Rapid Automated High Sensitivity Enzyme Immunoassay of C-Reactive Protein, Julie Wilkins, 1 J. Ruth Gallimore, 2 Edwin G. Moore, 1 and Mark B. Pepys 2*

The concentration of C-reactive protein (CRP), the classic acute phase plasma protein, increases rapidly in response to most forms of tissue injury, infection, and inflammation (1–3). CRP is secreted only by hepatocytes, where its synthesis is regulated by cytokines, of which IL-6 is the most important. The t₁/₂ of CRP in the circulation (~19 h) is independent of the plasma concentration and is remarkably constant in all conditions (4). Thus, the only determinant of the plasma concentration is the rate of CRP production, which usually reflects closely the extent and activity of the current pathology. Furthermore, the CRP response is very sensitive, and although it is nonspecific and can never on its own be diagnostic, it provides an extremely useful objective marker in clinical practice, both in screening for organic disease and in monitoring disease activity and response to therapy (3).

CRP concentrations in ostensibly healthy subjects have a very skewed distribution. In the first study with a sufficiently sensitive radioimmunoassay to detect CRP in all nondiseased sera tested, the median value was 0.58 mg/L (range, 0.068–8.2 mg/L) among 153 healthy blood donors (5). In our own original study of 468 volunteer blood donors, using the same 100% pure CRP as standard that was subsequently used to calibrate the World Health Organization International Standard for Human CRP (85/506) (6), the median value was 0.8 mg/L, and there was a tail of higher values, with the 90th percentile at 3 mg/L and the 99th percentile at 10 mg/L (7). In a more recent study based on the WHO standard and examining 143 apparently healthy blood donors, the median CRP value was 0.64 mg/L, and the 97.5th percentile was 3.11 mg/L (8). Routinely available immunochemical assay methods for plasma proteins have limited sensitivity, and until recently, CRP concentrations below ~10 mg/L could not be measured precisely, leading to widespread adoption of this value, or even higher, as the upper limit of the health-associated reference range. This is satisfactory for some purposes in general medicine because the marked acute phase responses that characterize bacterial infections, ischemic necrosis of tissue, and most active inflammatory conditions usually lead to much greater CRP values, sometimes reaching hundreds of milligrams per liter. However, in neonatal pediatric practice, a high-sensitivity CRP immunoassay shows that health-associated reference values are below 1–2 mg/L and that any rise above such values is associated with serious disease, usually bacterial infection (9).

More recently, application of sensitive CRP assays to studies of adult cardiovascular disease has revealed important prognostic relationships between modest increases of CRP and the occurrence, progression, and thrombo-occlusive complications of atherosclerosis. This was first demonstrated, using the present assay, in patients admitted to hospital with severe unstable angina (10) and in the European Concerted Action on Thrombosis and Disabilities (ECAT) Angina Pectoris Study (11, 12). Our findings have subsequently been widely confirmed and extended (13–18). Remarkably, the association extends even to apparently healthy asymptomatic individuals. These observations clearly have profound implications for risk assessment, for prophylaxis, and for routine clinical management of coronary heart disease. They also open new avenues for exploration of the pathogenesis of atheroma and its complications.

The present high-sensitivity automated enzyme immunoassay for CRP was developed specifically to measure the large number of samples in the ECAT study (11, 12). It has also been used for research in osteoarthritis (19) and neonatal infection, as well as for other coronary heart disease studies (20, 21). The speed and flexibility of the immunoassay should facilitate additional research and also the practical clinical applications of these important new findings.

CRP was isolated and purified as previously described (4), and 100% pure material, used as the reference analyte, was standardized on the WHO Standard (85/506) (6) (prepared at the Royal Postgraduate Medical School and available from the National Institute for Biological Standards and Control, Herts EN6 3QG, UK). Mouse monoclonal anti-CRP antibodies were raised against the 100% pure CRP; 95% pure CRP, diluted in Tris buffer, pH 7.5, with stabilizers and preservatives, was used for calibrators and controls.

The IMx™ (Abbott Laboratories) (22) CRP research assay is an automated microparticle capture enzyme immunoassay (MEIA). Two mouse anti-CRP monoclonal antibodies (Abbott Laboratories) were used, one immobilized on microparticles (Seradyn, Indianapolis, IN), the other conjugated to alkaline phosphatase (23). The IMx carousel containing 24 reaction cells was loaded with 20 samples consisting of sera diluted 1:50 with MEIA buffer. A zero blank and three controls containing low, medium, and high concentrations of CRP at 1, 5, and 20 mg/L,
respectively, were also included. The microparticles, conjugate, substrate, and MEIA buffer were placed in the instrument with the loaded carousel; the assay was then run automatically, yielding results after 30 min. Sample concentration is determined from a stored 6-point calibration curve, values 0, 0.5, 2.5, 10, 30, and 60 mg/L, referenced to pure CRP.

Sera were routinely assayed at dilutions of 1:50, and the detection limit was determined as 0.05 mg/L CRP, based on the value for 3-SD above the mean signal obtained in 10 replicates of a zero sample, consisting of MEIA buffer with 10 g/L casein and 2 mmol/L CaCl₂. To confirm this, 10 replicates of the zero CRP calibrator and of a 0.05 mg/L CRP calibrator were tested. The 0.05 mg/L calibrator gave a mean value of 0.054 mg/L, with 95% confidence limits of 0.0432–0.0648, compared with the zero calibrator, with a mean of 0.014 mg/L (0.0032–0.0248). This difference was highly significant, $P = 0.0013$ (Bonferroni t-test), and the IMx assay can thus detect this concentration without overlapping the zero calibrator. Sera containing 30 mg/L or more gave CVs of the IMx readout >10% and were, therefore, re-run at higher dilutions. When samples of acute phase serum or the purified CRP used as calibrators and controls, were diluted 1:5–1:400 with wash solution, linear regression analysis of the CRP results yielded $r = 0.995$ and slope = 1.050. When 10 replicate assays of the three purified CRP controls, low (CRP = 1 mg/L), medium (CRP = 5 mg/L), and high (CRP = 20 mg/L), were run on six successive days, the intra- and interassay CVs, respectively, were as follows: low, 6.3% and 6.0%; medium, 3.5% and 1.9%; and high, 5.3% and 3.3%. A set of calibrators was also run once on each of 9 separate days and gave CVs of 8.7% for the 0.05 mg/L calibrator and ≤5% for the other calibrators; 2.5, 10, 30, and 60 mg/L. No interference was detected in lipemic, icteric, or hemolyzed sera or samples containing rheumatoid factor. Serum and citrated plasma from the same blood sample gave identical results.

Purified CRP was added to two sera containing, respectively, 0.5 and 6 mg/L of CRP, to increase the concentration by 1, 2, 4, and 8 mg/L; the sera were then re-assayed in two separate assay runs. For the serum with 0.5 mg/L, the mean recovery (SD) was 100% (14%), and for the serum with 6 mg/L, it was 107% (16%). No difference was observed in assay performance when calibrators, controls, and antibody reagents were tested before and after incubation for 7 days at 37 °C or for 3 days at 45 °C. After storage of all these materials at 4 °C for 5 months or at −20 °C for 16 months, re-assayed serum samples gave results that were 96% of their original values. Two single batch assays of a set of 10 sera, at an interval of 25 months, gave essentially identical results.

Serum samples (CRP concentration range, 0.1–234 mg/L; n = 130) from renal transplant patients were measured in parallel in the IMx assay and the Abbott TD™ assay. Comparison of the results by the Bland-Altman method gave a mean difference (TDx − IMx) of 9.63 with 95% limits of agreement of −15.79 to 35.05, reflecting in part the known poor precision of the TDx method at low concentrations. A separate set of clinical samples with CRP concentrations up to 372 mg/L (n = 97) were compared in the IMx and Beckman methods. Interestingly, 18 sera were recorded as <6 mg/L in the Beckman method and had values in the IMx in the range <0.05–11 mg/L. An additional sample with a Beckman value of 11 mg/L contained <0.05 mg/L in the IMx. These findings reflect the known limited sensitivity and poor low range precision of direct light-scattering immunoassays. Values in the remaining 78 samples were log-transformed for comparison by the Bland-Altman method because plots of the differences increased as the size of the measurements increased. The mean difference expressed as the ratio Beckman/IMx was 0.85, with 95% limits of agreement of 0.44 to 1.64.

Comparisons were also made with two in-house sensitive immunoassays capable of detecting CRP within the same range as the IMx. A series of 359 samples with values between 0.05 and 19.4 mg/L were measured in parallel in our Sepharose-phosphoethanolamine ligand-binding monoclonal antibody radiometric assay (9). A separate series of 80 samples with values between 0.05 and 8.4 mg/L was measured in a microtiter plate immunoradiometric assay with immobilized polyclonal and labeled monoclonal antibodies. Both data sets were log-transformed for Bland-Altman comparison and gave mean ratios as follows: ligand-binding/IMx = 1.05, 95% limits of agreement, 0.47–2.35; immunoradiometric/IMx = 1.04, with 95% limits of agreement, 0.54–2.00.

Among 100 healthy subjects whose sera were stored at −20 °C before assay in a single batch, the median CRP value measured in the IMx was 0.7 mg/L; the range was 0.1–8.9 mg/L; the 90th percentile was at 3 mg/L; and the 97.5th percentile was 4.5 mg/L (Fig. 1A). These results are very close to those previously published (5, 7, 8). Serial monthly measurements for 10 months in 10 healthy individuals, whose samples were stored at −20 °C and assayed in a single batch, demonstrated CRP concentrations clustered on most occasions around a typical low value characteristic for each subject, with occasional higher outliers (Fig. 1B). These presumably reflect intercurrent minor acute phase responses to silent or subclinical pathological events and account for the 10% of values above 3 mg/L regularly seen in cross-sectional studies. The remaining values were remarkably constant in each subject and clustered around a value characteristic for that individual. On the basis of this and very similar results obtained by Macy et al. (8), it is likely that subjects with CRP values persistently >3 mg/L are mounting a substantial acute phase response to some underlying pathological process. Indeed, 3 of the 26 individuals followed for 6 months by Macy et al. (8) are probably in this group, and they may fall into the higher risk group for coronary heart disease identified in recent studies (16–18).

The recent descriptions of the prognostic relationship of increased CRP production to coronary heart disease indicate that sensitive CRP assays are now a necessary part of the investigation and management of patients with ischemic heart disease. More work is required to establish how often and at what intervals CRP should be measured.
to provide the relevant information, but the present serial data and those of Macy et al. (8) suggest that three assays at monthly intervals may be sufficient to characterize an individual’s typical “normal” range, provided there is no overt intercurrent pathology. The capacity to measure CRP on the widely available IMx instrument will facilitate development of this new aspect of acute phase protein measurement. However, it is essential to recognize that the CRP response is nonspecific and that, if low-level acute phase responses are to be interpreted clinically with respect to a particular disorder, then intercurrent acute phase-stimulating processes must be sought and identified. In the absence of confounding pathology, modestly increased CRP production is potentially of considerable clinical importance. For example, it has confirmed the existence of an inflammatory component in osteoarthritis and identified patients whose osteoarthritis is more likely to progress (19), findings that may have important therapeutic implications.

The IMx assay detects and precisely quantifies a CRP concentration of 0.05 mg/L, which is below the lowest value observed in previous large scale studies of ostensibly healthy adult subjects (5,7), although Macy et al. (8) report a lowest value of 0.02 mg/L. CRP concentrations in nondiseased cord blood and in healthy neonates are lower, but the present cutoff is sufficiently low to include all clinically useful information in this group of patients (9). The clinical need for highly sensitive CRP assay was first recognized in neonatal pediatric practice (9). Although the CRP acute phase response to infection is as vigorous in infants as it is in adults, the speed with which bacterial disease can overwhelm neonates is such that there is often not time for the CRP concentration to reach values that can be detected in routine systems. Furthermore, CRP values always rise, usually by >10-fold, during infective episodes, even when they do not exceed the health-associated reference range (9).

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References

Fig. 1. CRP concentrations.
(A) Frequency distribution of CRP concentrations in sera from 100 healthy adults. (B) Serum CRP concentration in monthly samples over 10 months in 10 healthy subjects.


