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Complement-Dependent Acute-Phase Expression of C-Reactive Protein and Serum Amyloid P-Component[1]

Alexander J. Szalai,[2]* Frederik W. van Ginkel,[†] Yue Wang,* Jerry R. McGhee,[†] and John E. Volanakis*[‡]

The acute-phase response (APR) is regulated by TNF-α, IL-1β, and IL-6 acting alone, in combination, or in concert with hormones. The anaphylotoxin C5a, generated during complement activation, induces in vitro the synthesis of these cytokines by leukocytes and of acute-phase proteins by HepG2 cells. However, there is no clear evidence for a role of C5a or any other complement activation product in regulation of the APR in vivo. In this study, using human C-reactive protein (CRP) transgenic mice deficient in C3 or C5, we investigated whether complement activation contributes to induction of the acute-phase proteins CRP and serum amyloid P-component (SAP). Absence of C3 or C5 resulted in decreased LPS-induced up-regulation of the *CRP* transgene and the mouse *SAP* gene. Also, LPS induced both the *IL-1β* and *IL-6* genes in normocomplementemic mice, but in complement-deficient mice it significantly induced only *IL-6*. Like LPS injection, activation of complement by cobra venom factor led to significant elevation of serum CRP and SAP in normocomplementemic mice but not in complement-deficient mice. Injection of recombinant human C5a into human CRP transgenic mice induced the *IL-1β* gene and caused significant elevation of both serum CRP and SAP. However, in human CRP transgenic IL-6-deficient mice, recombinant human C5a did not induce the *CRP* nor the *SAP* gene. Based on these data, we conclude that during the APR, C5a generated as a consequence of complement activation acts in concert with IL-6 and/or IL-1β to promote up-regulation of the *CRP* and *SAP* genes. *The Journal of Immunology,* 2000, 165: 1030–1035.

T issue injury results in a systemic reaction termed the acute-phase response (APR)[3] (1), that includes changes in the serum levels of several plasma proteins produced primarily by hepatocytes (2). Regulation of the APR is largely mediated by the proinflammatory cytokines TNF-α, IL-1β, and IL-6 acting alone, in combination, or in concert with various steroid hormones (3–5). A convenient murine model for studying induction of acute-phase protein genes is i.p. injection of LPS (6), which reproducibly elicits increased expression of proinflammatory cytokines (6, 7). LPS also activates the classical and alternative pathways of complement (8, 9), leading to generation of the bioactive peptides C3a and C5a (10). The anaphylotoxin C5a elicits from target cells a wide range of activities (10), including the synthesis of TNF-α, IL-1-β, and IL-6 by leukocytes (11–13), which amplify the inflammatory process. The biological activity of C5a is mediated through the C5a receptor (C5aR, CD88), a G-protein-coupled seven-transmembrane-domain protein expressed on cells of myeloid origin (10, 14), and also on nonmyeloid cells in the liver and lung (15–17). In mice, hepatic expression of C5aR is increased by

administration of LPS (17). Furthermore, C5a has been shown to induce the synthesis and secretion of acute-phase proteins by HepG2 cells (16, 17). Thus, it seems likely that by binding to C5aR on liver cells and/or by stimulating secretion of proinflammatory cytokines by myeloid cells, C5a generated as a consequence of complement activation contributes to regulation of the APR.

The pentraxins C-reactive protein (CRP) and serum amyloid P-component (SAP) (18, 19) are evolutionarily conserved, Ca^{2+}-binding proteins that participate variably in the APR in different species (3). For example, CRP is a major acute-phase protein in humans (20), while in the mouse it is a trace plasma component and only a minor acute-phase protein (21–23). In contrast, SAP is highly inducible during the APR in mice but not in humans (24). Numerous studies using freshly isolated human hepatocytes and hepatoma cell lines have established that IL-6 is the major inducer of the *CRP* gene and that IL-1 and glucocorticoids act in synergy with IL-6 to enhance *CRP* gene induction (25–29). Similar studies using primary mouse hepatocytes (30) have shown that the mouse *SAP* gene can be induced directly by either IL-1 or IL-6. We have shown (31) that in mice constitutive and IL-6-induced acute-phase expression of a human *CRP* transgene requires testosterone, whereas testosterone does not influence expression of the mouse *SAP* gene, which requires IL-1 plus IL-6 for acute-phase expression.

Horowitz et al. (32) reported an association between in vivo activation of the alternative complement pathway by inulin and increased serum CRP levels in rabbits, but the observation was not pursued further. Shortly thereafter, Pepys and Rogers reported (33) that in mice depleted of complement by i.p. injection of cobra venom factor (CoVF), induction of SAP by LPS was unaffected. However, the impact of complement activation by CoVF per se on SAP expression was not addressed. Recently, we reported (34) that 24 h after injection of CoVF into human CRP transgenic (CRPtg) mice, serum levels of human CRP and mouse SAP are elevated.

[3] Abbreviations used in this paper: APR, acute-phase response; C3D, C3 deficient; C5aR, C5a receptor (CD88); C5D, C5 deficient (DBA/2J mice); CRP, C-reactive protein; CRPtg, human CRP transgenic C57BL/6J mice; IL-6D, IL-6 deficient; rC5a, recombinant human C5a; SAP, mouse serum amyloid P-component; CoVF, cobra venom factor.

In the present study, we generated by selective breeding CRPtg mice deficient in C3 (C3D) or C5 (C5D) to investigate directly the contribution of these complement proteins to the induction of the *CRP* and *SAP* genes during the APR. We show that absence of either C3 or C5 attenuates the response of the human *CRP* transgene and mouse *SAP* gene to LPS. Injection of CoVF alone led to significant elevation of serum CRP and SAP in normocomplementemic mice, but not in either complement deficient strain. Importantly, injection of recombinant human C5a (rC5a) mimicked the effect of complement activation by CoVF, i.e., it caused elevation of serum CRP and SAP. Injection of rC5a into C3D or C5D mice also elicited elevation of CRP and SAP. In complement-deficient mice, the reduced acute-phase expression of the *CRP* and *SAP* genes after LPS administration was paralleled by the absence of significant elevation of serum IL-1β. Conversely, induction of the *CRP* and *SAP* genes in these mice by injection of rC5a was associated with elevation of serum IL-1β, but not of IL-6. Nevertheless, an absolute requirement for IL-6 in the induction of CRP and SAP by rC5a was indicated by the failure of rC5a to induce either gene in IL-6-deficient (IL-6D) mice. The combined data support the notion that C5a generated during complement activation contributes to up-regulation of the *CRP* and *SAP* genes during the APR. C5a does not act directly to mediate expression of these acute-phase genes, rather its effect is mediated in concert with IL-6 and/or of IL-1β.

Materials and Methods

Animals

All mice used in this study were fed and watered ad libitum and barrier maintained under a 12-h light-dark cycle according to protocols established by the Animal Resources Program at the University of Alabama at Birmingham. We have previously described the establishment of a breeding colony of CRPtg C57BL/6J congenic mice (35). CRPtg mice carry a 31-kb *Cla*I fragment of human genomic DNA comprised of the *CRP* gene, 17 kb of 5′-flanking sequence, and 11.3 kb of 3′ flanking sequence (36). After injection of LPS into CRPtg mice, peak levels of serum IL-1 and IL-6 are attained by 2 h, followed by a human CRP response with peak serum levels reached by 18 h (31). The generation and genetic background of IL-6D and complement C3D mice has also been described (37, 38). IL-6D mice are homozygous for a disruption of the fourth exon of the murine *IL-6* gene and produce no serum IL-6 after LPS injection (37). C3D mice produce no serum complement C3 due to targeted disruption of the murine *C3* gene promoter (38) and lack the ability to generate C3-convertases through the alternative pathway and C5-convertases through either the classical or alternative mutation in exon 7 of the murine *C5* gene (39). DBA/2J mice are complement C5D due to a spontaneous mutation in exon 7 of the murine *C5* gene (39).

Female CRPtg mice were crossed with IL-6D males to produce hybrids, and CRPtg hybrids were backcrossed to the IL-6D parent to generate CRPtg/IL-6D mice. The same breeding system was used to generate CRPtg/C3D and CRPtg/C5D mice. C3D and IL-6D mice were backcrossed to C57BL/6J for at least five generations before hybridization with CRPtg. Mice were screened for presence of the *CRP* transgene and for inheritance of the *C3* or *C5* mutant alleles using PCRs specific for human *CRP* (40), mouse *C3* (38), or mouse *C5* (39). IL-6D, C3D, and C5D vs sufficient progeny were obtained in the expected Mendellian ratios. The *CRP* transgene was responsive to induction by LPS in all three types of CRPtg F$_1$ hybrids. Male F$_2$ mice (8–12 wk old) were used in experiments, and all appeared normal and healthy.

Administration of LPS, CoVF, and rC5a

LPS from *Eschericia coli* (serotype 026:B6) was purchased from Sigma-Aldrich (St. Louis, MO), resuspended in sterile 0.9% NaCl, and injected i.p. at a dose of 25 μg per mouse. CoVF from *Naja naja* (Quidel Corporation, San Diego, CA) was dissolved in sterile pyrogen-free 0.9% NaCl (Abbott Laboratories, North Chicago, IL) and also injected i.p. at 25 μg per mouse. *N. naja* CoVF is known to support the formation of potent C3- and C5-convertases (41, 42). We have shown (34) that single i.p. injection of 25 μg CoVF reduces mouse serum C3 to <3% of initial values within 4 h and that the hypocomplementemia lasts several days. rC5a (Sigma-Aldrich) was reconstituted in 0.25% (w/v) BSA in sterile pyrogen-free 0.9% NaCl and injected i.p. at 2.5–20 μg/mouse. rC5a has been shown to acti-

vate various mouse cells including mast cells (43), astrocytes (44), and monocytes and macrophages (10, 45). *Limulus amebocyte* assays confirmed that CoVF and rC5a was not contaminated with endotoxin.

Measurement of serum IL-1β, IL-6, CRP, and SAP

Sera from blood samples (50 μl) collected before and 2 and 18 h after injection of stimulants were used to measure the concentration of IL-1β and IL-6 (2 h sera) and CRP and SAP (18 h sera). Mouse IL-1β and IL-6 ELISAs were performed exactly as described (31, 37) using rat mAb MP5-20F3 and biotinylated mAb MP5-32C11 (PharMingen, San Diego, CA) for IL-6 detection and rat mAb 13A10 and biotinylated mAb 13D11 (Biosource International, Camarillo, CA) for IL-1β detection. Peroxidase-labeled goat anti-biotin (Vector Laboratories, Burlingame, CA) was used as the reporter, and recombinant mouse IL-1β and recombinant mouse IL-6 (Genzyme, Cambridge, MA) were used to generate standard curves. The lower limit of detection in each case was 10 pg of cytokine/ml. CRPtg/IL-6D mice were identified by absence of detectable IL-6 in sera collected 2 h after LPS injection (31). ELISA for CRP used sheep anti-human CRP serum (Cappel, Durham, NC) and anti-CRP mAb HD2-4 (46) as the capture and detection Ab, respectively, and affinity-purified human CRP (47) as the standard. The assay does not detect mouse CRP and has a lower limit of detection of 20 ng of human CRP per ml of mouse serum. ELISA for mouse SAP was performed as described (48) using sheep and rabbit anti-SAP serum as the capture and detection Ab, respectively, and mouse SAP reference standards all from Calbiochem-Novabiochem (San Diego, CA). The lower limit of detection is 25 μg of SAP per ml serum.

Statistical analysis

All values are reported as the mean $\pm$ SEM of at least three experiments. Comparisons of means were performed using Student's t tests with $p <$ 0.05 considered significant.

Results

To investigate the possible role of complement in up-regulation of the *CRP* transgene and *SAP* gene during the APR, CRP and SAP serum levels were measured in age-matched male CRPtg/C3D and CRPtg/C5D mice before and after LPS injection. Normocomplementemic CRPtg and CRPtg/IL-6D mice were used as controls. Injection of LPS caused an ~12-fold increase of serum human CRP in normocomplementemic CRPtg mice but only an ~4-fold increase in CRPtg/C3D and CRPtg/C5D mice (Fig. 1*A*). The difference between normocomplementemic and complement-deficient mice was significant. Concomitant induction of the mouse *SAP* gene followed the same pattern, i.e., serum SAP was increased ~20-fold in normocomplementemic controls as compared with ~10-fold in complement-deficient mice. However, the difference in SAP increase between the two groups did not achieve statistical significance (Fig. 1*B*). In accordance with our earlier report (31), the *CRP* transgene was unresponsive and the *SAP* gene was induced only ~2.5-fold by LPS in CRPtg/IL-6D mice (Fig. 1, *A* and *B*). These data suggest that an intact complement system is required for full LPS-induced acute-phase expression of the *CRP* transgene and probably also of the *SAP* gene.

Because IL-6 is absolutely required for LPS induction of the *CRP* transgene and IL-1 together with IL-6 is required for full LPS induction of the *SAP* gene (31), we investigated whether induction of these cytokines by LPS is altered in complement-deficient mice. As shown in Fig. 1*C*, LPS administration evoked significant elevation of serum IL-6 in all mice carrying a normal *IL-6* gene, although levels were significantly lower in C5D compared with normocomplementemic ($p = 0.007$) or C3D ($p = 0.014$) mice (Student's t tests). In contrast, LPS caused a significant increase of serum IL-1β only in normocomplementemic animals (Fig. 1*D*). Thus, a normal complement system is necessary for LPS induction of the *IL-1β* but not of the *IL-6* gene.

The combined data suggested that complement activation products act together with IL-6 and probably also IL-1β to enhance induction of the *CRP* transgene by LPS. In the case of SAP, the
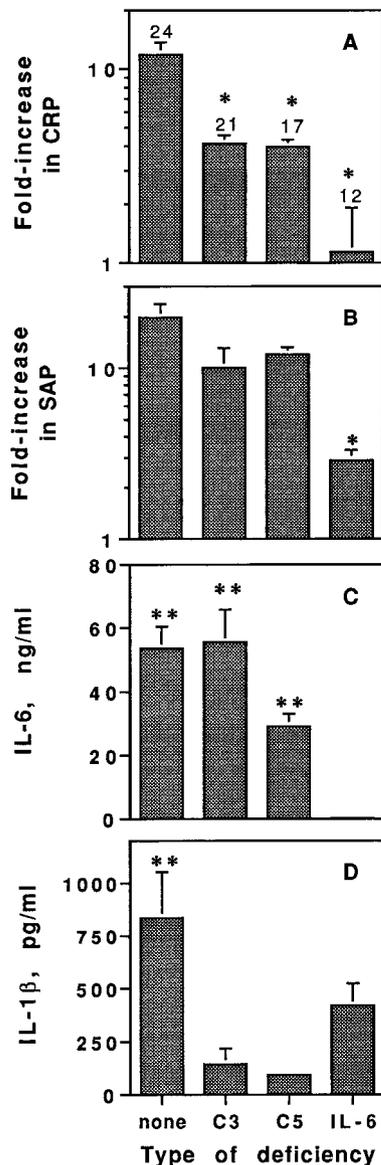
FIGURE 1. Effect of LPS injection on serum levels of CRP (*A*), SAP (*B*), IL-6 (*C*), and IL-1β (*D*). CRPtg mice and CRPtg mice deficient in C3, C5, or IL-6 were injected i.p. with 25 μg of LPS. Human CRP and mouse SAP were quantitated in sera collected before and 18h after LPS injection, and IL-1β and IL-6 were quantitated before and 2 h after injection. The total number of mice analyzed in each group is indicated above the columns in *A*. The results (mean + SEM for at least three experiments) are expressed on the vertical axis as fold increases over baseline values of CRP and SAP and as 2-h serum concentrations of IL-6 and IL-1β. Single asterisks indicate a statistically significant difference in fold increases of CRP or SAP compared with CRPtg mice, and double asterisks indicate a significantly greater than constitutive level of IL-6 or IL-1β (*, *p* < 0.05; **, *p* < 0.005; Student's *t* tests). The average constitutive level of each cytokine was 186 ± 41 pg IL-1β/ml of serum (all mice combined) and 9.6 ± 1.4 ng IL-6/ml (excluding IL-6D mice).

enhancing effect of complement activation was less pronounced and probably could also be attributed to interaction with IL-6 and IL-1β. To further investigate the effects of complement activation on the APR, we measured the cytokine and acute-phase protein responses of mice injected with the potent complement activator CoVF. As shown in Fig. 2, injection of CoVF in normocomplementemic mice reproducibly induced significant elevation of both CRP and SAP. Neither acute-phase protein was elevated substan-
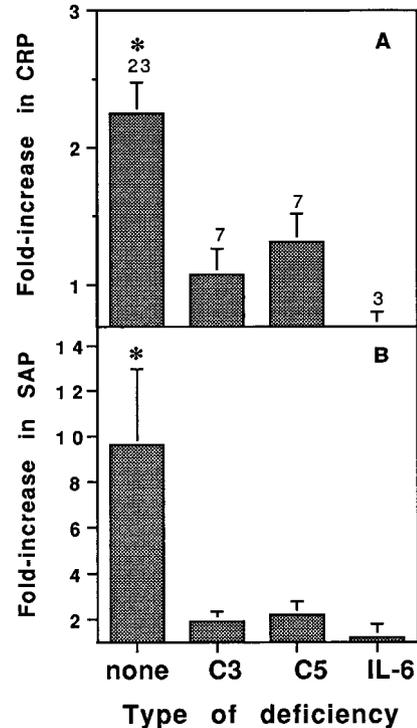
FIGURE 2. Effect of CoVF injection on serum levels of the acute-phase proteins CRP (*A*) and SAP (*B*). CRPtg mice and CRPtg mice deficient in C3, C5, or IL-6 were injected i.p. with 25 μg of CoVF. Human CRP and mouse SAP were quantitated in sera before and 18 h after CoVF injection, and the results (mean + SEM for at least three experiments) are expressed on the vertical axis as fold increases over baseline values. The total number of mice analyzed in each group is indicated above the columns in *A*. The asterisks indicate fold increases significantly greater than unity (*p* < 0.05; Student's *t* tests).

tially following CoVF injection in C3D or C5D mice, indicating that CoVF-induced up-regulation was mediated by complement activation products and not directly by CoVF or by a possible contaminant in the CoVF preparation. Injection of CoVF did not induce the *IL-1β* or the *IL-6* gene (data not shown) and failed to up-regulate CRP and SAP in IL-6D mice. The results are consistent with the view that complement activation products act in concert with IL-6 to enhance the APR.
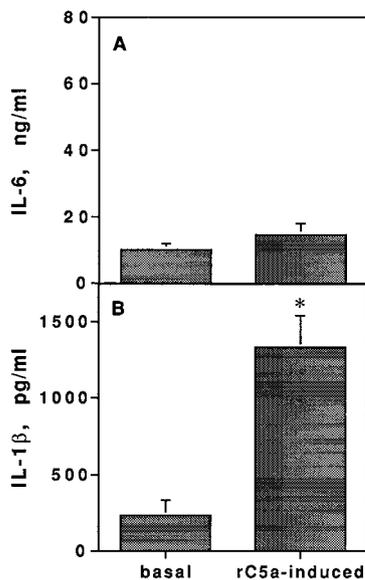
Because C5a has been shown to induce expression of some acute-phase proteins in vitro (17) and it cannot be produced by C5D mice and likely is not generated in CoVF-injected C3D mice, we tested directly if C5a can induce CRP and SAP acute-phase responses. Injection of rC5a into CRPtg mice elicited significant elevation of both CRP and SAP in a dose-dependent manner (Fig. 3). In fact, at the highest dose used (20 μg of rC5a) the magnitude of the CRP and SAP responses (about 2- and 12-fold, respectively) were comparable to those elicited by CoVF (Fig. 2, *A* and *B*). Furthermore, injection of rC5a elicited significant elevation of serum IL-1β but not IL-6 (Fig. 4). Conclusive evidence that C5a participates in acute-phase regulation of the *CRP* transgene and *SAP* gene was obtained by experiments using C3D and C5D mice. Injection of rC5a in either strain evoked a small but significant elevation of serum CRP (Fig. 5A). A parallel elevation of SAP did not achieve statistical significance (Fig. 5B). Although serum IL-6 was not increased after injection of rC5a, a requirement for the cytokine was indicated by the failure of rC5a to cause an increase in serum CRP in IL-6D mice (Fig. 5A).
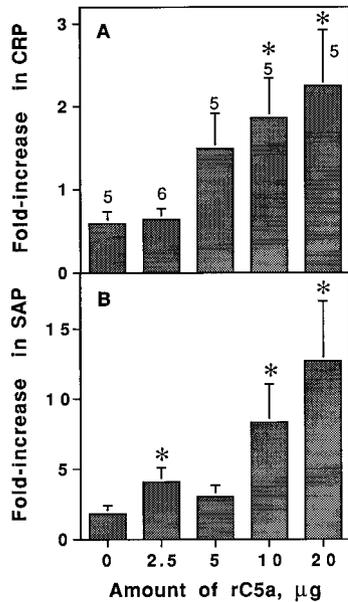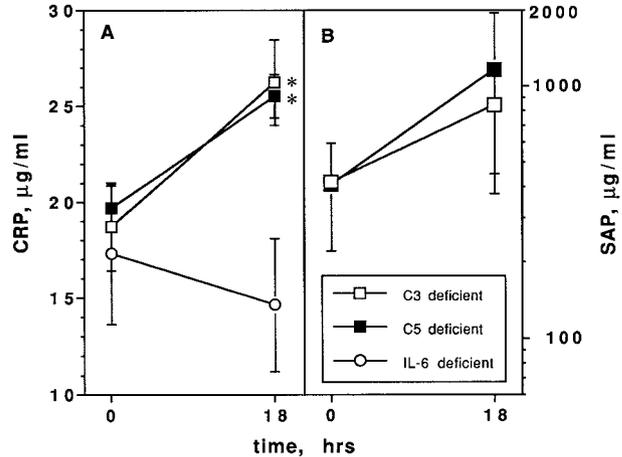
FIGURE 3. Dose-response of CRP (*A*) and SAP (*B*) induction by rC5a. CRPtg mice were injected with the indicated amounts of rC5a suspended in 0.25% BSA, 0.9% NaCl. Human CRP and mouse SAP were quantitated in sera collected before and 18 h after injecting rC5a. Results (mean + SEM for three experiments) are expressed on the vertical axis as fold increases over baseline values. The total number of mice analyzed in each group is indicated above the columns in *A*. The asterisks indicate a fold increase significantly greater than unity ($p < 0.05$; Student's *t* tests).

FIGURE 5. Serum concentration of human CRP (*A*) and mouse SAP (*B*) in C3D (□), C5D (■), and IL-6D (○) CRPtg mice measured before and 18 h after injection i.p. of 20 μg of rC5a. The asterisks indicate a significant elevation of serum CRP above preinjection levels ($p < 0.05$; Student's *t* tests).

Discussion

The APR is an essential manifestation of innate host defense against pathogens, its protective effect being dependent upon recognition of conserved repeating microbial structural elements (e.g., endotoxin, teichoic acids, or surface polysaccharides) by acute-phase proteins (e.g., CRP, mannose-binding lectin, LPS-binding protein) (1–3, 49). Also, certain acute-phase proteins are known to influence the initiation and type of adaptive immune responses (50, 51). Thus, identifying the factors that regulate synthesis of acute-phase proteins is an important step toward understanding host defense mechanisms as well as the pathophysiology of infectious and certain autoimmune diseases. A widely used in vivo model to study the APR is injection of LPS into mice (6), a treatment that elicits a constellation of inflammatory responses that mimics the endotoxin-induced APR in humans (52). Extensive studies in both species reveal that LPS-induced expression of acute-phase protein genes is largely controlled by IL-6, IL-1β, and hormones (2, 49). Combinations of these mediators have additive, inhibitory, or synergistic effects on the APR (2), and studies using cytokine-deficient mice (37, 53–55) show that the APR is not dependent on a single cytokine or hormone but uses multiple overlapping and redundant pathways.

The data we have generated using LPS-injected CRPtg mice and their IL-6D counterparts (Fig. 1) reinforce these concepts and are in agreement with our previous finding that in male CRPtg mice IL-6 is essential for acute-phase induction of CRP while maximal induction of SAP requires both IL-6 and IL-1β (31, 35). A novel finding of the present study is that in complement-deficient mice receiving LPS, up-regulation of CRP and SAP is blunted (Fig. 1, *A* and *B*) despite significant up-regulation of serum IL-6 (Fig. 1*C*). Furthermore, following LPS injection there is no significant elevation of IL-1β in the sera of complement-deficient mice (Fig. 1*D*). As it is known that in addition to being a strong inducer of cytokine synthesis (7) LPS is an efficient activator of the complement alternative and classical pathways (8, 9), we speculated that LPS-mediated complement activation contributed to the observed up-regulation of the *IL-1β, CRP,* and *SAP* genes. To further test this concept, we measured the APR of mice injected with CoVF, a protein that activates the alternative complement pathway (41, 42). We found that CoVF injection caused significant elevation of both CRP and SAP serum levels in CRPtg mice, but not in their C3D, C5D, or IL-6D counterparts (Fig. 2). Furthermore, CoVF induced CRP and SAP without eliciting significant increase of serum IL-1β or IL-6. The combined results strongly support the concept that complement activation and up-regulation of serum CRP and SAP are causally related, as originally proposed by Horowitz et al. (32).

FIGURE 4. Changes in concentration of serum cytokines induced by rC5a. CRPtg mice were injected with 20 μg of rC5a. IL-6 (*A*) and IL-1β (*B*) were quantitated in sera collected before (basal) and 2 h after rC5a injection (rC5a-induced). Representative results (mean + SEM) from one of three experiments using five mice are shown. The asterisk indicates a significantly greater than basal concentration of IL-1 ($p < 0.05$; Student's *t* tests).

Because there was no substantial difference between C3D and C5D mice in terms of their attenuated acute-phase responses to either LPS or CoVF (Figs. 1 and 2), we reasoned that probably the crucial defect was their common inability to generate C5a during complement activation. Direct proof for this hypothesis was provided by experiments using rC5a. Injection of rC5a led to significant elevation of CRP and SAP serum levels in CRPtg mice in a dose-dependent manner (Fig. 3). Also, injected rC5a elicited significant elevation of serum CRP in both C3D and C5D mice (Fig. 5). Injection of rC5a also induced significant elevation of IL-1β but not of IL-6 (Fig. 4). However, the *CRP* transgene was not responsive to rC5a in IL-6D mice (Fig. 5). Thus, the C5a effect on CRP expression apparently requires the presence of IL-6, albeit in low concentrations such as those expected to be expressed constitutively in CRPtg mice.

It has been shown that in vitro C5a induces the synthesis and release of leukocyte-derived cytokines (11–13) known to participate in the regulation of the APR (3–5) and the expression of the acute-phase proteins α_1-antitrypsin, α_1-antichymotrypsin, C3, and complement factor B by HepG2 cells (16, 17). The current investigation using CRPtg mice has provided the first direct evidence that C5a contributes to the regulation of the APR in vivo. The significance of these findings is underlined by the fact that complement activation is an early consequence of most if not all forms of tissue injury that cause an APR. Included are bacterial infections, burns, ischemic necrosis, and immune-complex-mediated injury. Therefore, we propose that C5a generated as a consequence of complement activation following tissue damage or necrosis cooperates with proinflammatory cytokines and stress hormones to ensure maximal acute-phase expression of CRP, SAP, and perhaps also additional acute-phase proteins. Thus, complement activation products are an integral component of the highly complex network of mediators that interact to ensure appropriate expression of genes during the APR.

Acknowledgments

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