Proinflammatory Effects of Bacterial Recombinant Human C-Reactive Protein Are Caused by Contamination With Bacterial Products, Not by C-Reactive Protein Itself

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Abstract—Intravenous administration to human volunteers of a commercial preparation of recombinant human C-reactive protein (CRP) produced in *Escherichia coli* was recently reported in this journal to induce an acute phase response of serum amyloid A protein (SAA) and of CRP itself, and to activate the coagulation system. The authors concluded that CRP is probably a mediator of atherothrombotic disease. Here we confirm that this recombinant CRP preparation was proinflammatory both for mouse macrophages in vitro and for mice in vivo, but show that pure natural human CRP had no such activity. Furthermore mice transgenic for human CRP, and expressing it throughout their lives, maintained normal concentrations of their most sensitive endogenous acute phase reactants, SAA and serum amyloid P component (SAP). The patterns of in vitro cytokine induction and of in vivo acute phase stimulation by the recombinant CRP preparation were consistent with contamination by bacterial products, and there was 46.6 EU of apparent endotoxin activity per mg of CRP in the bacterial product, compared with 0.9 EU per mg of our isolated natural human CRP preparation. The absence of any proinflammatory activity in natural CRP for macrophages or healthy mice strongly suggests that the in vivo effects of the recombinant preparation observed in humans were attributable to proinflammatory bacterial products and not human CRP. (*Circ. Res. 2005;97:e97-e103.*)

Key Words: C-reactive protein ■ inflammation ■ atherosclerosis ■ atherothrombosis cardiovascular disease

There is enormous current interest in the predictive association between the circulating concentration of C-reactive protein (CRP) and future coronary events. This followed our original description of such a relationship in patients with established coronary disease,1 and the subsequent finding by others that single baseline measurements of CRP are also significantly associated with risk of future coronary events in individuals in the general population. With increasing recognition that atherosclerosis and atherothrombosis are inflammatory processes, the capacity to precisely measure a sensitive systemic marker of inflammation has proved irresistible. Unfortunately, enthusiasm for what was widely perceived as an important new risk marker has swept away decades old knowledge of CRP and submerged critical analysis of both the epidemiological evidence and in vitro studies with CRP.

CRP, the classical acute phase protein, is an extremely sensitive nonspecific systemic marker of toxicity, inflammation, infection, and tissue damage.2,3 There are very few inflammatory and tissue damaging conditions in which CRP production does not increase, and there is no specific association of CRP with cardiovascular disease. CRP is an invariant protein that has long been assayed with high sensitivity,4,5 and there is no novel entity of high sensitivity CRP (hs-CRP) that is somehow specific for cardiovascular disease. Furthermore all other nonspecific systemic markers of inflammation that have been measured are similarly associated with future atherothrombotic events.6–8 With more than 7000 coronary events now analyzed in prospective studies in general populations, it has become clear that the strength of the association with CRP is notably less than was apparent from the smaller initial studies,8,9 and there are compelling arguments against both the rationale for, and utility of, measuring CRP as a risk marker in healthy individuals.10,11 Nevertheless, the intense focus on CRP has provoked pervasive speculation that the epidemiological association between CRP values and atherothrombotic events implies a causal relationship.12,13

The idea that CRP might contribute to the pathogenesis of atherosclerosis and atherothrombotic events is not new. We first suggested it more than 20 years ago when we discovered the specific binding of CRP to low density lipoproteins (LDL) and very low–density lipoproteins (VLDL),14,15 and it
is supported by the potentially proinflammatory capacity of human CRP to activate complement
16,17 and the presence of CRP in atherosclerotic plaques.18 However, many plasma proteins enter the plaques,
19,20 and presence at the scene of a crime is not proof of guilt or complicity; CRP in plaques could equally well have an atheroprotective function.21 Transgenic expression of human CRP in apoE knockout mice does not affect development or severity of atherosclerosis or the nature of the lesions,22 but because spontaneous atherothrombotic arterial occlusion does not occur in this model it is not informative about a possible role of CRP in atherothrombosis. A variety of proinflammatory and prothrombotic effects of human CRP preparations, mostly obtained from commercial suppliers, have been reported on cultured cells in vitro (reviewed in refs 12,13). However none of the preparations were properly characterized, nor did these studies include adequate controls. Bisoendial et al have now reported that intravenous infusion into human subjects of recombinant human CRP, produced in bacteria for in vitro use in CRP immunoassays, elicited a massive acute phase response and activated the coagulation system.23 Are these really the effects of human CRP?

Materials and Methods

CRP

Human CRP produced in recombinant Escherichia coli (Oriential Yeast Co Ltd, Shiga, Japan)24 was supplied in solution at 1 mg/mL in 140 mmol/L NaCl, 2 mmol/L CaCl2, 20 mmol/L Tris, 0.05% NaN3, pH 7.5 (BiosPacific). Our natural human CRP, purified from malignant ascitic fluid,25 and finally in solution at 140 mmol/L NaCl, 10 mmol/L Tris, 2 mmol/L CaCl2, 0.1% NaN3, pH 8.0, was >99% pure by SDS reduced 8% to 18% PAGE (50 μg sample loading, Brilliant blue R-350 stain, single band sensitivity limit ~0.1 μg). Both CRP preparations at 1 mg/mL, confirmed by latex enhanced immunoturbidimetric assay (Roche)26 and by BCA protein assay (Pierce), were extensively dialyzed against the same buffer; a fourth, applied by BiosPacific, this product after extensive dialysis, and natural human CRP preparations, mostly obtained from commercial sources, were properly characterized, nor did these studies include adequate controls. Bisoendial et al have now reported that intravenous infusion into human subjects of recombinant human CRP, produced in bacteria for in vitro use in CRP immunoassays, elicited a massive acute phase response and activated the coagulation system.23 Are these really the effects of human CRP?

Endotoxin Assay

Endotoxin was assayed, according to the 2005 European Pharmacopoeia monograph 2.6.14, by Limulus kinetic turbidimetric method (Cambrex), in pyrogen-free tubes with pyrogen-free water as diluent, at final CRP concentrations of 100, 10, and 1 μg/mL. Results in two separate assays were consistent.

Mice

Mice deficient respectively in Toll-like receptor 4 (TLR4), TLR2, and myeloid differentiation factor 88 (MyD88)27 were bred under specific pathogen-free conditions (Bundesinstitut für Risikobewertung, Berlin, Germany). Conventional female wild type C57BL/6 mice aged 7 weeks, C57BL/6 mice transgenic for human CRP,22 and their wild-type litter mates, were used for in vivo experiments.

Cell Lines, Reagents, and In Vitro Assays

Bone marrow–derived macrophages obtained respectively from wild-type, TLR2−/−, TLR4−/−, and MyD88-deficient mice, were seeded at 50,000 per well in 96-well plates in DMEM containing 10% FCS, 5% horse serum, 10 mmol/L Hepes, 1 mmol/L pyruvate, and 10 mmol/L L-glutamine at 37°C and 5% CO2.28 After adherence overnight, stimulation was compared in 200 μL fresh medium containing 10% FCS using highly purified Shigella flexneri lipopolysaccharide (LPS),29 bacterial lipopeptide, PamC3S-Lys-Lys (BLP; EM Microcollections), and the CRP preparations. Supernatants collected after 24 hours were frozen until assayed for murine TNF-α by ELISA (R&D Systems), with a lower detection limit of 10 pg/mL.

In Vivo Acute Phase Responses

Two days after bleeding for baseline values, 3 different groups of mice (n = 7 to 8) received intravenous injections of 100 μg of, respectively, commercial recombinant human CRP exactly as supplied by BiosPacific, this product after extensive dialysis, and natural human CRP dialyzed extensively against the same buffer; a fourth, control, group received buffer alone. All mice were bled 24 hours later for measurement of human CRP by latex enhanced immunoturbidimetric assay (Roche).26 mouse serum amyloid P component (SAP), and complement component C3 by electroimmunoassay,30,31 and serum amyloid A protein (SAA) by ELISA (BioSource UK, Nivelles, Belgium). An otherwise identical preliminary experiment but with 3 mice per group gave closely similar results.

Results

Authenticity of the CRP Preparations

The recombinant and the natural human CRP preparations were both composed entirely of intact native pentamers by analytical gel filtration chromatography and were 100% functionally intact by specific calcium-dependent binding to phosphocholine. In both preparations the CRP protomer mass was 23,028 Da by electrospray mass spectrometry. Soluble CD14 and LPS binding protein (LBP) were from Biometec GmbH.

Endotoxin Content of the CRP Preparations

Assays of the natural CRP, with acceptable recoveries (106.5 to 146.6%) of spiked endotoxin at all dilutions of CRP tested, detected 0.9080 endotoxin units (EU)/mg of CRP when tested at 100 μg/mL of CRP; nothing was detected at higher dilutions of CRP. Recovery of spiked endotoxin was also acceptable (126.7 to 138.3%) at all dilutions of recombinant CRP tested, but the apparent endotoxin content was substantial: 46.62 EU/mg of CRP at 100 μg/mL of CRP, 30.58 EU/mg of CRP at 10 μg/mL of CRP, and 15.13 EU/mg of CRP at 1 μg/mL of CRP. The falling apparent endotoxin content at increasing dilutions of CRP is bizarre as the endotoxin value per mg of CRP should remain constant regardless of dilution. Either interfering material is present, despite the acceptable recoveries observed, or the activity in the assay does not behave like authentic bacterial LPS. Whatever the explanation, there is a very striking difference from the natural human CRP preparation.
Transgenic Production of Human CRP Is Not Proinflammatory in Mice In Vivo

Male mice transgenic for human CRP, with median human CRP concentrations of ∼2 to 5 mg/L throughout their lives, maintained normal values of their exquisitely sensitive autologous acute phase proteins, mouse SAP, and SAA, apart from rare spikes caused by occasional fighting injury (Table 2).

Pure Natural Human CRP Is Not Proinflammatory In Vitro

LPS-induced TNF-α production is dependent on TLR4 whereas TLR2 induces TNF-α release in response to bacterial lipopeptides (BLP), and both pathways involve activation of NF-κB through the adaptor MyD88. Therefore TLR4−/− and MyD88−/− macrophages do not produce significant amounts of TNF-α in response to LPS, whereas TLR2−/− and MyD88−/− macrophages do not respond to BLP (Figure 1A).

TABLE 1. Effect of Intravenous Injection of Different Human CRP Preparations on Acute Phase Proteins in Mice

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|-----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Protein, mg/L                  | CRP (n=7) | SAP (n=7) | SAA (n=7) | CRP (n=7) | SAP (n=7) | SAA (n=7) | CRP (n=7) | SAP (n=7) | SAA (n=7) | CRP (n=7) | SAP (n=7) | SAA (n=7) |
| Control Buffer Only                  | Not detected | 3.22 (2.16, 3.98) | 2.78 (1.74, 3.22) | 2.56 (1.76, 4.40) | 0.39 (excluding control group) |
| Bacterial Recombinant CRP (n=7)     | 12.9 (12.5, 16.9) | 14.0 (11.0, 16.0) | 16.6 (11.8, 20.2) | 22.3 (16.8, 32.2) | 0.0009* |
| Dialyzed Bacterial Recombinant CRP (n=7) | 12.2 (7.3, 15.3) | 10.6 (7.1, 22.5) | 460.0 (319.2, 847.1) | 410.8 (248.4, 741.2) | 0.0001 |
| Difference in SAA (24 hours to baseline) | −2.0 (−7.9, 2.7) | −2.3 (−8.4, 11.2) | 443.7 (301.3, 830.5) | 391.4 (223.7, 709.0) | 0.0001 |
| SAP at baseline                  | 7.4 (6.6, 11.3) | 7.05 (5.6, 8.3) | 6.8 (5.0, 11.7) | 8.15 (6.0, 11.9) | 0.13 |
| SAP at 24 hours                  | 4.8 (4.2, 12.8) | 4.4 (2.3, 11.6) | 14.2 (13.1, 19.3) | 17.35 (12.5, 23.4) | 0.0001 |
| Difference in SAP (24 hours to baseline) | −2.5 (−7.1, 5.4) | −2.55 (−3.7, 3.3) | 7.8 (2.5, 10.3) | 9.35 (3.0, 13.6) | 0.0003 |
| C3 at baseline                  | 513 (457, 651) | 410 (292, 504) | 499 (355, 621) | 471 (443, 626) | 0.05+ |
| C3 at 24 hours                  | 611 (448, 778) | 343 (319, 494) | 523 (436, 576) | 471 (373, 669) | 0.002+ |
| Difference in C3 (24 hours to baseline) | 45 (−99, 321) | −65 (−145, 41) | 15 (−121, 192) | −34 (−214, 149) | 0.12 |

P values by Kruskal-Wallis test.

Table 2 Mouse SAP and SAA Values in Wild-Type and Human CRP Transgenic Mice

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|-----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Age (months)                      | 12 months | 14 months | 16 months | 18 months |
| Protein, mg/L                  | CRP | SAP | SAA | CRP | SAP | SAA | CRP | SAP | SAA | CRP | SAP | SAA |
| Wild-type (n=7)                  | Median | 0 | 6.3 | 6.7 | 0 | 4.2 | NA | 0 | 7.1 | NA | 0 | 8.9 | 12.5 |
|                                 | Range | 0–0 | 5.7–11.9 | 4.2–16.6 | 0–0 | 3.0–8.9 | 0–0 | 5.2–18.0 | 0–0 | 6.0–35.1 | 10.7–171.0 |
| hCRP transgenic (n=10)           | Median | 2.4 | 7.0 | 7.6 | 4.9 | 5.2 | NA | 1.5 | 7.1 | NA | 2.8 | 8.0 | 20.4 |
|                                 | Range | 0.7–9.1 | 5.2–10.3 | 1.4–16.2 | 1.9–79.3 | 3.6–90.2 | 0.7–10.2 | 4.1–12.7 | 0.8–14.2 | 5.3–47.9 | 10.7–141.7 |

NA indicates not assayed.

There were no significant differences between wild-type and human CRP transgenic mice with respect to SAP and SAA concentrations at any time point; range of P values (Mann–Whitney U test) at various ages, 0.52 to 0.77.
The recombinant CRP preparation elicited substantial production of TNF-α from both wild-type and TLR4−/− cells but not from TLR4−/− and MyD88−/− macrophages, regardless of whether or not it was dialysed before testing (Figure 1B). In marked contrast, our natural human CRP elicited no detectable production of TNF-α from any of the cells tested (Figure 1). NF-κB was activated in THP-1 cells by the recombinant CRP preparation in the presence of soluble CD14 and LBP, consistent with the presence of LPS, but the natural human CRP had no effect (Figure 2). Stimulation by the recombinant CRP was unaffected by dialysis before testing. HEK293 cells stably transfected with human TLR2 showed activation of NF-κB by synthetic BLP at doses as low as 0.1 ng/mL, but there was no response to either CRP preparation at any concentration up to 100 μg/mL (Figure 3). These results are all compatible with the presence of LPS but not BLP in the recombinant preparation. In other experiments, not shown here, the recombinant CRP also induced IL-1β production by human peripheral blood monocytes, whereas the natural CRP did not.

**Discussion**

Intravenous injection into mice of highly purified, structurally intact, and fully functionally active human CRP, isolated from human subjects, did not induce an acute phase response of either mouse SAP or SAA. In marked contrast, injection of the same quantity of CRP from the commercial bacterial recombinant preparation induced a dramatic increase in the
circulating concentration of both these proteins, compatible with the well known exquisitely sensitive murine acute phase response to endotoxin.\textsuperscript{31,34,35} The recombinant material also triggered proinflammatory cytokine and signaling responses in mouse macrophages in vitro, consistent with the presence of Gram-negative bacterial LPS in the preparation, whereas our natural human CRP had no effect in these assays. We did not detect bacterial lipopeptides in the recombinant preparation, but did not exclude the possible presence of bacterial peptidoglycans. Formal in vitro testing for endotoxin by the Limulus kinetic turbidimetric assay detected just 0.9 EU/mg of CRP in the natural human CRP preparation, compared with 46.6 EU/mg in the recombinant product. However the activity in the commercial material behaved anomalously on dilution, and it was not possible to assign an estimate of endotoxin content compatible with European Pharmacopoeia guidelines. Bisoendial et al report that the Limulus assay detected $<$1.5 EU/mL in their material but they do not state at what CRP concentration this result was obtained.\textsuperscript{23} Because both the recombinant and the natural human CRP molecules were structurally and functionally intact, and were indistinguishable from each other, the acute phase response and transient neutrophilia observed in humans by Bisoendial et al\textsuperscript{23} are very unlikely to have been caused by CRP itself. These effects are consistent with contamination by bacterial or other extraneous material, despite the apparent lack of pyrogenicity or increased TNF-$\alpha$ production. The present completely negative result with authentic highly purified human CRP places the onus on others to prove that the effects observed with recombinant bacterial or other preparations are really attributable to CRP itself.

Bisoendial et al\textsuperscript{23} subjected their material to a single gel filtration chromatography step to purify it before intravenous injection, but they showed that thereafter it still remained proinflammatory both to cells in vitro and to mice as well as humans in vivo. This is not surprising because LPS, in particular, is known to bind avidly and nonspecifically to proteins and surfaces, so that once present as a contaminant it is exceptionally difficult to remove from biological systems. Indeed in our experiments, extensive dialysis against physiological buffer, which is likely to be more effective than a single gel filtration step for removal of low molecular weight solutes, had no effect on the stimulatory activity of the recombinant CRP preparation either in vitro or in vivo. The recombinant CRP provided by BiosPacific is manufactured by the Oriental Yeast Company in Japan, and, to our knowledge, this is the only commercial source of bacterial recombinant human CRP. CRP released by the recombinant \textit{E. coli} is isolated from the culture medium by nonspecific calcium-dependent affinity chromatography on EACA-Sepharose.\textsuperscript{24} Although this captures the human CRP, it would be astonishing if all bacterial proinflammatory products were removed, and we show here that they definitely are not. This does not matter for the intended in vitro use of the product as a calibrator for CRP immunoassays, but it is critical for experiments on living cells in culture and evidently even more important when administered in vivo. Furthermore, different batches of the recombinant CRP might be variably contaminated with different proinflammatory bacterial products, leading to varying biological effects. The present results are consistent with recent evidence that most of the proinflammatory activities ascribed to CRP, on the basis of in vitro studies with commercial CRP preparations, are caused by either contamination with LPS or by failure to remove the sodium azide that is universally present as a preservative.\textsuperscript{25,36–41} In particular van den Berg and colleagues have shown that CRP isolated carefully from human malignant ascites or produced recombinantly in mammalian cells had no proinflammatory effects on endothelial cells, and that these effects of the bacterial recombinant product could all be replicated by addition of either LPS or azide.\textsuperscript{41} These results, together with the present findings, also demonstrate that earlier observations of cytokine induction by preparations of natural human CRP in the prerecombinant era, for example Ballou et al,\textsuperscript{42} must have been caused by contamination with proinflammatory substances other than CRP.

Human CRP concentrations encompass a 10 000-fold dynamic range from $\approx$0.05 to $\approx$500 mg/L, usually reaching a peak around 24 to 48 hours after a single acute phase stimulus.\textsuperscript{3,5,43} This behavior, which has been known for decades, does not suggest a role for CRP in modulation of systemic inflammatory, vascular, or coagulation responses. Patients with many different acute and chronic diseases display the whole range of CRP concentrations without associated coagulopathy, vascular dysregulation, or global upregulation of inflammation. Although CRP and SAA values generally correlate, there can be substantial divergence between them\textsuperscript{44} that is inconsistent with the idea that CRP itself triggers SAA production as proposed by Bisoendial et al.\textsuperscript{23} Also, when the stimulus for acute phase CRP production terminates, for example, after acute myocardial infarction\textsuperscript{41} or with successful antimicrobial treatment of sepsis,\textsuperscript{45–47} the plasma CRP concentration falls exponentially with a half time of $\approx$24 hours, which is almost as fast as the rate of CRP clearance from the circulation.\textsuperscript{48} There is never a secondary biphasic spike in CRP concentration as would be expected if CRP itself were a trigger for its own production as suggested by Bisoendial et al\textsuperscript{23}

Transgenic expression of human CRP evokes no acute phase response in mice (Table 2) and intravenous or subcutaneous injection into rats of 40 mg/kg of pure natural human CRP affects neither acute phase proteins (unpublished observations) nor blood pressure.\textsuperscript{25} In sharp contrast, despite the lack of clinical symptoms or major signs other than transient neutrophilia, the bacterial recombinant CRP preparation induced a massive acute phase response in humans. The SAA values in the range of 50 to $>$2000 mg/L\textsuperscript{23} are comparable to the SAA responses to severe sepsis, acute myocardial infarction, major surgery, and massive trauma. SAA is the precursor of AA type amyloid fibrils which cause the serious and often lethal disease of AA amyloidosis that complicates chronic inflammatory diseases.\textsuperscript{49} It would be highly undesirable if the therapeutic use of a bacterial recombinant protein incurred the risk of such a grave complication, and activation of coagulation is also potentially harmful. The observations of Bisoendial et al therefore suggest that in addition to pyrogenicity, safety screening of recombinant proteins for
human administration should include testing for acute phase responses.

The present demonstration that natural human CRP is not proinflammatory in vivo in healthy animals does not conflict with the compelling evidence that human CRP can contribute significantly to the severity of ischemic tissue damage,\(^{50,51}\) and possibly also other forms of tissue injury associated with major acute phase responses in diseased subjects. The presence in damaged tissues of ligands for CRP, coupled with its greatly increased concentration, evidently enables CRP to exert pathogenic effects that are absent in healthy undamaged individuals. Human CRP is thus a valid therapeutic target, as we originally suggested,\(^{50}\) but the only responsible basis for development and application of CRP-inhibitor treatments must be rigorous evidence specifically implicating CRP itself in pathogenesis.

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