. Maenhaut, M.J. Simons, J.E. Dumont, and G. Vasssert. 1989. Selective amplification and cloning of four new members of the G-protein coupled receptor family. *Science (Wash. DC).* 244:569–572.
25. Hershey, A.D., and J.E. Krause. 1990. Molecular characterization of a functional cDNA encoding the rat substance P receptor. *Science (Wash. DC).* 247:958–962.
26. Molmenti, E.P., D.H. Perlmutter, and D.C. Rubin. 1993. Cell-specific expression of α1-antitrypsin in human intestinal epithelium. *J. Clin. Invest.* 92:2022–2034.
27. Perlmutter, D.H., G.I. Glover, M. Rivetna, C.S. Schasteen, and R.J. Fallon. 1990. Identification of a serpin-enzyme complex receptor on human hepatoma cells and human monocytes. *Proc. Natl. Acad. Sci. USA.* 87:3753–3757.
28. Molmenti, E.P., T. Ziambaras, and D.H. Perlmutter. 1993. Evidence for an acute phase response in human intestinal epithelial cells. *J. Biol. Chem.* 268:14116–14124.
29. Kushner, I., and A. Makiewicz. 1993. The acute phase response. *In* Acute Phase Proteins: Molecular Biology, Biochemistry and Clinical Applications. A. Mackiewicz, I. Kushner, and H. Baumann, editors. CRC Press, Boca Raton, FL. pp. 3–19.
30. Won, K.-A., S.P. Campos, and H. Baumann. 1993. Experimental systems for studying hepatic acute phase response. *In* Acute Phase Proteins: Molecular Biology, Biochemistry and Clinical Applications. A. Mackiewicz, I. Kushner, and H. Baumann, editors. CRC Press, Boca Raton, FL. pp. 255–271.
31. Marinkovic, S., G.P. Jahreis, G.G. Wong, and H. Baumann. 1989. IL-6 modulates the synthesis of a specific set of acute phase plasma proteins in vivo. *J. Immunol.* 142:808–812.
32. Kaiser, E., R. Forster, I. Wolf, C. Ebensperger, W.M. Kuehl, and M. Lipp. 1993. The G protein-coupled receptor BLR1 is involved in murine B cell differentiation and is also expressed in neuronal tissues. *Eur. J. Immunol.* 23:2532–2539.
33. Kouba, M., M. Vanetti, X. Wang, M. Schafer, and V. Holt. 1993. Cloning of a novel putative G-protein-coupled receptor (NLR) which is expressed in neuronal and lymphatic tissue. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 321:173–178.
34. Loetscher, M., T. Geiser, T. O'Reilly, R. Zwahlen, M. Bag-

giolini, and B. Moser. 1994. Cloning of a human seven-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes. *J. Biol. Chem.* 269:232–237.
35. Federspiel, B., I.G. Melhado, A.M.V. Duncan, A. Delaney, K. Schappert, I. Clark-Lewis, and F.R. Jirik. 1993. Molecular cloning of the cDNA and chromosomal localization of the gene for a putative seven-transmembrane segment (7-TMS) receptor isolated from human spleen. *Genomics.* 16:707–712.
36. Gauldie, J., C. Richards, D. Harnish, et al. 1987. Interferonβ2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proc. Natl. Acad. Sci. USA.* 84:7251–7255.
37. Perlmutter, D.H., C.A. Dinarello, P.I. Punsal, and H.R. Colten. 1986. Cachectin/tumor necrosis factor regulates hepatic acute-phase gene expression. *J. Clin. Invest.* 78:1349–1354.
38. Darlington, G.J., W.D. Wilson, and L. Lachman. 1986. Monocyte-conditioned medium, interleukin-1 and tumor necrosis factor stimulate the acute phase response in human hepatoma cells in vitro. *J. Cell. Biol.* 103:787–793.
39. Baumann, H., S.F. Ziegler, B. Mosley, K.K. Morella, S. Pajovic, and D.P. Gearing. 1993. Reconstitution of the response to leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor in hepatoma cells. *J. Biol. Chem.* 268:8414–8417.
40. Richards, C.D., T.J. Brown, M. Shoyab, H. Baumann, and J. Gauldie. 1992. Recombinant oncostatin M stimulates the production of acute phase proteins in HepG2 cells and rat primary hepatocytes in vitro. *J. Immunol.* 148:1731–1736.
41. Baumann, H., and H. Schendel. 1991. Interleukin-11 regulates the hepatic expression of the same plasma protein genes as interleukin-6. *J. Biol. Chem.* 266:20424–20427.
42. Kopf, M., H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, R. Ainkernagel, H. Bluethmann, and G. Kohler. 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature (Lond.).* 268:339–342.
43. Gao, J.-J., E.L. Becker, R.J. Freer, N. Muthukumaraswamy, and P.M. Murphy. 1994. A high potency nonformylated peptide agonist for the phagocyte N-formylpeptide chemotactic receptor. *J. Exp. Med.* 180:2191–2197.