

Originals

Effects of Cytokines and Immunosuppressants on the Production of Serum Amyloid A Protein and C-reactive Protein in HepG2 Cells

Naoto Shimetani, Midori Suzuki, Keiko Shimetani*, Takashi Nishikawa** and Mikio Mori

*Department of Clinical Laboratory, Koshigaya Hospital, Dokkyo University School of Medicine,
Koshigaya, Saitama, 343 - 8555 Japan*

** Department of Clinical Pathology, Kitasato University School of Medicine, Sagami-hara, Kanagawa, 228 - 8555 Japan*

*** Division of Medico-chemical Analysis, Kyoritsu College of Pharmacy, Minato-ku, Tokyo, 105 - 8512 Japan*

SUMMARY

C-reactive protein (CRP) and serum amyloid A protein (SAA) are acute-phase proteins produced by the liver in response to inflammatory cytokines. The concentrations of these proteins in serum vary in parallel in most pathological conditions, but sometimes vary independently. CRP and SAA were determined in HepG2 cell culture medium supplemented with five immunosuppressants (corticosteroid, gusperimus hydrochloride, cyclosporin A, mizoribine and tacrolimus hydrate), with or without interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). We also examined the effects of the immunosuppressants on the production of cytokines and changes in CRP and SAA production in HepG2 cells stimulated with the culture fluid from lipopolysaccharide (LPS)-treated monocytes. In HepG2 cells, production of CRP and SAA was greatly affected by IL-6 and IL-1 β , respectively. Prednisolone (PSL) suppressed CRP production, while it enhanced SAA production. The other four immunosuppressants did not affect CRP production, but inhibited SAA production. PSL significantly inhibited cytokine production in monocytes, while the other immunosuppressants enhanced it. In HepG2 cells incubated with the culture fluid from LPS-stimulated monocytes, CRP production was suppressed, while SAA production was enhanced. PSL suppressed CRP production in HepG2 cells by inhibiting IL-6 production in monocytes, whereas PSL increased SAA production through a direct action on the hepatoma cells. In contrast, the other immunosuppressive agents enhanced IL-1 β production in monocytes. The agents induced SAA production in the HepG2 cells but did not affect CRP production.

Key Words : C-reactive protein, serum amyloid A protein, cytokines, HepG2 cell, Immunosuppressants

INTRODUCTION

C-reactive protein (CRP) and serum amyloid A protein (SAA) are acute-phase proteins commonly monitored as indicators of allograft rejection and infections

after organ transplantation. In allografts infection and acute inflammation accompanied by tissue damage, cytokines implicated in inflammatory reactions such as interleukin-1 (IL-1), tumor necrosis factor- α (TNF α) and interleukin-6 (IL-6) act on hepatocytes to induce the production of acute-phase proteins. Among the acute-phase proteins CRP and SAA are regarded as the most sensitive indicators¹⁾. Although the two proteins in serum generally show a close correlation and similar patterns of change, their dispositions differ in specific disor-

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Reprint requests to : Naoto Shimetani

Department of Clinical Laboratory, Koshigaya Hospital, Dokkyo University School of Medicine, Koshigaya, Saitama, 343 - 8555 Japan

ders. For example, in viral infections, increase in the SAA is greater than that of CRP, better reflecting the state of disease²). In renal and liver allograft rejections, SAA markedly increases, but CRP frequently shows no significant increase^{3,4}).

We previously demonstrated that administration of PSL in daily doses of 30 mg or more induced rapid reductions in CRP compared with SAA, and that SAA increased earlier than CRP in pneumonia or other inflammatory complications associated with the use of steroids⁵). These clinical findings indicate that SAA is a more sensitive indicator than CRP in patients under steroid therapy.

In this study, we examined the effects of various cytokines and five immunosuppressive agents (corticosteroid, gusperimus hydrochloride, cyclosporin A, mizoribine and tacrolimus hydrate) on the production of CRP and SAA by a human hepatoma cell line, HepG2, in an attempt to clarify the difference in expression patterns of the two proteins.

MATERIALS AND METHODS

1. Hepatoma cells and culture conditions.

HepG2 human hepatoma cells (obtained from the Riken Cell Bank, Tsukuba, Japan) were cultured at 37 °C, 5 % CO₂ in Dulbecco's MEM (DMEM; Nissui Pharmaceutical Co., Ltd. Tokyo, Japan), supplemented with 10 % fetal calf serum (Gibco, Grand Island, NY, USA).

2. Effects of IL-1 β and IL-6 on the production of CRP and SAA by HepG2 cells.

To assess the production of CRP and SAA in HepG2 cells, the hepatocytes were plated in 24-well tissue culture plates at a density of 5×10^4 cells/well and incubated for 5 days. The supernatant was discarded, and the cells were washed with DMEM three times. Then, the cells were incubated for 48 h in 1 ml of DMEM supplemented with recombinant human (rh) IL-1 β (Immugenex Co., LA, USA) or recombinant human (rh) IL-6 (Gibco) at various concentrations (0 - 50 ng/ml). The concentrations of CRP and SAA in each medium were determined.

3. Effects of immunosuppressive agents on the production of CRP and SAA by HepG2 cells.

To study the effects of immunosuppressive agents on the production of CRP and SAA in HepG2 cells, cells were

incubated in growth media supplemented with 25 ng/ml rhIL-1 β , 25 ng/ml rhIL-6, and either prednisolone (PSL) (Sigma Chemical St Louis, MO, USA), gusperimus hydrochloride (Spanidin), cyclosporin A (Sandimmune), mizoribine (Bredinin) or tacrolimus hydrate (Prograf), at concentrations of 1 μ M and/or 5 μ M. After incubation for 48-72 h, the concentrations of CRP and SAA in culture supernatant were determined.

4. Preparation of monocyte-conditioned media (MOCM).

Heparinized peripheral venous blood samples (50 ml each) were collected from 5 healthy volunteers and subjected to gradient centrifugation to prepare peripheral blood monocytes. The monocytes were suspended in DMEM at a density of 3×10^6 cells/ml and incubated in plastic tissue culture flasks at 37 °C for 2 h; the flasks were washed twice with PBS to remove non-adherent cells⁶). Adherent cells were incubated for 48 h in growth medium supplemented with 10 μ g/ml *E. coli* lipopolysaccharide (LPS) (Difco, Detroit, MI, USA). The media (MOCM-a) were recovered, centrifuged and stored at -80 °C until use. The monocytes prepared by this procedure showed a trypan blue staining ratio of 90 % or more.

5. Effects of immunosuppressive agents on the production of cytokines in monocytes.

The concentrations of cytokines (IL-1 α , IL-1 β , IL-6, and TNF α) were determined in MOCM-a, which was prepared by stimulating human monocytes with LPS, and in culture supernatants (MOCM-b) of monocytes incubated in the medium containing one of the 5 immunosuppressive agents for 48 and 72 h.

6. Induction of CRP and SAA production in HepG2 cells by addition of MOCM.

HepG2 cells were incubated at a density of 5×10^4 cells/well in 24-well plates. On the fifth day of incubation, the supernatants MOCM-a or -b were added to the medium to stimulate the cells. After incubation for an additional 48 and 72 h, CRP and SAA in each supernatant were determined.

7. Determination of CRP, SAA, and cytokines.

CRP in culture fluid was determined using the enzyme immunoassay described by Janssen et al⁷). SAA was determined by a sandwich enzyme immunoassay as fol-

lows. Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with monoclonal anti-human SAA antibodies (Dako, CA, USA) diluted in 50 mM, pH 9.6 sodium carbonate buffer for 1 h at 37 °C. Standard solutions of SAA (Eiken Chemical Co., Tokyo, Japan) or culture fluid samples were added and the plates were incubated at 37 °C for 1 h. After washing, the plates were allowed to react with polyclonal rabbit anti-human SAA antibodies (Eiken Chemical Co.) at 37 °C for 1 h, with peroxidase-labelled goat anti-rabbit IgG antibodies (Dako) at 37 °C for 1 h, and with a substrate of ammonium-2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS ; Sigma) at 37 °C for 30 min. The absorbance at 405 nm was read using a multiscan plate reader (BioRad, Richmond, CA, USA). The SAA concentration in the sample was determined from a calibration curve derived from the standard solution. Cytokine concentrations were determined by two methods : an EIA for IL-1 α , IL- β and TNF α and an enzyme-linked immunosorbent assay for IL-6⁸⁾.

8. Statistical analysis.

Data on the concentrations of cytokines, CRP, and SAA produced under various culture conditions were subjected to statistical analysis using the Student's t-test. A p value less than 0.05 was considered statistically significant.

RESULTS

1. Induction of CRP and SAA production in HepG2 cells by IL-1 β and IL-6.

HepG2 cells were cultured various concentration of rhIL-6 at constant concentration of 25 ng/ml rhIL-1 β . The production of CRP and SAA was highest when both 25 ng/ml rhIL-1 β and 25 ng/ml rhIL-6 were present. Addition of rhIL-1 β alone resulted in a modest production of CRP ; rhIL-1 β plus 25 ng/ml rhIL-6 increased CRP production 2.6 times more of the control. SAA production was induced with rhIL-1 β alone, and showed only a modest increase to approximately 1.3 times of the control, even when 25 ng/ml rhIL-6 was added (Fig. 1).

These results indicate that the optimum concentrations of rhIL-1 β and rhIL-6 for induction of SAA synthesis are both 25 ng/ml. CRP production was increased by rhIL-6. In contrast, SAA production was enhanced by rhIL-1 β but not significantly affected by rhIL-6. At a high concentration of 50 ng/ml rhIL-6, the production of both CRP

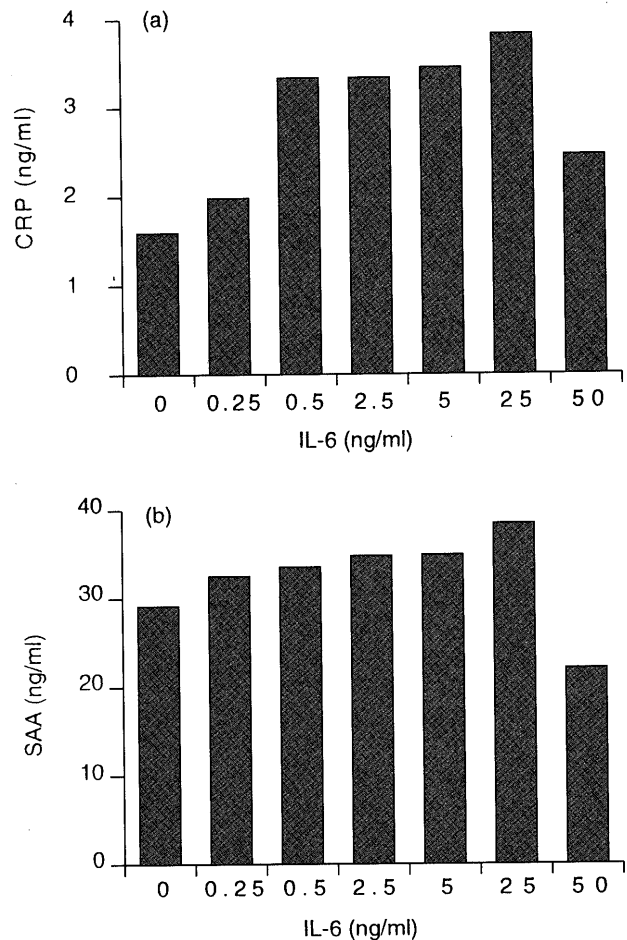


Fig. 1 HepG2 cells were cultured various concentration of recombinant human (rh) IL-6 at constant concentration of 25 ng/ml rhIL-1 β . Production of C-reactive protein (CRP) and serum amyloid A protein (SAA) by HepG2 cells induced with rhIL-6 (0-50 ng/ml) and rhIL-1 β (25 ng/ml). Values expressed are the means of three experiments. Interexperimental variation was less than 25%. (a) CRP produced. (b) SAA produced.

and SAA have decreased.

2. Effects of 5 immunosuppressive agents on CRP and SAA production in cytokine-supplemented HepG2 cell cultures.

The concentrations of CRP and SAA were determined in HepG2 cells incubated in the medium supplemented with one of the 5 immunosuppressive agents in the presence or absence of 25 ng/ml rhIL-1 β and 25 ng/ml rhIL-6 (Fig. 2, 3).

Addition of rhIL-1 β and rhIL-6 to the cell culture medium in the absence of any drug did not affect CRP production between 48 and 72 h of incubation (3.7 ng/ml ; Fig. 2-b). CRP production was not significantly

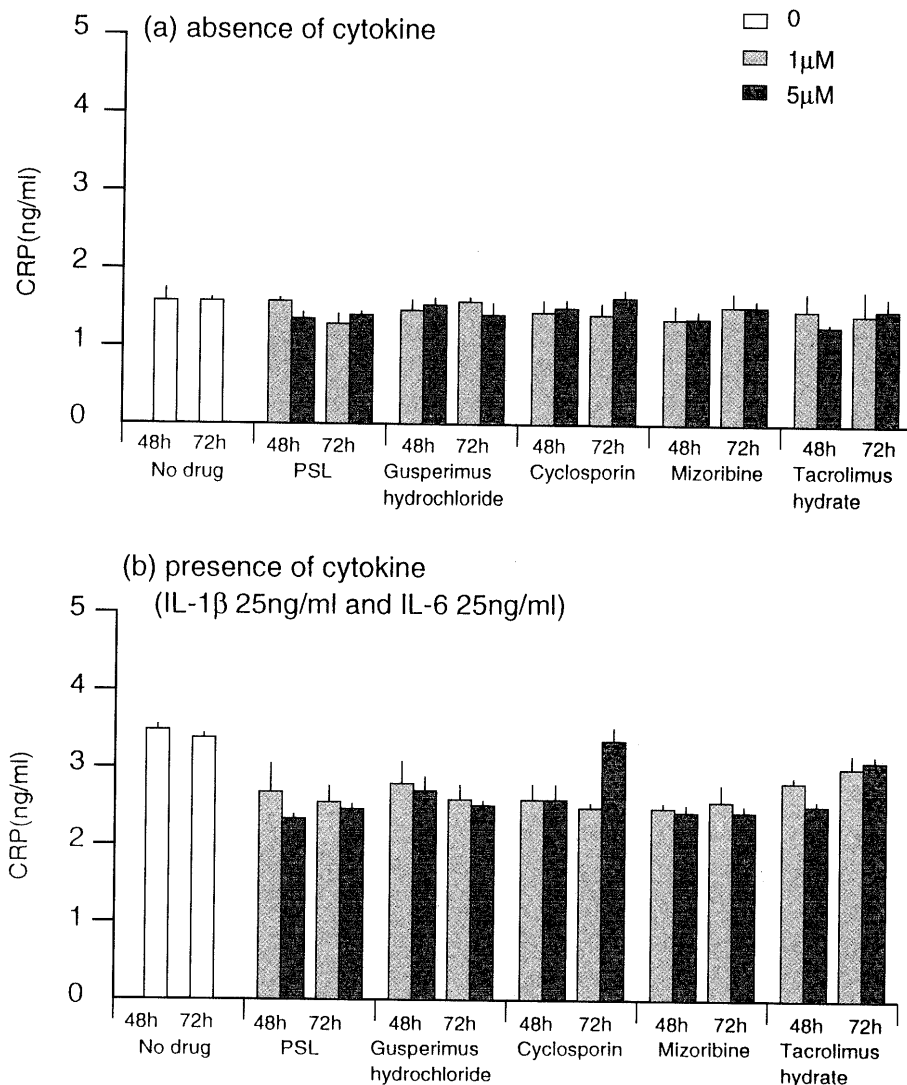


Fig. 2 Production of C-reactive protein (CRP) by HepG2 cells induced with different concentrations of prednisolone and 4 immunosuppressants in the presence or absence of recombinant human (rh) IL-1 β and rhIL-6 (both 25 ng/ml). Values expressed are the means of three experiments. Interexperimental variation was less than 25%. CRP produced during incubation (48–72 h) with prednisolone (0, 1 and 5 μ M) in medium. (a) CRP concentration in the absence of cytokine. (b) CRP concentration in the presence of rhIL-1 β and rhIL-6 (both 25 ng/ml).

affected by addition of PSL. The time courses of CRP in the cell cultures supplemented with PSL did not show a significant difference between the two concentrations, regardless of the presence of cytokines (Fig. 2 - a, b).

By contrast, SAA increased to 80 ng/ml after incubation for 72 h in the presence of 1 μ M PSL and 25 ng/ml rhIL-1 β and 25 ng/ml rhIL-6, indicating approximately a two-fold or greater increase, compared with the control without PSL (Fig. 3 - b). Addition of 1 μ M PSL produced the highest increase in SAA production, and 5 μ M PSL led to a smaller increase (Fig. 3 - b).

Production of CRP and SAA was examined in cell cultures supplemented with various concentrations of gusperimus hydrochloride, cyclosporin A, mizoribine or tacrolimus hydrate after 48 and 72 h of incubation in the presence or absence of 25 ng/ml rhIL-1 β and 25 ng/ml rhIL-6 in combination. The amount of CRP produced was almost constant with any of the 4 immunosuppressive agents added at concentrations of 1 and 5 μ M, or between the two different periods of incubation (Fig. 2 - a, b). The amount of SAA produced was not significantly affected by rhIL-1 β and rhIL-6, although it was decreased by

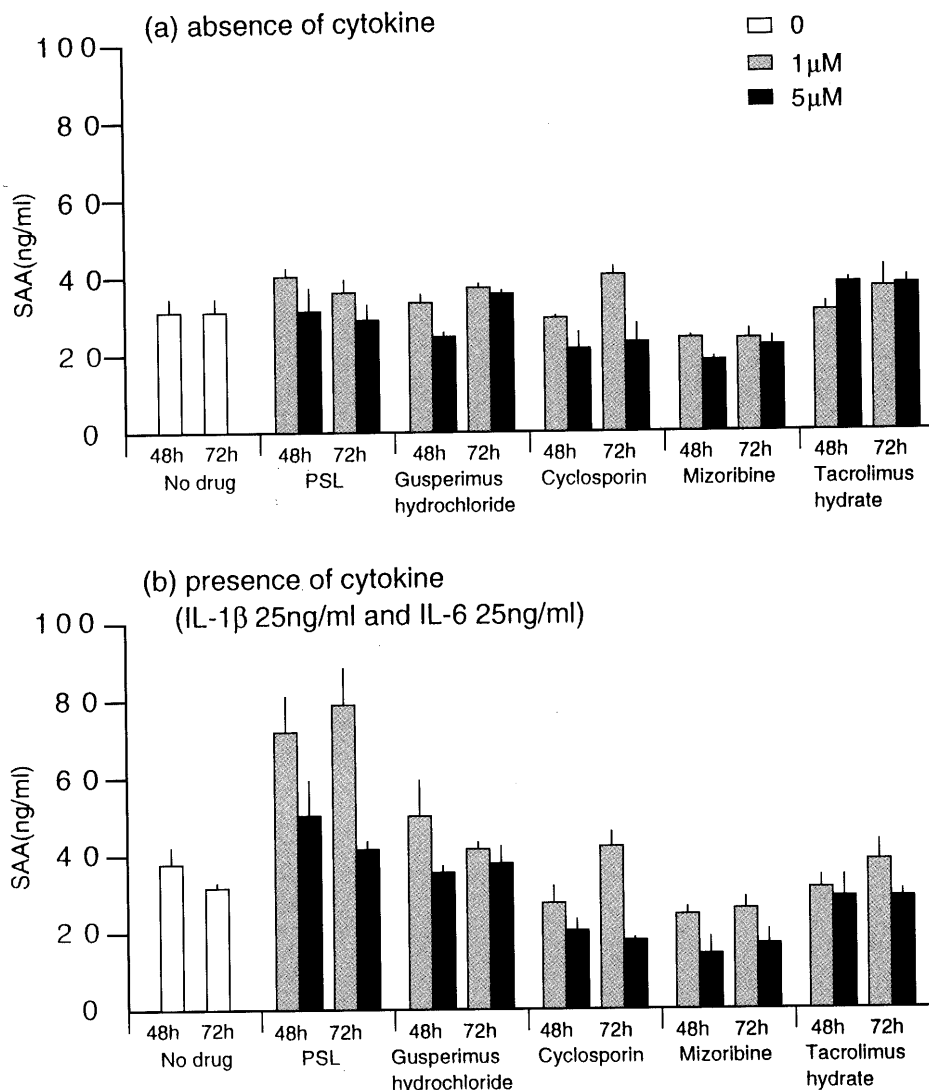


Fig. 3 Production of serum amyloid A protein (SAA) by HepG2 cells induced with different concentrations of prednisolone and 4 immunosuppressants in the presence or absence of recombinant human (rh) IL-1 β and rhIL-6 (both 25 ng/ml). Values expressed are the means of three experiments. Interexperimental variation was less than 25%. SAA produced during incubation (48-72 h) with immunosuppressants (0, 1 and 5 μ M) in medium. (a) SAA concentration in the absence of cytokine. (b) SAA concentration in the presence of rhIL-1 β and rhIL-6 (both 25 ng/ml).

each of the 4 agents at 5 μ M both after 48 and 72 h of incubation (Fig. 3-a, b).

Thus, PSL reduced CRP production in HepG2 cells. Addition of 1 μ M PSL provided maximum production of SAA, and 5 μ M PSL induced less SAA production. The 4 immunosuppressive agents had no significant effect on CRP production but reduced SAA production at 5 μ M.

3. Effect of 5 immunosuppressive agents on the production of cytokines by monocytes.

The production of IL-1 α , IL-1 β , IL-6, and TNF α was

examined in LPS-stimulated and non-stimulated monocytes incubated for 48 h in the presence or absence of immunosuppressive agents (Table 1). Although the production of these cytokines by monocytes was observed even in the absence of LPS stimulation, monocytes were stimulated by LPS in MOCM induced a marked increase in the production of IL-1, IL-1 β , IL-6, and TNF α (Table 1). PSL, 10 μ g/ml, caused a decrease in the production of each cytokine to 68 % (IL-1 α), 71 % (IL-1 β), 52 % (IL-6) and 50 % (TNF α) of the control levels.

The 4 immunosuppressive agents induced definitive

Table 1 Effect of prednisolone and 4 immunosuppressants on the production of IL-1 α , IL-1 β , IL-6 and TNF α by monocytes#.

LPS (μ g/ml)	MOCM preparation											
	No drug		Prednisolone		Gusperimus hydrochloride		Cyclosporin		Mizoribine		Tacrolimus hydrate	
	0	10	0	10 ⁺	0	10 ⁺⁺	0	10 ⁺⁺	0	10 ⁺⁺	0	10 ⁺⁺
IL-1 α (pg/ml)	25.4	441.5 \pm 249.5 (100%)	3.9	301 \pm 136 (68%)	57.1	602 \pm 137 (136%)	91.3	631 \pm 249 (143%)	67.2	488.5 \pm 0.5 (111%)	76.5	460 \pm 10 (104%)
IL-1 β (pg/ml)	22.6	1645.5 \pm 704.5 (100%)	6.3	1170 \pm 360 (71%)	57.4	1725 \pm 135 (105%)	44.4	2900 \pm 1500 (176%)*	62.2	1760 \pm 200 (107%)	60.9	1750 \pm 220 (106%)
IL-6 (pg/ml)	1230	7090 \pm 210 (100%)	383	3650 \pm 1120 (52%)*	708	8465 \pm 1235 (120%)	2355	8955 \pm 1005 (126%)	1285	9830 \pm 130 (139%)	1239.5	7405 \pm 325 (104%)
TNF α (pg/ml)	6.3	179.5 \pm 20.5 (100%)	< 2.0	90 \pm 32 (50%)*	9.4	279.5 \pm 65.5 (156%)	7.5	181.5 \pm 50.5 (102%)	9.3	309 \pm 131 (172%)*	6.5	188.5 \pm 27.5 (105%)

Monocyte conditioned media (MOCM) from control and treated monocytes was analyzed for IL-1 α , IL-1 β , IL-6 and TNF α at 48h of incubation with lipopolysaccharide (LPS).

+ Prednisolone and LPS were added to the medium at same time.

++ Immunosuppressants (final concentration of 1 μ M) were added at the same time of LPS addition.

Values are expressed as mean \pm S.D. (concentration in the medium) for three experiments.

* Significantly different at $p < 0.05$.

increases in the production of IL-1 α , IL-1 β , IL-6 and TNF α in LPS-stimulated monocytes, compared with non-stimulated controls.

4. Effects of 5 immunosuppressive agents added to MOCM on CRP and SAA production by HepG2 cells.

The production of CRP and SAA was examined in HepG2 cells incubated with MOCM from LPS-stimulated or non-stimulated monocytes prepared in the presence or absence of 5 immunosuppressive agents (Table 2). While CRP production was not affected, SAA production increased in HepG2 cells incubated with the MOCM from cells treated with one of the 5 agents (1.0 μ M) in the absence of LPS stimulation. By contrast, in HepG2 cells incubated with MOCM from LPS-stimulated cells treated with PSL or one of the 4 agents, CRP production tended to be decreased while SAA production tended to be increased, as compared with the non-drug treated MOCM. A marked increase to 172% in SAA production occurred in the hepatoma cells incubated with MOCM treated with PSL, while only modest increases occurred

with the other drugs.

DISCUSSION

Acute-phase proteins are produced in the liver in response to inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, and TNF α ; IL-6 is regarded as the most potent inducer for the production of CRP and SAA⁹⁾. Plasma CRP and SAA levels usually show parallel increases and decreases in pathological conditions, however, they sometimes do not show parallel changes²⁻⁵⁾.

The present study has shown the effects of cytokines and immunosuppressive agents on the production of CRP and SAA in the HepG2 cell line. In HepG2 cells, CRP and SAA were produced by IL-1 β stimulation, but addition of IL-6 increased their production. IL-6 alone caused a higher production of CRP, which was further increased by the addition of IL-1 β . SAA production was enhanced potent by IL-1 β even in the absence of IL-6, and addition of IL-6 to IL-1 β further increased the production of SAA. In HepG2 cells, IL-6 was a significant inducer of CRP production and IL-1 β was a more effective inducer of

Table 2 Production of serum amyloid A (SAA) and C-reactive protein (CRP) by HepG2 cells stimulated with monocyte conditioned medium (MOCM) produced in the presence or absence of prednisolone and 4 immunosuppressants#

LPS ($\mu\text{g/ml}$)	MOCM preparation												
	No drug		Prednisolone		Gusperimus hydrochloride		Cyclosporin		Mizoribine		Tacrolimus hydratee		
	0	10	0	10 ⁺	0	10 ⁺⁺	0	10 ⁺⁺	0	10 ⁺⁺	0	10 ⁺⁺	
CRP (ng/ml)	48 hr.	2.2 \pm 0.1 (100%)	2.8 \pm 0.3 (82%)	2.6 \pm 0.3 (82%)	2.3 \pm 0.3 (82%)	2.5 \pm 0.6 (86%)	2.4 \pm 0.6 (86%)	2.5 \pm 0.2 (86%)	2.4 \pm 0.2 (86%)	2.3 \pm 0.1 (82%)	2.3 \pm 0.1 (82%)	2.5 \pm 0.2 (86%)	2.4 \pm 0.2 (86%)
	72 hr.	2.2 \pm 0.8 (100%)	2.5 \pm 0.8 (88%)	2.5 \pm 0.8 (88%)	2.2 \pm 0.8 (88%)	2.4 \pm 0.6 (92%)	2.3 \pm 0.6 (92%)	2.5 \pm 0.5 (96%)	2.4 \pm 0.5 (96%)	2.5 \pm 0.5 (92%)	2.3 \pm 0.5 (92%)	2.7 \pm 0.5 (96%)	2.4 \pm 0.5 (96%)
SAA (ng/ml)	48 hr.	29 \pm 8.5 (100%)	48.5 \pm 8.5 (100%)	50 \pm 0.5 (118%)	57 \pm 0.5 (118%)	47.5 \pm 8 (101%)	49 \pm 8 (101%)	39.5 \pm 3 (103%)	50 \pm 3 (103%)	33 \pm 4 (109%)	53 \pm 4 (109%)	37 \pm 7 (103%)	50 \pm 7 (103%)
	72 hr.	38 \pm 6.5 (100%)	57.5 \pm 6.5 (100%)	56.5 \pm 16 (172%)*	99 \pm 16 (172%)*	65 \pm 11 (114%)	66 \pm 11 (114%)	56 \pm 4 (101%)	58 \pm 4 (101%)	43.5 \pm 2 (104%)	60 \pm 2 (104%)	39 \pm 6 (101%)	58 \pm 6 (101%)

Values are concentration of CRP or SAA in the tissue culture fluid that had been incubated with MOCM for 48 or 72 h.

+ Prednisolone was added at the same time as LPS addition.

++ Immunosuppressants (final concentration of 1 μM) were added at the same time of LPS addition.

Values are expressed as mean \pm S.D. for three experiments.

* Significantly different at $p < 0.05$.

SAA production, which indicates possible differences in the regulation of CRP and SAA.

CRP production in HepG2 cells was decreased by the high concentrations of PSL. By contrast, SAA production in the HepG2 cells was increased with PSL, but was smaller with 5 μM PSL than with 1 μM PSL; these results differ from a report that a corticosteroid (dexamethasone) caused dose-dependent increases in SAA production in Hep3B cells¹⁰. Recently, the effects of corticosteroids have been studied in various hepatoma-derived cell lines including Hep3B and HepG2^{6,10,11}. It was reported that CRP and SAA production by HepG2 cells exhibited different sensitivities to stimulation by corticosteroids added alone or in combination with cytokines⁶. It was reported that dexamethasone reduced and delayed CRP release from HepG2 cells and that use of dexamethasone and IL-6 in combination increased CRP accumulation in HepG2 cells¹¹. SAA production was induced by corticosteroids⁶; the effects of corticosteroids on cytokine-stimulated CRP and SAA production may reflect different actions on DNA transcription¹².

Little has been reported on the effects of drugs including immunosuppressive agents other than corticosteroids on CRP and SAA production by hepatocytes. Although the immunosuppressive agents used in our present study had no significant effects on CRP production, a clear inhibition of SAA production in hepatoma cells was observed at a concentration of 5 μM . These results indicate that the production of CRP and SAA differ in response to different immunosuppressive agents.

When PSL was added to the culture medium, the production of cytokines was affected, of which reductions in IL-6 and TNF α were marked. By contrast, the production of cytokines tended to be increased by the other 4 immunosuppressive agents. These findings show that the production rate of cytokines by monocytes depends on the drug used.

In HepG2 cells stimulated with MOCM from LPS-treated monocytes, SAA production was significantly increased by PSL, while CRP production tended to be decreased. PSL reduced the production of cytokines, especially IL-6 in monocytes, which appears to cause

secondary inhibition of CRP production in HepG2 cells. The PSL-induced SAA production could be explained by a direct action on HepG2 cells. Another possible mechanism might involve the promotion of receptor binding for IL-1 β in hepatocytes by SAA, because two kinds of IL-1 receptors (IL-1 α and IL-1 β receptors) are suggested to be present in HepG2 cells¹³⁾, and IL-1 α may be substituted for IL-1 β in these receptors¹⁴⁾.

The present experiments, adding PSL or one of the 4 immunosuppressive agents in culture media of monocytes and hepatoma cells, demonstrate that CRP and SAA differ in their production. The results could account for the different patterns of CRP and SAA in diseases. Maury et al.¹⁵⁾ observed that CRP and SAA exhibited different patterns of changes in different stages of diseases. Smith et al.¹⁶⁾ showed that SAA increased, while CRP significantly decreased, in cystic fibrosis patients treated with corticosteroids, compared with those treated with non-steroidal anti-inflammatory drugs (NSAIDs). SAA is a useful indicator in monitoring allograft rejection after kidney and liver transplantation. SAA and CRP are reported to exhibit different patterns based on the finding that SAA levels were significantly increased while CRP were hardly increased in allograft rejection^{3,4,17)}. We also observed that in patients treated with high-dose corticosteroids, SAA levels decreased, reflecting the state of disease, while CRP levels decreased rapidly even to the normal range⁵⁾. The results demonstrated that responses of CRP and SAA level to immunosuppressants are different.

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