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

Mark B. Pepys, Philip N. Hawkins, Melvyn C. Kahan, Glenys A. Tennent, J. Ruth Gallimore, David Graham, Caroline A. Sabin, Arturo Zychlinsky, Juana de Diego

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, 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. 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, 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. 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, and there are compelling arguments against both the rationale for, and utility of, measuring CRP as a risk marker in healthy individuals. Nevertheless, the intense focus on CRP has provoked pervasive speculation that the epidemiological association between CRP values and atherothrombotic events implies a causal relationship.

The idea that CRP might contribute to the pathogenesis of atherosclerosis and atherothrombosis 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), 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. Bisoeidential 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 (Oriental Yeast Co Ltd, Shiga, Japan)24 was supplied in solution at 1 mg/mL in 140 mM NaCl, 2 mM L-CaCl2, 20 mM Tris, 0.05% NaN3, pH 7.5 (BiosPacific). Our natural human CRP, purified from malignant ascitic fluid,25 and finally in solution in 140 mM NaCl, 10 mM Tris, 2 mM 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 batch of 140 mM NaCl, 10 mM Tris, 2 mM L-CaCl2, pH 8.0, to remove azide and any other dialyzable substances, and their structural and functional integrity were confirmed by gel filtration on Superose-12 (Amersham), calcium dependent binding to immobilized phosphocholine, and electrospray mass spectrometry.

Endotoxin Assay
Endotoxin was assayed, according to the 2005 European Pharmacopeia 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 mM L-HEPES, 1 mM L-pyruvate, and 10 mM 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 lipopoly saccharide (LPS),29 bacterial lipopeptide, PamCysSerLys4 (BLP; EMC 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. The NF-κB–dependent luciferase reporter assay30 used human embryonic kidney (HEK) 293 cells that stably express human TLR2 or that had been transfected with empty vector as a control. The THP-1–IκB enhanced green fluorescent protein (EGFP) cell line was generated by stable transfection of THP-1 with the pIκB–EGFP expression cassette (Clontech) and selected for neomycin resistance at a final concentration of 0.5 µg/mL. A stable population of cells was activated by fluorescence activated cell sorting. THP-1–IκB–EGFP cells remained fluorescent when the NF-κB pathway was inactive whereas degradation of IκB–EGFP in response to NF-κB activators was quantifiable by fluorescence activated cell sorting analysis. Soluble CD14 and LPS binding protein (LBP) were from Biometec GmbH.

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 diazylized 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.40

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.
The residual concentrations of human CRP were as expected for the control, were injected intravenously into each mouse 48 hours after bleeding for baseline values. After 24 hours each animal was bled for assay of human CRP and of P.

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

<table>
<thead>
<tr>
<th>CRP at 24 hours</th>
<th>Control Buffer Only</th>
<th>Bacterial Recombinant CRP (n=7)</th>
<th>Dialyzed Bacterial Recombinant CRP (n=7)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP at 24 hours</td>
<td>Not detected (n=7)</td>
<td>3.22 (2.16, 3.98)</td>
<td>2.78 (1.74, 3.22)</td>
<td>2.56 (1.76, 4.40)</td>
</tr>
<tr>
<td>SAA at baseline</td>
<td>12.9 (12.5, 16.9)</td>
<td>14.0 (11.0, 16.0)</td>
<td>16.6 (11.8, 20.2)</td>
<td>22.3 (16.8, 32.2)</td>
</tr>
<tr>
<td>SAA at 24 hours</td>
<td>12.2 (7.3, 15.3)</td>
<td>10.6 (7.1, 22.5)</td>
<td>460.0 (319.2, 847.1)</td>
<td>410.8 (248.4, 741.2)</td>
</tr>
<tr>
<td>Difference in SAA (24 hours to baseline)</td>
<td>-2.0 (-7.9, 2.7) (n=6)</td>
<td>-2.3 (-8.4, 11.2)</td>
<td>443.7 (301.3, 830.5)</td>
<td>391.4 (223.7, 709.0)</td>
</tr>
<tr>
<td>SAP at baseline</td>
<td>7.4 (6.6, 11.3)</td>
<td>7.05 (5.6, 8.3)</td>
<td>6.8 (5.0, 11.7)</td>
<td>8.15 (6.0, 11.9)</td>
</tr>
<tr>
<td>SAP at 24 hours</td>
<td>4.8 (4.2, 12.8)</td>
<td>4.4 (2.3, 11.6)</td>
<td>14.2 (13.1, 19.3)</td>
<td>17.35 (12.5, 23.4)</td>
</tr>
<tr>
<td>Difference in SAP (24 hours to baseline)</td>
<td>-2.5 (-7.1, 5.4) (n=7)</td>
<td>-2.55 (-3.7, 3.3)</td>
<td>7.2 (2.5, 10.3)</td>
<td>9.35 (3.0, 13.6)</td>
</tr>
<tr>
<td>C3 at baseline</td>
<td>513 (457, 651)</td>
<td>410 (292, 504)</td>
<td>499 (355, 621)</td>
<td>471 (443, 626)</td>
</tr>
<tr>
<td>C3 at 24 hours</td>
<td>611 (448, 778)</td>
<td>343 (319, 494)</td>
<td>523 (436, 576)</td>
<td>471 (373, 669)</td>
</tr>
<tr>
<td>Difference in C3 (24 hours to baseline)</td>
<td>45 (-99, 321)</td>
<td>-65 (-145, 41)</td>
<td>15 (-121, 192)</td>
<td>-34 (-214, 149)</td>
</tr>
</tbody>
</table>

Preparations containing 100 μg of the different human CRP preparations shown, in solution in Tris-buffered saline containing calcium, or this buffer only as a control, were injected intravenously into each mouse 48 hours after bleeding for baseline values. After 24 hours each animal was bled for assay of human CRP and murine SAP and SAA. The residual concentrations of human CRP were as expected for the ~4 hour plasma half life of human CRP in mice.52

P values by Kruskal-Wallis test.

*Although the baseline SAA values were significantly different between the groups, the absolute difference was trivial and they were all within the normal range.

†Baseline and 24 hour C3 values differed between groups but no treatment produced a significant positive or negative acute phase response.

### Pure Natural Human CRP Is Not Proinflammatory In Vivo

There was no increase in concentration of mouse SAP, SAA, or C3 at 24 hours after intravenous injection into mice of pure natural human CRP (Table 1), at the time when the acute phase responses of these proteins to a single inflammatory or toxic stimulus is usually maximal.31,33 The ≈4.00 mg/kg dose of CRP was >3-fold higher than given to human subjects by Bisoendial et al,23 but it did not provoke any acute phase response and its effect was indistinguishable from injection of buffer alone (Table 1). In dramatic contrast, injection of this same dose of recombinant CRP, whether or not dialyzed beforehand, induced a highly significant 2- to 5-fold rise in mouse SAP and a 20- to 40-fold rise in mouse SAA (Table 1). This startling acute phase response is comparable to that produced in humans by the putatively “highly purified” recombinant CRP preparation of Bisoendial et al.23

### 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 injury22 (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 2 Mouse SAP and SAA Values in Wild-Type and Human CRP Transgenic Mice

<table>
<thead>
<tr>
<th>Age</th>
<th>12 months</th>
<th>14 months</th>
<th>16 months</th>
<th>18 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, mg/L</td>
<td>CRP</td>
<td>SAP</td>
<td>SAA</td>
<td>CRP</td>
</tr>
<tr>
<td>Wild-type (n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>6.3</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>Range</td>
<td>0–0</td>
<td>5.7–11.9</td>
<td>4.2–16.6</td>
<td>0–0</td>
</tr>
<tr>
<td>hCRP transgenic (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>2.4</td>
<td>7.0</td>
<td>7.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Range</td>
<td>0.7–9.1</td>
<td>5.2–10.3</td>
<td>1.4–16.2</td>
<td>1.9–79.3</td>
</tr>
</tbody>
</table>

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 TLR2−/− 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.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.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 al23 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-α 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 al23 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 E. coli is isolated from the culture medium by nonspecific calcium-dependent affinity chromatography on EACA-Sepharose.24 Although this captures the human CRP, it would be astonishing if all bacterial proinflammatory products were clear from each other, the acute phase response and transient neutrophilia observed in humans by Bisoendial et al23 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-α 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.

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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.

Acknowledgments

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