Background—C-reactive protein (CRP) plasma levels have been associated with short- and long-term occurrence of coronary events. We investigated whether circulating inflammatory cell responsiveness to low-grade stimuli could contribute to the reported association between CRP and coronary events.

Methods and Results—We studied 32 patients with unstable angina who were followed for 24 months and were free of symptoms for 6 months (group 1): 19 patients had persistently high CRP levels (>0.3 mg/dL) (group 1A); 13 patients had normal CRP levels (group 1B). During the follow-up, 12 (63%) group 1A but no group 1B patients developed an infarction or recurrence of unstable angina (P<0.001). Eighteen patients with chronic stable angina (group 2) and 18 healthy subjects (group 3) were studied as controls. Interleukin (IL)-6 production (median, range) by peripheral blood mononuclear cells after 4 hours of in vitro stimulation with 1 ng/mL lipopolysaccharide (LPS) was significantly higher in group 1A (4526 pg/mL, 3042 to 10 583 pg/mL) than in group 1B (1752 pg/mL, 75 to 3981 pg/mL), group 2 (707 pg/mL, 41 to 3275 pg/mL), and group 3 (488 pg/mL, 92 to 3503 pg/mL) (all P<0.001). No significant differences were observed among the other groups. IL-6 production after LPS-challenge was correlated with baseline CRP levels (r=0.42, P=0.005).

Conclusions—Mononuclear cells of patients with recurrent phases of instability exhibit an enhanced production of IL-6 in response to low-dose of LPS, correlated with baseline CRP levels, 6 months after the last acute event. This persisting enhanced acute-phase responsiveness may help explain the association between CRP and acute coronary events. 

(Circulation. 2001;103:2236-2241.)

Key Words: angina ■ inflammation ■ interleukins ■ leukocytes ■ prognosis
inflammatory cells, we assessed the in vitro production of IL-6, the major inducer of CRP, by peripheral blood mononuclear cells in response to lipopolysaccharide (LPS)-challenge in patients with a history of UA, with and without elevated levels of CRP at 6 months after the waning of symptoms.

Methods

Population

Protocol A: Responsiveness of Whole Blood Cells to LPS Challenge

Three groups of subjects were prospectively studied. Group 1 included 32 of 65 consecutive patients with a history of UA (26 men; mean age, 63±8 years) who were followed for a mean period of 29±4 months (minimum, 24 months) after coronary care unit (CCU) admission, who were part of a previous study on the long-term prognostic role of acute-phase proteins in UA, and who were free of symptoms from at least 6 months. Group 2 included 18 patients (17 men; mean age, 61±9 years) with stable angina (SA) lasting >6 months, and group 3 included 18 healthy volunteers (13 men; mean age, 53±6 years) without coronary risk factors.

Protocol B: Responsiveness of Isolated Monocytes to LPS-Challenge

To assess the direct effects of LPS on isolated monocytes, a smaller study was designed including 14 group 1 patients (9 men; mean age, 62±11 years), 8 group 2 patients (6 men; mean age, 61±8 years), and 8 group 3 subjects (5 men; mean age, 50±7 years). All subjects were prospectively enrolled according to protocol A inclusion criteria.

All subjects gave their written, informed consent. The Ethics Committee of the Catholic University of Rome approved the study.

Design of the Study

Long-Term Follow-up and Longitudinal Monitoring of CRP Levels in UA

The recurrence of new acute coronary events (MI, new CCU admission for UA) was recorded. Venous blood samples were taken at (1) hospital admission, (2) discharge, and (3) every 3 months after discharge. Coded plasma samples were stored at -70°C and were analyzed for CRP in a single batch at the end of the follow-up period; all categorization and management of patients were independent of these results.

At the end of the follow-up period, 32 protocol A and 14 protocol B patients were identified who fulfilled the following inclusion criteria: (1) no symptoms in the previous 6 months; (2) no evidence of inflammatory or infectious diseases, malignancies, or immunologic or hematologic disorders; (3) no treatment with antiinflammatory drugs other than low-dose aspirin; (4) no depressed cardiac function (ejection fraction <40%); (5) <75 years of age.

Experimental Design

For each blood sample, plasma levels of CRP and IL-6 and total and differential white blood cell counts were assessed. Heparinized (10 U/mL) blood was used for in vitro assessment of IL-6 production. In 7 of 18 healthy individuals, an additional blood sample was taken after 3 months to assess the direct modulation of IL-6 production by CRP.

Whole Blood Assay: Effects of CRP

In 7 healthy individuals, an additional blood sample was taken after 3 hours to assess the direct modulation of IL-6 production by CRP. Aliquots of 1 mL of heparinized whole blood were incubated for 3 hours with LPS alone (1 ng/mL), with CRP alone (5 µg/mL or 50 µg/mL), or with both LPS and CRP and processed as described above. Highly purified (>99%) human CRP was obtained from Sigma Chemical Co. The whole blood cultures stimulated with CRP alone were coincubated with Polymixin B (10 µg/mL) to insure that CRP-induced IL-6 production was distinct from that mediated by LPS. The CRP doses of 5 µg/mL and 50 µg/mL corresponded to CRP plasma concentrations detected in UA patients.

Monocyte Isolation and Stimulation

Isolation of monocytes from heparinized blood was performed by layering over Nycodenz (Life Technologies). Isolation of ≥80% pure monocytes was obtained, as evaluated by using flow cytometry (data not shown). Freshly isolated monocytes (5×10^6) were incubated for 4 hours at 37°C in RPMI-1640 supplemented with 10% fetal calf serum, 50 U/mL penicillin, 50 µg/mL streptomycin, and 0.2 mmol/L L-glutamine with or without LPS (1 ng/mL). After 4 hours of incubation, the culture supernatant was removed and stored at -80°C for further analysis.

Laboratory Assays

High-sensitivity CRP was measured with a latex-enhanced immunonephelometric assays on a BN II analyzer (Dade Behring). The median normal value for CRP is 0.18 mg/dL, with 90% of normal values <0.3 mg/dL.

IL-6 was measured with a commercial assay kit (Quantikine human IL-6, R and D System). IL-6 measurements were performed in duplicate, and the intra- and interassay variability was <10%. The range of values detected by the assay was 3 to 300 pg/mL.

Total and differential white blood cell counts were obtained with a Bayer H*3 hematology analyzer by using automated cytochemistry in flow.

Statistical Analysis

Because CRP and IL-6 values do not follow a normal distribution, nonparametric tests were used (the Kruskal-Wallis test for comparisons between groups and the Friedman test and the Wilcoxon test with the Bonferroni correction for comparisons within groups). Correlations were determined with the Spearman rank correlation test. The remaining continuous variables were compared by using t tests for paired and unpaired variables, as appropriate. Proportions were compared with the χ^2 test. CRP and IL-6 values are expressed as medians and ranges; the remaining variables are expressed as mean±SD. P<0.05 (two-tailed) was considered statistically significant.

Results

Protocol A

During the 24 months of follow-up, 19 of 32 group 1 patients had CRP levels persistently >0.3 mg/dL (group 1A), and 13 group 1 patients had CRP levels persistently below this value (group 1B). Twelve group 1A patients (63%) had new coronary events (4 had an acute MI and 8 had a new CCU admission for recurrent UA), but no group 1B patients had coronary events (P<0.001). Demographic and clinical data for group 1A and group 1B patients are summarized in Table 1.

Sigma Chemical Co), which reflects the LPS concentration detected during clinical infections. After 4 hours of incubation, samples were removed, placed in ice to terminate the stimulation, and immediately processed. The plasma supernatant was removed and stored at -80°C for further analysis.
Persistent Elevation of IL-6 Plasma Levels in Patients With Recurrent UA

Plasma levels of IL-6 were undetectable in the 18 healthy volunteers (group 3), in 16 (89%) of 18 stable patients (group 2), and in 9 (69%) of 13 unstable patients with normal levels of CRP (group 1B), but they were elevated in 14 (74%) of 19 unstable patients with CRP levels \(>0.3\) mg/dL (group 1A) (Figure 1A).

Table 1. Demographic and Clinical Characteristics of UA Patients

<table>
<thead>
<tr>
<th></th>
<th>Group 1A</th>
<th>Group 1B</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>63±9</td>
<td>62±8</td>
<td>0.88</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>14:5</td>
<td>12:1</td>
<td>0.39</td>
</tr>
<tr>
<td>Risk factors, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history of IHD</td>
<td>11 (58)</td>
<td>6 (46)</td>
<td>0.77</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>7 (37)</td>
<td>3 (23)</td>
<td>0.66</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (16)</td>
<td>2 (15)</td>
<td>0.64</td>
</tr>
<tr>
<td>Hypertension</td>
<td>11 (58)</td>
<td>3 (23)</td>
<td>0.11</td>
</tr>
<tr>
<td>Smoking</td>
<td>12 (63)</td>
<td>7 (54)</td>
<td>0.67</td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrates</td>
<td>7 (37)</td>
<td>4 (31)</td>
<td>0.98</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>6 (32)</td>
<td>6 (46)</td>
<td>0.64</td>
</tr>
<tr>
<td>Calcium blockers</td>
<td>14 (74)</td>
<td>7 (54)</td>
<td>0.43</td>
</tr>
<tr>
<td>Aspirin</td>
<td>11 (58)</td>
<td>9 (69)</td>
<td>0.78</td>
</tr>
<tr>
<td>Other antiplatelet agents</td>
<td>6 (32)</td>
<td>2 (15)</td>
<td>0.53</td>
</tr>
<tr>
<td>Lipid-lowering agents</td>
<td>7 (37)</td>
<td>3 (23)</td>
<td>0.66</td>
</tr>
<tr>
<td>PTCA/CABG</td>
<td>11 (58)/5 (26)</td>
<td>4 (31)/6 (46)</td>
<td>0.25/0.43</td>
</tr>
<tr>
<td>Multi-vessel disease</td>
<td>10 (53)</td>
<td>8 (62)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Values are mean±SD, ratio, or number (percent).

Group 1A includes 19 patients with persistently elevated levels of CRP (>0.3 mg/dL); group 1B, 13 patients with normal levels of CRP. IHD indicates ischemic heart disease.

LPS-Induced Production of IL-6

Incubation of whole blood for 4 hours at 37°C in the absence of LPS induced a slight but significant increase of IL-6 in group 1A from 6.4 pg/mL (range, 0 to 28.1) to 4526 pg/mL (range, 3042 to 10 583) in group 1A (\(P<0.001\)) from 0 to 1752 pg/mL (range, 75 to 3981) in group 1B (\(P=0.002\)); from 0 to 707 pg/mL (range, 41 to 3275) in group 2 (\(P=0.002\)); and from 0 to 488 pg/mL (range, 92 to 3503) in group 3 (\(P<0.001\)).

LPS-stimulated production of IL-6 was markedly and significantly higher in patients with UA and CRP levels >0.3 mg/dL (group 1A) and not significantly different in the other groups (Table 2), independently of monocyte number (Figure 1B).

In the overall population of 68 subjects, IL-6 production induced by LPS was linearly correlated with baseline levels of CRP (\(r=0.42, P=0.005\)) and IL-6 (\(r=0.62, P<0.001\)) (Figures 2A and 2B). A linear correlation between LPS-stimulated production of IL-6 and circulating levels of CRP was also observed in the 43 subjects with CRP levels within the normal range, belonging to group 1B (13 patients), group 2 (14 patients), and group 3 (16 subjects) (\(r=0.47, P=0.015\); Figure 2C).

Protocol B

Although in our experimental condition monocytes were the only cells producing IL-6, we could not exclude the possibility that LPS might affect other cells in whole blood and that these cells in turn might activate monocytes. Therefore, we assessed the direct effects of LPS on isolated monocytes in a smaller study (protocol B).

The 14 UA patients included in this protocol were subgrouped according to CRP levels during the follow-up: 8 patients had CRP levels persistently >0.3 mg/dL (group 1A), and 6 patients had CRP levels persistently below this value (group 1B).

Figure 1. IL-6 production in response to LPS-challenge is enhanced in recurrent UA. A, Plasma levels of IL-6 were assessed in 4 groups of subjects: 19 patients with a history of UA, persistently elevated levels of CRP, and recurrent phases of instability during 24 months of follow-up (group 1A); 13 patients with a history of UA and normal levels of CRP who were free of symptoms during the follow-up period (group 1B); 18 patients with chronic SA (group 2); and 18 healthy volunteers (group 3). IL-6 levels were significantly higher in group 1A patients (\(P<0.001\), Kruskal-Wallis one-way ANOVA). No differences were observed among the other groups. B, IL-6 production by peripheral blood mononuclear cells was assessed after stimulation of 1 mL of whole blood with 1 ng of LPS for 4 hours. LPS-stimulated production of IL-6 was significantly higher in group 1A patients (\(P<0.001\), Kruskal-Wallis one-way ANOVA). No differences were observed among the other groups. IL-6 production was normalized for the number of monocytes per liter.
LPS-induced IL-6 production by isolated monocytes was significantly higher in group 1A (1931 pg/mL; range, 994.3 to 3863) than in group 1B (573.3 pg/mL; range, 97 to 1613), group 2 (468.3 pg/mL; range, 103.9 to 2248), and group 3 (325.8 pg/mL; range, 79.3 to 1332). The differences among the other groups were not significant (Figure 3).

Effects of CRP on LPS-Induced IL-6 Production
To investigate whether the increased circulating levels of CRP in group 1A themselves represent a sufficient explana-

### TABLE 2. Biological and Experimental Parameters

<table>
<thead>
<tr>
<th></th>
<th>Group 1A</th>
<th>Group 1B</th>
<th>Group 2</th>
<th>Group 3</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP, mg/dL</td>
<td>0.62 (0.34–3.3)</td>
<td>0.18 (0.06–0.27)</td>
<td>0.18 (0.06–0.32)</td>
<td>0.12 (0.01–0.33)</td>
<td>...</td>
</tr>
<tr>
<td>IL-6 plasma levels, pg/mL</td>
<td>6.4 (0–28.1)†</td>
<td>0 (0–12.4)</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 after LPS challenge, pg/mL</td>
<td>4526 (3042–10 583)‡</td>
<td>1752 (75–3981)</td>
<td>707 (41–3275)</td>
<td>488 (92–3503)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 after LPS challenge, monocytes/L</td>
<td>10.9 (5.9–21.6)‡</td>
<td>4.1 (11–7.4)</td>
<td>2.6 (0.88–11.3)</td>
<td>1.4 (0.21–9.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBC, 10⁹/L</td>
<td>7.5 (5.5–10.5)</td>
<td>6.7 (5.4–9.2)</td>
<td>5.8 (4.5–9.5)</td>
<td>5.9 (4.8–7.5)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Neutrophils, 10⁹/L</td>
<td>4.5 (3.3–6.96)</td>
<td>4 (3.4–5.5)</td>
<td>3.6 (2.4–6.9)</td>
<td>3.7 (2.1–4.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes, 10⁹/L</td>
<td>2 (1.2–3.2)</td>
<td>1.9 (1.2–3.1)</td>
<td>1.6 (1.1–2.4)</td>
<td>1.9 (1.2–2.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Monocytes, 10⁹/L</td>
<td>0.45 (0.27–0.86)</td>
<td>0.46 (0.29–0.81)</td>
<td>0.32 (0.22–0.52)</td>
<td>0.34 (0.15–0.47)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Eosinophils, 10⁹/L</td>
<td>0.12 (0.05–1.4)</td>
<td>0.14 (0.05–1.4)</td>
<td>0.1 (0.02–0.28)</td>
<td>0.1 (0.07–0.31)</td>
<td>NS</td>
</tr>
<tr>
<td>Basophils, 10⁹/L</td>
<td>0.06 (0.02–0.15)†</td>
<td>0.04 (0.01–0.09)</td>
<td>0.03 (0.01–0.06)</td>
<td>0.03 (0.02–0.16)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are presented as median (ranges). Group 1A includes 19 patients with UA and persistently elevated levels of CRP (>0.3 mg/dL); Group 1B, 13 patients with UA and normal levels of CRP; Group 2, 18 patients with SA; Group 3, 18 healthy volunteers. WBC indicates white blood cells.

*Group 1A versus group 2 and group 3.
†P<0.05 group 1A versus group 1B.
‡P<0.001 group 1A versus group 1B.

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Correlation between LPS-induced IL-6 production and plasma levels of CRP and IL-6. A and B, In the overall population of 68 subjects, IL-6 production in response to LPS (normalized for the number of monocytes per liter) was linearly correlated with plasma levels of CRP ($r=0.42$, 95% confidence interval [CI], 0.13 to 0.63; $P=0.005$) and IL-6 ($r=0.62$, 95% CI, 0.42 to 0.76; $P=0.001$). C, Linear correlation between LPS-stimulated production of IL-6 and plasma levels of CRP was also observed in the 43 subjects with CRP levels within the normal range ($r=0.47$, 95% CI, 0.17 to 0.66; $P=0.015$). The experimental conditions are described in Figure 1.
Our study shows that monocytes from patients with recurrent phases of UA and persistent elevation of acute phase reactants exhibit a greatly enhanced production of IL-6 in response to LPS challenge 6 months after the last acute event. The magnitude of this response is linearly related to circulating levels of CRP also persistently elevated in patients with history of UA and elevated levels of CRP and IL-6 may play a major role in determining the magnitude of the inflammatory reaction and clinical outcome.

Discussion

Figure 3. IL-6 production by isolated monocytes in response to LPS challenge. IL-6 production by $5 \times 10^5$ isolated monocytes was assessed after stimulation with 1 ng/mL LPS for 4 hours. No differences were observed among the other groups. Data are presented as medians, 25th and 75th percentiles (boxes), and 10th and 90th percentiles (whiskers). Group 1A: 8 patients; group 1B: 6 patients; group 2: 8 patients; group 3: 8 healthy volunteers.

As expected, LPS alone induced a significant increase of IL-6 to 1636 pg/mL (range, 72 to 4260 pg/mL); the LPS-induced IL-6 production assessed at this time point did not differ from the previous results in the same subjects (1295 pg/mL; range, 325 to 3503 pg/mL; $P=0.61$), demonstrating the reproducibility of our experimental results. The simultaneous addition of both CRP and LPS induced a significant further increase in IL-6 production only for the CRP dose of 50 $\mu$g/mL to 3418 pg/mL (range, 901 to 8753 pg/mL; $P=0.016$ versus LPS alone) (Figure 4B).

The study of LPS-induced IL-6 production in whole-blood cultures avoided possible artifacts related to cell isolation techniques and reduced the likelihood of contamination requiring minimal handling, while maintaining the integrity of the cellular interactions. In this experimental condition, monocytes are the only cells producing IL-6. However, to exclude the possibility that LPS might affect other cells in whole blood capable of activating monocytes, we designed a smaller study to assess the direct effects of LPS on isolated monocytes. Preliminary time-course studies have shown that IL-6 production increases very rapidly at 4 to 8 hours of stimulation and thereafter continues to rise at a slower rate, reaching a peak at 24 hours. In this study, whole blood samples, as well as isolated monocytes, were incubated for 4 hours with a low dose of LPS, which reflects LPS concentration detected during clinical infections.

LPS-stimulated IL-6 production in whole blood was, on average, 3 times higher in patients with history of UA and persistently elevated levels of CRP than in those with CRP levels in the normal range. It was linearly correlated with plasma CRP and IL-6 values, and the differences persisted after normalization for the number of circulating monocytes. Moreover, isolated monocytes from UA patients with high CRP exhibited a greatly enhanced response to LPS-challenge.

A genetically determined variability of response was reported for cytokine production by human monocytes after endotoxin stimulation in vitro and for inflammatory responses to oxidized lipoproteins in inbred mouse strains. Alternatively, monocytes and granulocytes of patients with recurrent phases of UA and elevated levels of CRP and IL-6 may be primed to produce more cytokines and reactive oxygen species in response to subliminal stimuli. Indeed, interferon (IFN)-$\gamma$, TNF-$\alpha$, and macrophage colony-stimulating factor (MCSF) induce a rapid priming of human monocyte functions. We have recently shown that UA patients are characterized by the expansion of an unusual subset of T cells committed to IFN-$\gamma$ production. The chronic up-regulation of IFN-$\gamma$ could lead to subsequent activation of monocytes/macrophages in the circulation as well as in tissue lesions.

Moreover, high doses of CRP induce a significant production of IL-1$\beta$, TNF-$\alpha$, and IL-6 and have a synergistic effect on LPS-dependent IL-1$\beta$ production by human peripheral blood monocytes. However, we found synergistically increased IL-6 production only when LPS was combined with...
CRP doses of 50 µg/mL, which are quite uncommon in UA patients. How circulating monocyte activation relates to inflammatory stimuli at the sites of atherosclerotic lesions and whether it reflects the local inflammation that predisposes to plaque instability or results from inflammation elsewhere in the body remain unknown. Oxidized-LDL or chronic infections may initiate and sustain local endothelial damage, leading to a low-grade chronic inflammatory condition.11,12,27 However, no association was found between cytomegalovirus, Helicobacter pylori, and Chlamydia pneumoniae serum antibody titers and acute MI and UA, and there is controversy about such association with chronic atherosclerotic syndromes.9,10,28–30

Finally, a polymorphism in the CD14 receptor on monocytes, an important mediator for monocyte activation by LPSs, was recently found to be associated with MI.29 Our findings concur, suggesting that an enhanced reactivity of monocytes to a variety of infectious and noninfectious stimuli may contribute to phases of coronary instability.

Clinical Implications
Although the mechanisms responsible for the association between CRP and acute coronary events remain speculative, an enhanced acute-phase responsiveness of circulating monocytes to low-grade inflammatory stimuli may help explain the greater predictive value of CRP than that of seropositivity to chronic infectious agents, oxidized LDL antibodies, and homocysteine. It may also help explain the long-term predictive value of CRP levels within the accepted normal range in low-risk individuals.6–8

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Enhanced Response of Blood Monocytes to In Vitro Lipopolysaccharide-Challenge in Patients With Recurrent Unstable Angina
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