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Unstimulated Peripheral Blood Mononuclear Cells from Patients with the Hyper-IgD Syndrome Produce Cytokines Capable of Potent Induction of C-Reactive Protein and Serum Amyloid A in Hep3B Cells

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The hyper-IgD and periodic fever syndrome (HIDS) and familial Mediterranean fever (FMF) are both characterized by attacks of periodic fever accompanied by acute phase responses that are substantially higher in HIDS than in FMF. To determine whether this difference could be due to differences in production of acute phase protein-inducing mediators, we studied PBMC from HIDS and FMF patients in the inactive phase of disease. Unstimulated PBMC from patients with inactive HIDS released significantly more IL-1 β , IL-6, and TNF- α than did PBMC from patients with FMF, but unstimulated PBMC from the latter group released significantly more IL-1 β and IL-6 compared with controls. Conditioned medium (CM) derived from PBMC of patients with inactive HIDS induced significantly greater CRP production and significantly higher mRNAs for CRP and SAA in Hep3B cells than did CM derived from the PBMC of patients with inactive FMF. Stimulation of PBMC with LPS led to further increases in cytokine production and in acute phase protein-inducing ability in both patient groups and in controls. These findings suggest that the greater acute phase response seen in HIDS compared with FMF reflects greater production of acute phase protein-inducing cytokines in the former patients and indicates that PBMC from inactive HIDS patients are already activated *in vivo*. Finally, the finding of both quantitative and qualitative differences in cytokine production by unstimulated PBMC from HIDS and FMF patients supports the likelihood of different pathogenesis of these diseases. *The Journal of Immunology*, 1996, 157: 400–404.

The hyper-IgD and periodic fever syndrome (HIDS)³ is a rare disease characterized by recurrent febrile attacks with abdominal complaints, lymphadenopathy, skin lesions, and joint involvement. Up to now, some 65 patients, mainly of European origin, have been recognized (1). A clinically similar entity, familial Mediterranean fever (FMF), is an autosomal recessive inflammatory disorder characterized by short episodes of fever, peritonitis, pleuritis, and arthritis (2), which predominantly affects non-Ashkenazi Jews, Armenians, and Arabs. The pathogenesis of these periodic fever syndromes is unknown, but it is probable that attacks arise from dysregulation of the inflammatory response in both. For example, a lack of C5a inhibitor has been incriminated as a cause of the recurrent attacks of serositis in FMF (3).

Inflammatory states are often accompanied by increased plasma concentrations of a group of acute phase proteins, including C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, and α_1 -acid glycoprotein (AGP) (4). A number of inflammation-associated cytokines, particularly IL-1, IL-6, and TNF- α are considered the main inducers of their synthesis (5). Febrile attacks in both HIDS and FMF are accompanied by an acute phase response, which, however, differs in intensity. In HIDS, substantially higher serum concentrations of acute phase proteins are achieved during attacks than are seen in FMF (6–8). This variation in acute phase response between the two syndromes could theoretically be due to a different quality of a hypothetical inflammatory stimulus, to differences in production of inflammatory mediators influencing plasma protein synthesis, or to different responses of hepatocytes.

PBMC produce a wide range of cytokines capable of acute phase protein induction after exposure to bacterial LPS (9), and conditioned medium (CM) from such LPS-stimulated PBMC has been shown to induce acute phase reactants in human hepatoma cell lines (10, 11). Accordingly, we explored possible differences between these two periodic fever syndromes in production of acute phase protein-inducing mediators by comparing the abilities of PBMC from HIDS and FMF patients to produce three inflammation-associated cytokines. In addition, we compared the abilities of their CM both to induce production of the protein CRP and to induce expression of the mRNAs for the two major human acute phase protein genes, CRP and SAA, in the human hepatoma cell line Hep3B.

Materials and Methods

Patients

Eight patients with HIDS (four male, mean age: 25.9 yr); six patients with FMF (three male, mean age: 25.3 yr), and seven healthy Dutch volunteers (seven males, mean age: 30.3 yr) took part in the study. The diagnoses of HIDS and FMF were made according to standard criteria listed elsewhere

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³ Abbreviations used in this paper: HIDS, hyper-IgD periodic fever syndrome; FMF, familial Mediterranean fever; CM, conditioned medium; CRP, C-reactive protein; SAA, serum amyloid A; AGP, α_1 -acid glycoprotein; FMF-CM, conditioned medium prepared from peripheral blood mononuclear cells derived from patients with familial Mediterranean fever; C-CM, conditioned medium prepared from peripheral blood mononuclear cells derived from control adults; HIDS-CM, conditioned medium prepared from peripheral blood mononuclear cells derived from patients with hyper-IgD syndrome; TBS, Tris-buffered saline; OPD, *o*-phenylenediamine.

(1, 2). The patients with HIDS were all of Dutch origin, while five patients with FMF came from Turkey and one from Greece. The patients refrained from taking any medication during the study, and all patients were asymptomatic at the time of blood drawing. The FMF patients had normal renal function and were, apart from the study period, receiving continuous treatment with colchicine (1–1.5 mg/day).

Isolation of PBMC and preparation of CM

Peripheral blood was drawn into sterile 10-ml tubes containing 0.2 mg EDTA. PBMC were isolated by buoyant density gradient centrifugation on Percoll (12). The cells from the interphase were aspirated and washed twice in sterile saline. After the last washing, cells were resuspended at a concentration of 5×10^6 /ml in RPMI 1640 medium (Dutch modification; Flow Labs, Irvine, Scotland) supplemented with 2 mM L-glutamine, 1 mM pyruvate, and 500 μ g/ml gentamicin. Medium was loaded onto a 50-ml syringe and forced through a 0.2- μ m filter by manual force to remove endotoxin and other cytokine-inducing material (13). Heat-inactivated (30 min at 56°C) pooled human sera (5%) was added after filtration. PBMC were resuspended at a concentration of 5×10^6 /ml in round-bottom 96-well plastic tissue culture plates and incubated either with or without 100 ng/ml *Escherichia coli* LPS at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. Fifty microliters of the PBMC suspension was applied to cytospin centrifugation, and the contribution of monocytes to the PBMC solution was assessed microscopically under high powered view. The contribution of monocytes to the PBMC solution was similar for each group (HIDS, $17.5 \pm 5.8\%$; FMF, $16 \pm 6\%$; controls, $18 \pm 8.6\%$). After incubation, the culture plates were centrifuged at $4000 \times g$ for 10 min to remove cellular material. Cell supernatants (CM) were then aspirated and stored at -70°C . A baseline value for CRP was determined for the CM of the patients and controls. This baseline value was subtracted from the total amount of CRP subsequently released into the medium by Hep3B cells to calculate actual CRP production.

Cultures of Hep3B cells

Human hepatoma cells, Hep3B, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were subcultured weekly after trypsinization. Induction of acute phase proteins was conducted when cells were confluent, usually on day 6 after subculture. Hep3B cells were incubated at 37°C in 35-mm dishes containing a total of 1 ml RPMI 1640 supplemented with 1 μ M dexamethasone and 10% CM. The medium was collected after 24 h and stored at -70°C until assay.

ELISAs

CRP and SAA concentrations were measured in duplicate in sera obtained from patients and healthy controls at the time of isolation of the PBMC and in media from Hep3B cells by ELISAs as previously reported (14, 15). Immulon III Dividastrip wells were coated overnight at 4°C with 1:1000 dilution of goat anti-human CRP, IgG fraction (Atlantic Antibodies no. 89134) in 100 μ l Tris-buffered saline (TBS). After three washes with 200 μ l TBS containing 0.05% Tween-20, 200 μ l of 1% BSA in TBS was added to each well to block nonspecific binding. Samples were diluted in glass tubes in RPMI 1640 with 1% BSA. Wells were washed before 100- μ l aliquots of samples and standards were added and incubated at 37°C for 2 h. After three washes, 100 μ l of PBS containing 1 ng/ml biotinylated, sheep anti-human CRP (Cappel Laboratories, West Chester, PA; no. 55122) was added. This Ab was biotinylated using Pierce (Rockford, IL) Immunopure NHS-LC Biotin according to the manufacturer's protocol. After incubation for 1 h at 37°C and washing with TBS-Tween, 100 μ l of streptavidin-horseradish peroxidase conjugate (diluted 1:1000 in PBS) was added and incubated for 1 h at 37°C. Wells were washed before the substrate was added (OPD kit; Sigma Chemical Co., St. Louis, MO). The reaction was stopped by 2 M H₂SO₄ and absorbance was measured at 490 nm.

TNF- α and IL-1 β were assayed by ELISAs developed in our laboratory in Cleveland, OH. Immulon III plates were coated for 2 h at 37°C with 100 μ l anti-TNF- α mAb (Boehringer Mannheim, Indianapolis, IN) diluted 1:40 in a bicarbonate buffer, pH 9.0, and placed overnight at 4°C. After five washes with TBS containing 0.05% Tween-20, nonspecific binding of proteins to the plastic was blocked by 3 h of incubation with 200 μ l nonfat dry milk in bicarbonate buffer. After five washes, 100- μ l samples and standards were allowed to incubate for 2 h at 37°C. Each sample was assayed in duplicate. After five washes, polyclonal rabbit anti-human TNF- α (Endogen, Boston, MA), diluted 1:1000 in PBS containing 2% nonfat dry milk was incubated for 2 h at 37°C. After incubation with horseradish-conjugated goat anti-rabbit antiserum (Tago Immunochemicals, Burlingame, CA) diluted 1:1000 in PBS with 2% nonfat dry milk, OPD substrate (Sigma) was used. After a few minutes, the color development was stopped

with 50 μ l 2N sulfuric acid and absorbance measured at 490 nm. The lower limit of detection was 15 pg/ml.

The ELISA for IL-1 β involved coating Immulon III plates for 24 h at 4°C with polyclonal anti-IL-1 β Ab diluted 1:9000 in PBS. The IL-1 β polyclonal Ab was purchased from Cistron Biotechnology (Pine Brook, NJ) and does not react with pro-IL-1 β . The plates were washed three times with PBS containing 0.05% Tween-20 and were incubated for 90 min at room temperature with PBS containing 5% nonfat dry milk. After three washes, 100- μ l samples and standards were added and incubated for 90 min at room temperature. After washing again, polyclonal rabbit anti-human IL-1 β antiserum diluted 1:4000 in PBS containing 1% nonfat dry milk was added, and after 90 min at room temperature, horseradish peroxidase-conjugated goat anti-rabbit antiserum (Tago) diluted 1:3000 was added and the plate was incubated for an additional hour. After washing, OPD solution (Sigma) was added, and the color development terminated after approximately 5 min with 50 μ l 2N sulfuric acid. Optical density was read at 490 nm. The lower limit of detection with this assay is 7 pg/ml.

IL-6 was measured using a commercial sandwich ELISA (R&D Systems, Inc., Minneapolis, MN). The assays were conducted according to the manufacturer's instructions and all samples were tested in duplicate.

RNA isolation and analysis

Total RNA was isolated from stimulated Hep3B cells by guanidinium isothiocyanate lysis following a modification of the method of Chomczynski and Sacchi (16). RNA samples (15 μ g per lane) were heat denatured at 65°C in a solution consisting of 50% deionized formamide, 2 M formaldehyde in 0.2 M 3-(*N*-morpholino)propane sulfonic acid buffer, pH 7.0, with 10 mM EDTA, and then fractionated on 1% agarose gels containing 2.2 M formaldehyde. After transfer to MagnaGraph nylon (Micron Separations Inc., Westboro, MA) by 3 h of vacuum blotting, RNA was UV cross-linked to the membrane. Dried membranes were photographed under UV transillumination. Prehybridization (4 h) and hybridization (18 h) with CRP and SAA cDNA probes labeled with [³²P]dCTP by the random primer method (10⁶ cpm) were performed at 42°C in hybridization buffer containing 50% deionized formamide, 5 \times standard saline solution (SSC), 1 \times Denhardt's solution, 1% SDS, and 10 mg/ml denatured salmon sperm DNA. Following hybridization, nonspecifically bound radioactivity was removed from membranes by washing twice with 6 \times SSC, 0.1% SDS at room temperature for 30 min, followed by two subsequent washes for 15 min with 0.1 \times SSC, 0.1% SDS at 62°C. The membranes were then exposed at -70°C to X-OMAT AR Kodak film. Membranes were stripped by 5 min of exposure to 95°C distilled H₂O containing 1% SDS before hybridization with the next labeled probe. The probes used were: 1) CRP, pCRP5, kindly provided by H. R. Colten, Washington University, St. Louis, MO (17); and 2) SAA, pA10 (capable of detecting all SAA isotypes) courtesy of J. Sipe, Boston University, Boston, MA (18).

The intensities of 18S RNA bands on photographs of ethidium bromide-stained formaldehyde gels and of mRNA bands on autoradiographs were measured by using a UMAX scanner (Data System Inc., Hsinchu, Taiwan) with the software Intelligent Quantifier for Macintosh (Bio Image, Ann Arbor, MI). Densitometry of 18S rRNA bands was used to correct for minor technical variations and the autoradiograph band intensities were adjusted appropriately.

Statistical analysis

All results are shown as the mean \pm SEM. Comparison between groups was assessed by the unpaired nonparametric Mann Whitney *U* test. A *p* value of <0.05 was considered to be the lowest level of significance.

Results

Serum concentrations of acute phase proteins

The CRP content in serum drawn from the patients was higher compared with the healthy control group, albeit the difference was not statistically significant (Table I). Serum from HIDS patients and FMF patients contained comparable quantities of SAA but the concentrations were (nonsignificantly) higher compared with the control group.

Cytokine production by PBMC

The concentrations of cytokines in CM, reflecting production by PBMC over 24 h of incubation, are indicated in Table II. CM derived from unstimulated PBMC from HIDS patients (HIDS-CM) contained significantly more IL-1 β , IL-6, and TNF- α than

Table 1. CRP and SAA content of serum from HIDS and FMF patients and from healthy controls as measured by specific ELISA assays^a

	HIDS	FMF	Controls
CRP (mg/L)	4.1 ± 1.8	5.9 ± 2.5	0.17 ± 0.03
SAA (mg/L)	6.9 ± 5.7	14.3 ± 7.9	0.27 ± 0.05

^a Data are expressed as mean ± SEM in ng/ml.

did CM derived from PBMC of FMF patients (FMF-CM). FMF-CM, in turn, contained significantly more IL-1 β and IL-6, but not TNF- α , than did CM derived from PBMC of healthy controls (C-CM). In all three groups, cytokine production by PBMC stimulated with 100 ng/ml LPS was significantly increased compared with unstimulated PBMCs (Table 1). Stimulated HIDS-CM contained threefold more IL-1 β and twofold more IL-6 than did stimulated FMF-CM. Unlike unstimulated PBMC, LPS-stimulated PBMC derived from HIDS and FMF patients produced comparable amounts of TNF- α . LPS-stimulated FMF-CM contained more IL-6 than did C-CM, while IL-1 and TNF- α production did not differ between these two groups.

Acute-phase response to CM

Unstimulated PBMC. Incubation of Hep3B cells for 24 h with unstimulated HIDS-CM resulted in fivefold greater accumulation of CRP in culture medium, reflecting greater production, than was observed with unstimulated FMF-CM ($p < 0.001$) or C-CM ($p < 0.005$) (Fig. 1). Unstimulated HIDS-CM also caused significantly greater increases in levels of mRNA in Hep3B cells for CRP (sevenfold) and SAA (50-fold) than did unstimulated FMF-CM (Fig. 2, A and B). Unstimulated HIDS-CM induced about 2.6-fold higher CRP mRNA and fivefold higher mRNA levels for SAA than did C-CM but neither of these differences could be shown to achieve statistical significance.

LPS-stimulated PBMC. For all three groups, stimulation with LPS led to production of CM capable of greater acute phase protein-inducing ability than was seen with unstimulated PBMC. Hep3B cells incubated with stimulated HIDS-CM secreted fivefold more CRP than did cells incubated with stimulated FMF-CM or C-CM (Fig. 3), and 2.4-fold more than cells incubated with unstimulated HIDS-CM ($p = 0.009$). Following incubation of Hep 3B cells with LPS-stimulated HIDS-CM, CPR and SAA mRNA levels were significantly greater than was observed following incubation with FMF-CM or C-CM (Fig. 2, C and D). Serum SAA and CRP concentrations (determined during disease inactivity) did not correlate with acute phase protein-inducing activity of both the stimulated and unstimulated samples we tested.

Discussion

The major purpose of the current investigation was to determine the extent to which clinically observed differences in the magnitude of the acute phase response in HIDS and FMF patients, as measured by serum levels of acute phase proteins, could be attributed to differences in release of inflammatory cytokines by PBMC from these patients (Refs. 6–8; and J. P. H. Drenth, personal observations). We found that unstimulated PBMC from patients with inactive HIDS released significantly more IL-1 β , IL-6, and TNF- α than did PBMC from patients with FMF, and that CM from these cells induced significantly greater CRP production and significantly more mRNA for CRP and SAA in Hep3B cells than did CM from FMF patients. These results support the hypothesis that dif-

ferences in the magnitude of the acute phase response between HIDS and FMF patients reflect differences in cytokine production by their PBMC and indicate that these effects on acute phase protein production are exerted at the pretranslational level. We studied PBMC rather than monocytes because isolation of monocytes involves an adherence step that may itself activate these cells, which we wanted to avoid. To the extent to which findings in PBMC reflect the behavior of monocytes and tissue macrophages, our findings raise the possibility that macrophages may also produce large amounts of these inflammatory cytokines in patients with HIDS. The results from our study cannot be explained by differences in monocyte content in the PBMC since this was similar for all studied groups.

Serum SAA and CRP concentrations (determined during disease inactivity) were relatively low in both patient groups, although greater than those seen in controls, and did not correlate with acute phase protein-inducing activity of the samples we tested. This came as no surprise, since in vivo and in vitro circumstances are quite different. Our in vitro assay is relatively insensitive, based on protracted exposure of Hepatoma cells to an unchanging test sample, while in vivo acute phase protein levels reflect effects of continuous flow of plasma on in situ hepatocytes and of clearance of acute phase proteins from plasma.

In addition, our findings also indicate that PBMC are already markedly activated in vivo in patients with inactive HIDS. In contrast, unstimulated PBMC from patients with inactive FMF produced substantially less IL-1 β and IL-6, and their TNF- α production was indistinguishable from that seen in PBMC from healthy controls. Overproduction of cytokines by unstimulated PBMC from HIDS patients could be due either to faulty regulation of autonomous cytokine production or to the effects of an exogenous stimulatory factor. Recent findings favor the latter explanation; IgD isolated from HIDS patients has been found to stimulate normal human PBMC to produce large quantities of IL-1 β , IL-6, TNF- α , and leukocyte inhibitory factor. (19). It is reasonable to conclude, in light of our current findings, that the high concentrations of IgD seen in these patients serve as a continuous stimulus to inflammatory cytokine production, even during clinically inactive periods. This conclusion is consistent with our previous observation that increased fucosylation of AGP, a phenomenon shown to be due to the effects of cytokines (20), is found in HIDS patients during asymptomatic intervals. The finding that CM derived from unstimulated PBMC from HIDS patients in the intercritical phase is able to induce an acute phase response in Hep3B cells, a relatively insensitive bioassay (21), supports this conclusion, as does the observation that serum AGP concentrations are persistently elevated between attacks and increase slightly during febrile attacks of HIDS (20).

There has been similar interest in the effect of CM derived from circulating cells on the synthesis of acute phase proteins in other inflammatory disorders. Patients with active Crohn's disease generally have higher circulating CRP concentrations compared with those with ulcerative colitis (22). IL-1 β and TNF- α production by unstimulated peripheral monocytes was not different in cells from patients with Crohn's disease than in those with ulcerative colitis, but LPS-stimulated cytokine production was significantly higher in monocytes from Crohn's disease patients (22) and this difference was even greater during active disease. CM from both unstimulated and stimulated monocytes from patients with active Crohn's disease induced greater release of CRP from PLC/PRF/5 human hepatoma cells than did CM from patients with active ulcerative colitis (23). Similarly, CM from unstimulated and LPS-stimulated

Table II. IL-1 β , IL-6, and TNF- α content of CM from PBMC of HIDS and FMF patients from healthy controls as measured by specific ELISA assays^a

	Unstimulated			LPS-Stimulated		
	HIDS	FMF	Controls	HIDS	FMF	Controls
IL-1 β	3.2 \pm 1.1 ^b	0.64 \pm 0.4 ^c	0.048 \pm 0.4	63 \pm 7.2 ^b	19.8 \pm 3.3	22 \pm 6.9
IL-6	11.7 \pm 4.6 ^b	5.1 \pm 2.8 ^c	0.068 \pm 0.017	150 \pm 23.7 ^b	73 \pm 4.1 ^c	15.3 \pm 8
TNF- α	1.5 \pm 0.75 ^b	0.072 \pm 0.04	0.065 \pm 0.012	7.4 \pm 2.4	5.5 \pm 2.9	1.4 \pm 0.8

^a CM was prepared from unstimulated PBMC or from PBMC stimulated with 100 ng/ml LPS. Data are expressed as mean \pm SEM in ng/ml.

^b Indicates difference ($p < 0.05$) compared to FMF.

^c Indicates difference ($p < 0.05$) compared to controls.

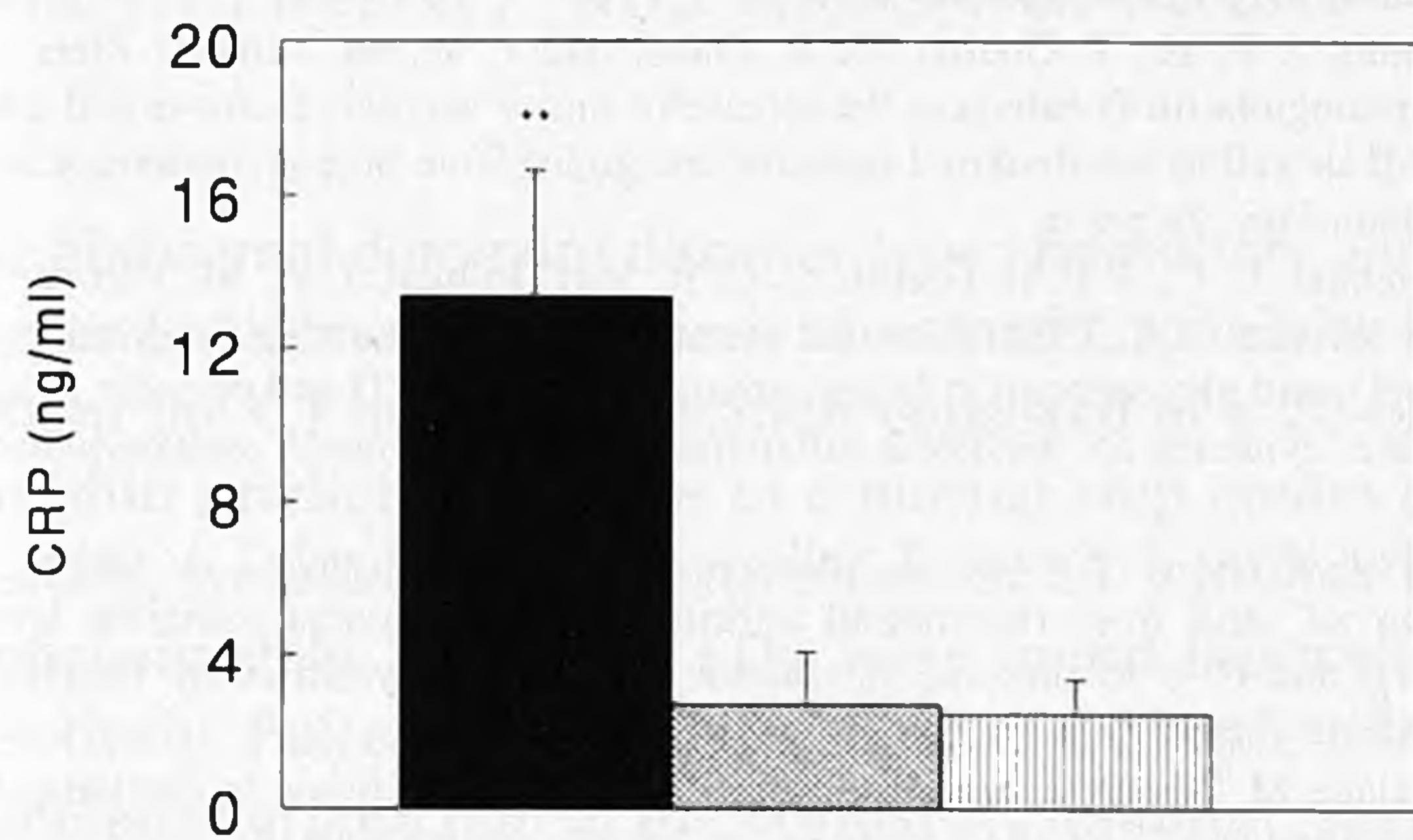


FIGURE 1. Effect of CM (10%) from unstimulated PBMC of patients with HIDS (■), FMF (■), and healthy controls (□) (C-CM) on the production of CRP from Hep3B cells. Supernatants were harvested after 24 h of incubation and CRP concentrations determined. Results are expressed as mean \pm SEM. HIDS-CM caused significantly more CRP production than did FMF-CM and C-CM (**, $p < 0.01$).

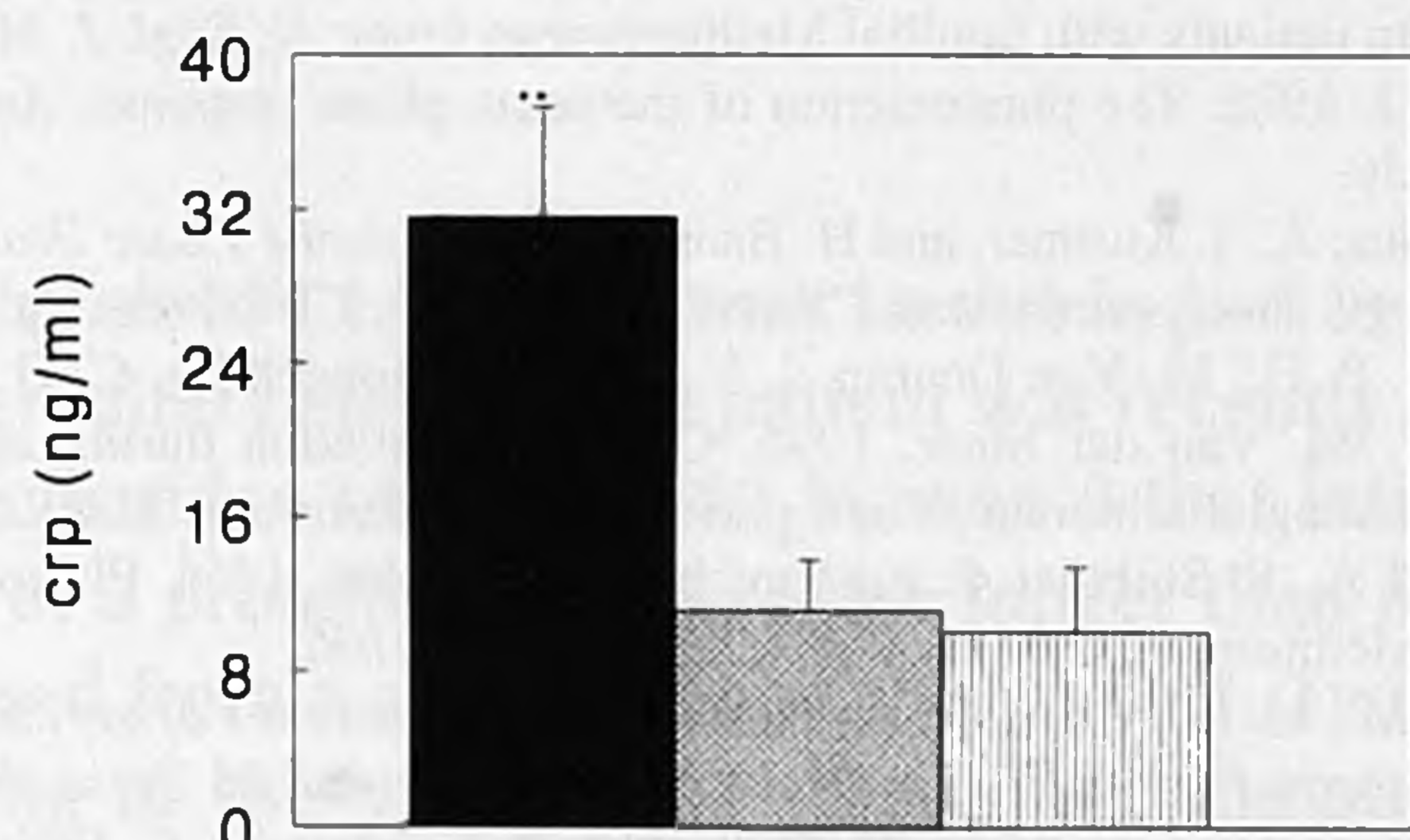


FIGURE 3. Effect of CM from LPS-stimulated PBMC of HIDS (■), FMF (■), and healthy controls (□) on production of CRP from Hep3B cells. Hep3B cells were incubated with 10% CM obtained from PBMC stimulated with 100 ng/ml *E. coli* LPS. After 24 h, supernatants were harvested and CRP concentrations determined. Results are expressed as mean \pm SEM. Stimulated HIDS-CM caused significantly more CRP production than did FMF-CM and C-CM (**, $p < 0.01$).

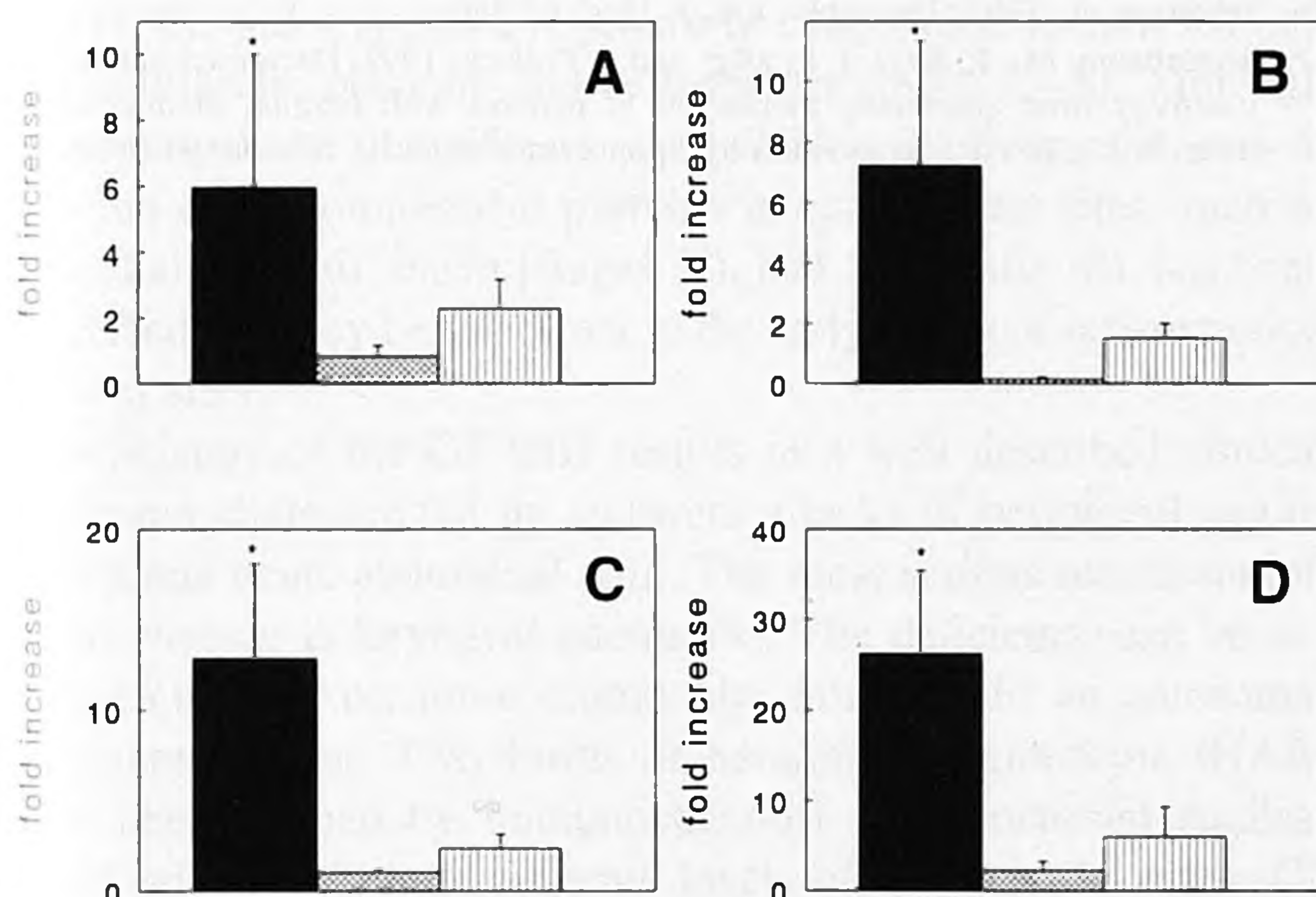


FIGURE 2. Effect of CM from unstimulated PBMC (upper panel) and LPS-stimulated PBMC (lower panel) of patients with HIDS (■), FMF (■), and healthy controls (□) on levels of mRNA in Hep3B cells for CRP (A (unstimulated) and C (stimulated)) and SAA (B (unstimulated) and D (stimulated)). Results are represented as fold increase over mRNA levels in unstimulated Hep3B cells. *, $p < 0.05$ for HIDS patients compared with FMF patients.

PBMC from patients with rheumatoid arthritis significantly increased production of α_1 -antichymotrypsin and α_1 -proteinase inhibitor by HepG2 cells over levels achieved by CM from PBMC of patients with active systemic lupus erythematosus (24).

The differences we observed in the profile of cytokines produced by unstimulated PBMC from patients with inactive HIDS and FMF indicate both qualitative and quantitative differences in cytokine production in patients with these two periodic inflammatory disorders during intercritical periods. Unstimulated PBMC from

FMF patients produced significantly more IL-6 and IL-1 β , but not TNF- α , than did PBMC from normal controls, while PBMC from HIDS patients produced significantly more of all three cytokines than did FMF PBMC. Taken together with the observation that supernatants from both unstimulated and LPS-stimulated PBMC from HIDS and FMF patients differed in their capacity to elicit CRP production and acute phase protein mRNA changes in Hep3B cells, these findings suggest differences in the pathogenesis of these two intermittent febrile disorders.

Finally, the observation that HIDS PBMC, although already activated, were able to respond to LPS stimulation with further increase in cytokine production and increased acute phase protein-inducing capability is consistent with the hypothesis that acute febrile attacks are precipitated by unknown stimuli capable of further increasing cytokine production. There is evidence that the ability to produce cytokines in HIDS patients increases during febrile attacks. In these patients, LPS-stimulated ex vivo production of TNF- α and IL-1 β , as measured in a whole blood culture system, was found to be significantly greater during acute attacks than following defervescence (6). In contrast, LPS-stimulated TNF- α and IL-1 β production in supernatants of PBMC from FMF patients was markedly decreased during febrile attacks but normalized during recovery (25–27). This apparent differential regulation of cytokines during attacks in these two syndromes might further explain the difference in the magnitude of the acute phase response observed in vivo.

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