Rapid Automated Enzyme Immunoassay of Serum Amyloid A

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Serum amyloid A (SAA), a sensitive acute-phase protein, is the precursor of AA fibrils in reactive amyloidosis. However, SAA is poorly immunogenic, and development and standardization of immunoassays of this protein have been difficult. We established an automated polyclonal/monoclonal microparticle capture enzyme immunoassay, standardized with the World Health Organization prospective reference standard for SAA. A stabilized concentrate of SAA was used for controls and calibrators. The assay range was 1–750 mg/L with CVs <7% throughout. Plasma and serum gave identical results and no interferences were observed. Linear regression against radial immunodiffusion assay gave a slope of 1.04 (95% confidence interval 0.99–1.10), intercept of −9 mg/L (95% confidence interval −14–3), and residual SD (SEE) of 20 for samples containing <200 mg/L (n = 173). In 105 apparently healthy adults the mean (SD) SAA concentration was 3.7 (3.6) mg/L, the median was 3.0 mg/L, and the range, 0.7–26.4 mg/L. In clinical acute-phase sera, values up to 2200 mg/L were seen. This method will facilitate measurement and investigation of SAA in clinical practice generally and in AA amyloidosis.

Indexing Terms: acute-phase proteins/amyloidosis/lipoproteins/apolipoproteins/C-reactive protein/reference material

Serum amyloid A (SAA) is an apolipoprotein of high-density lipoprotein (HDL) particles and is the polymorphic product of a set of genes located on the short arm of chromosome 11.5 SAA is highly conserved in evolution and is a major acute-phase reactant in all species in which it has been studied. Most of the SAA in plasma is synthesized in hepatocytes, under transcriptional regulation by cytokines, especially interleukin-1, interleukin-6, and tumor necrosis factor, acting via nuclear factor κB-like (and possibly other) transcription factors (1). After secretion, SAA rapidly associates with HDL, from which it displaces apolipoprotein AI (apoAI). Circulating concentrations of SAA can increase from normal amounts (~3 mg/L) to >1000 mg/L within 24–48 h of an acute stimulus; with ongoing chronic inflammation the concentration may remain high (2, 3). Certain isoforms of SAA, the products of different genes, are predominantly synthesized by macrophages, adipocytes, and other cells. Although these isoforms also associate with HDL, their acute-phase synthesis is stimulated differently and presumably they have different functions (4). There is also a closely related family of trace apoproteins of HDL that are not acute-phase reactants (5).

In most circumstances the serum concentration of SAA correlates with that of the classical and highly sensitive acute-phase reactant, C-reactive protein (CRP) (3), but SAA reaches higher concentrations and may respond more rapidly. The cytokine regulation of SAA production is also different from that of CRP, and the differential acute-phase responses of SAA and CRP to different stimuli may be clinically important (6–10). Most of the routinely available commercial assays for CRP detect only amounts >5 mg/L, whereas 50% of healthy subjects have CRP concentrations in the range of 0.07–0.8 mg/L, and 90% have <3 mg/L (11). Thus the concentration of CRP may increase up to 100-fold before this analyte is detected, let alone recorded as abnormal (12). A rapid, convenient immunoassay for SAA, covering its whole range from normal to peak acute-phase concentrations, would therefore be useful in clinical monitoring of the acute-phase response.

Circulating SAA is the precursor of amyloid A (AA), which forms the fibrils deposited in the tissues in reactive amyloidosis, complicating chronic inflammatory and infective conditions (13). Amyloidosis is a serious and usually fatal condition for which there is no specific therapy. Sustained increases in the production of SAA, with high circulating concentrations of the protein, are a necessary, though not sufficient, condition for development of AA amyloidosis. In patients with established AA amyloidosis, persistently high values of SAA are associated with progression of amyloid deposition, whereas regression can occur when SAA production is reduced to normal by spontaneous or therapeutically induced remission of the primary disease (14, 15). Thus the capacity to monitor serum SAA concentrations frequently and precisely could make a significant contribution to the management of patients with AA amyloidosis.

To date, studies of SAA have been confined to research laboratories, with variation in methodology and sample processing, and no uniformity in standardization. Assays for apolipoproteins are difficult to standardize because of the heterogeneity of lipoprotein particles, the insolubility in aqueous media of isolated apoproteins, and the variation in their epitope expression under different physiochemical conditions. We report here
the development on the Abbott IMx™ (Abbott Labs., N. Chicago, IL) of an automated enzyme immunoassay that is rapid, sensitive, precise, and reproducible, and overcomes the previously existing problems in clinical assays of SAA. Furthermore, at the invitation of the World Health Organization (WHO), we have produced a candidate material for an International Reference Standard for immunoassay of SAA, in which the SAA content has been precisely determined in mg/L, and which is about to undergo an international collaborative study for its validation. Our new assay has been standardized against this material.

Materials and Methods

Acute-phase serum, HDL, and SAA. Serum was obtained from patients 24–48 h after cardiac surgery. Each coded sample was tested for hepatitis B surface antigen and for anti-human immunodeficiency virus-1 and -2 antibodies with Food and Drug Administration-approved ELISAs (Abbott Labs.), and all gave negative results. A pool of ~2000 mL was constructed, the bulk of which was supplied to the National Institute for Biological Standards and Controls, Potters Bar, UK, for lyophilization and use as the candidate WHO International Reference Standard for SAA immunoassay, and a portion was retained as the primary standard for SAA immunoassay. HDL was isolated from the remainder by sequential flotation ultracentrifugation (16). Some of this acute-phase HDL was delipidated and the apolipoproteins were fractionated by gel filtration chromatography on a column of Sephacryl™ S-200 HR (Pharmacia Biotech, St. Albans, Herts, UK), eluted with 7 mol/L urea in 138 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L Tris, and 1 g/L NaN₃. The SAA eluted at a discrete symmetrical peak and fractions containing SAA were identified by electroimmunoassay with monospecific polyclonal rabbit anti-human SAA antisera under conditions precisely as described elsewhere for horse SAA immunoassay (17). Fractions were also tested for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were pooled conservatively to yield material in which only a single protein band was present. The pure SAA was then dialyzed exhaustively against pure water, with low-molecular-mass-cutoff benzoylated dialysis tubing (Sigma Chemical Co., Poole, UK), and was stored frozen at −70°C.

Determination of SAA content in acute-phase HDL. Two separate lots of pure SAA in water were exhaustively freeze-dried (Modulyo EF4; Edwards High Vacuum, Crawley, UK) and were kept under reduced pressure until immediately before being weighed (R160P microgram balance; Sartorius Instruments, Sulton, UK). The masses obtained were 3.55 and 3.29 mg, respectively, with precision of ± 10 μg. These absolute amounts of SAA were dissolved in known volumes of 20 mmol/L Tris-HCl, pH 8.4, and were run in duplicate in reduced SDS/8–18% gradient PAGE (Excelgel™; Pharmacia) at loadings ranging from 0.7 to 7 μg/lane. Duplicate dilutions of the acute-phase HDL preparation were run in adjacent and alternating lanes in the same gels. The gels were then stained with Brilliant Blue R-350, according to the Pharmacia protocol, and 0.5 × 1.0 cm areas of gel, containing only the SAA band, were excised from each lane and extracted for 18 h at room temperature in 1-mL volumes of 250 mL/L pyridine. Identical sections of the same gels cut from the same region as the SAA band but from lanes in which no protein had been loaded were used as blanks. The concentration of dye in each sample was determined by measurement of A₄₅₀ (Spectracon 2800 spectrophotometer; Ciba-Corning, Halstead, UK) A standard curve constructed from the SAA samples was linear with excellent agreement between the duplicates, and was used to quantify SAA concentration in the HDL.

Antibodies to SAA. Rabbits were immunized with whole acute-phase HDL rich in SAA in Freund’s complete adjuvant, and the antisera were extensively absorbed before use with whole normal human serum containing <3 mg/L SAA. IgG was then isolated by affinity chromatography on immobilized Protein A (Pharmacia). Monoclonal mouse antibodies to SAA were raised by immunization with purified SAA that had been coupled with glutaraldehyde to keyhole limpet hemocyanin as a carrier protein. The specificity of these reagents was established by using them to immunostain blots of whole acute-phase serum, normal serum, and pure SAA with an indirect immunoperoxidase technique. In addition, anti-SAA antibodies immobilized on CNBr-Sepharose (Pharmacia) or on IMx microparticles (see below) were incubated with acute-phase serum and the effects on SAA and cholesterol concentrations tested in the IMx assay and in the Abbott TDx system, respectively.

SAA assay. The IMx instrument for automated microparticle capture enzyme immunoassay (MEIA) has been reported previously (18). For the present assay, mouse monoclonal IgG anti-SAA antibodies (clone Reu 86.1) were covalently coupled to carboxylate-modified 0.194-μm latex microparticles (Seradyn, Indianapolis, IN) by using ethyl dimethylaminopropyl carbodiimide (Sigma, St Louis, MO), followed by overcoating with 10 g/L casein (Sigma). The microparticles were then diluted and stored in 0.06 mol/L Tris, 0.3 mol/L NaCl, 136 g/L sucrose, and 1 g/L NaN₃, pH 8.0, at a calculated antibody concentration of 7.5 mg/test. Rabbit IgG anti-SAA antibodies were covalently coupled to calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) by using a 30-atom extended heterobifunctional linker (19) and were stored in 0.1 mol/L Tris, 0.5 mol/L NaCl, 0.1 mmol/L MgCl₂, 1 mmol/L ZnCl₂, 30 g/L Brij-35, 5 g/L nonfat dry milk solids, 24 g/L fish skin gelatin, 30 g/L normal rabbit IgG, and 1 g/L NaN₃, pH 7.9, at a final concentration to provide 6.2 mg/test.

The IMx carousel containing 24 reaction cells is loaded with 20 samples consisting of serum diluted, according to their SAA concentration, either 1:50 or 1:5 with MEIA buffer containing 10 g/L casein. A zero blank and three controls containing low, medium, and high concentrations of SAA, ~20, 100, and 200 mg/L, respectively, are also included. The microparticles, conjugate, substrate, and MEIA buffer with 10 g/L casein are placed in the
instrument with the loaded carousel; the assay then runs automatically, yielding results after 40 min. In the first step of the assay the probe/electrode assembly of the IMx delivers anti-SAA microparticles and sample to the incubation well of the IMx reaction cell. An aliquot of the reaction mixture is then transferred to the glass matrix tab and washed before addition of anti-SAA–alkaline phosphatase conjugate and further incubation. After washing to remove unbound conjugate, the substrate 4-methylumbelliferyl phosphate is added to the matrix and the fluorescent product measured by the IMx front-surface fluorometer. Sample concentration is then determined from a stored 6-point calibration curve referenced to acute-phase serum of known SAA concentration.

**SAA calibrators and controls for the IMx assays.** A concentrate of SAA was extracted from pooled acute-phase material by hydrophobic interaction chromatography (20). This preparation in a solution of guanidine/ethylene glycol was exceptionally stable with respect to its SAA immunoactivity and was used at suitable dilutions in 0.06 mol/L Tris, 0.3 mol/L NaCl, 1 mL/L Tween 20, 1 mL/L Triton X-100, 10 g/L casein, and 1 g/L NaN₃, pH 7.5, to provide calibrators and controls for the IMx.

**Single radial immunodiffusion (RID) assay of SAA** (21). Agarose gel (Ionagar no. 2; Oxoid, Basingstoke, UK), 12 g/L in 70 mmol/L barbital, 2 mmol/L CaCl₂, 30 g/L polyethylene glycol 6000, and 1 g/L NaN₃, pH 8.6, containing 50 mL/L sheep anti-SAA serum, was cast at 1-mm thickness on Gelbond™ film (Flowgen Instruments, Sittingbourne, UK). Wells (2-mm diameter) were cut 14 mm apart in 5 rows of 11 on each 18.5 × 9.5 cm gel; they were loaded with 2-μL samples by using a positive-displacement pipette (Absoluter™; Alpha Labs., Eastleigh, UK). Triplicate series of five dilutions of standards or calibrators and two control acute-phase sera with known SAA concentrations were included in each gel. The gels were then incubated in a moist chamber at 37°C for 40 h before being washed and dried at 60 h at room temperature in 50 g/L NaCl and 1 g/L NaN₃, pressed, dried, and stained with Brilliant Blue R-250. Results were read both by eye and by an automated image analysis system (Gop302; Contextvision, Kista, Sweden). Standard curves were plotted manually and also spline-fitted with RIA Smart™ software (Canberra Packard, Pangbourne, UK). Both methods gave the same values, with an assay range of 1–300 mg/L and inter- and intraassay CVs <10% throughout the range.

**Determination of SAA concentration in the acute-phase serum primary standard.** Multiple-replicate different dilutions of the acute-phase serum pool were run as samples in a series of independent RID assays, with the use of separate, freshly prepared dilutions of the calibrated acute-phase HDL as the standard.

**Correlation between RID and IMx assays.** Serum samples from patients with rheumatoid arthritis, in which SAA had been measured by RID, were selected to provide a complete range of values and were then assayed in duplicate or triplicate on the IMx. The mean IMx value and the RID result for each sample were analyzed by linear regression.

**Assays in normal and patients’ sera.** Sera from 105 normal healthy volunteers were collected and stored frozen at −20°C before assay in one batch for SAA and CRP. CRP was determined by using a monoclonal antibody MEIA on the IMx (submitted for publication). Sera collected prospectively at monthly intervals from 31 patients with histochemically confirmed AA amyloidosis and extensive series of clinical serum samples from patients with rheumatoid arthritis, renal allografts, myocardial ischemia, and bacterial infections were also assayed for SAA and CRP. Results from the latter studies are to be reported elsewhere. Collection and use of all clinical sera reported here had been approved by the Institutional Ethical Committees at the Royal Postgraduate Medical School or at Abbott Labs.

**Results**

**Specificity of anti-SAA antibodies.** The polyclonal rabbit antiserum was monospecific for SAA in agarose gel diffusion immunoprecipitation testing against whole acute-phase serum. It also reacted specifically with AA deposits in immunohistochemical testing. Neither the rabbit nor the mouse monoclonal anti-SAA reagents reacted with normal serum in immunoblots, and they both identified just a single band in acute-phase serum, migrating in the position expected for SAA. They also gave strong positive reactions in immunoblot analysis of pure SAA. Absorption of acute-phase serum with immobilized rabbit or mouse anti-SAA antibodies resulted in dose-dependent depletion of SAA and was complete if sufficient antibody was used. However, such absorption had no effect on the serum cholesterol concentration, nor was any cholesterol detectable in the eluate from immobilized anti-SAA antibodies that had been incubated in acute-phase serum. The capture process thus involves just SAA and no other components of the HDL particles with which it is associated in serum. This is consistent with the results of preliminary experiments in which we attempted to capture acute-phase HDL for SAA assays by using immobilized antibodies to apoAI and in which no SAA was detectable.

**Establishment of the acute-phase serum primary standard for SAA.** The SAA content of an acute-phase HDL preparation was determined by the SDS-PAGE method of Godenir et al. (22). However, we directly measured the absolute mass of SAA used for calibration of the PAGE, in contrast to Godenir et al., who used a colorimetric protein assay based on an albumin standard under the unjustified assumption that SAA behaves identically to albumin in such assays. Extreme precautions were taken to maintain the dried SAA in an anhydrous state, and performance of rapid repeat weighings confirmed that there was no significant mass gain during the brief period between exposure to air and the initial mass determination.

On the basis of the calibrated acute-phase HDL as a standard, each separate measurements by RID assay of the pooled acute-phase serum primary reference material yielded a mean (SD) SAA concentration of 300 (18) mg/L. This value was therefore used in the IMx assays.
to assign the SAA concentration of appropriate dilutions of the guanidine/ethylene glycol SAA concentrate, which constituted the working calibrators and controls. 

**IMx assay for SAA.** Range: Serum samples were routinely run at a dilution of 1:50 and the detection limit was determined as 0.2 mg/L SAA, based on 3 SD above the mean signal obtained in 10 replicates of a zero sample consisting of MEIA buffer with 10 g/L casein. However, any samples giving signals this low were run again at a dilution of 1:5; thus, an actual SAA concentration of 1 mg/L in whole serum could be detected. The upper limit of precise quantification on the standard curve was 750 mg/L, and samples falling above this were rerun at higher dilutions.

Dilution linearity: When samples of either acute-phase serum or the SAA concentrate in guanidine/ethylene glycol used as calibrators and controls were diluted 1:5–1:200 with wash solution, normal human serum, or human serum with a high HDL content, the SAA results were linear, with \( r = 0.99 - 1.00 \) and slope 0.97–1.00.

Reproducibility: Ten replicate assays of two acute-phase serum samples containing, respectively, 160 and 56 mg/L SAA were run on 5 days successively. The inter- and intraassay CVs were, respectively, 4.8% and 7.0%, and 5.9% and 3.9%.

Recovery: Three acute-phase sera containing, respectively, 12, 39, and 80 mg/L SAA were assayed before and after addition of acute-phase serum (containing 13 or 202 mg/L SAA) and, separately, SAA concentrate containing 17 or 361 mg/L SAA). Recovery ranged from 83% to 108%, with overall mean of 92%.

Stability of calibrators, controls, and antibody reagents: Controls containing 56 and 180 mg/L SAA showed no significant change in immunoreactivity when they were subjected to three consecutive cycles of freezing and thawing. Similarly, controls containing 35 and 125 mg/L SAA showed no significant change in immunoreactivity over 3 days at 0, 24, 37, or 45°C. The antibody reagents showed no change in activity after 3 days at 45°C, and after 5 months of storage of all assay materials at 4°C recovery was 92%.

Interference: No interference was detected in lipemic, icteric, or hemolyzed sera or samples containing rheumatoid factor. Serum and plasma from the same blood sample gave identical results.

**Correlation between RID and IMx assays for SAA.** There was an excellent linear correlation between SAA values obtained by the RID reference method and by the IMx assay (Fig. 1, Table 1). The highest values, >300 mg/L, tended to be greater in the IMx than in the reference method, probably because of underestimation by immunodiffusion, but at values <300 mg/L the two methods gave very similar results.

**SAA concentration in sera of normal subjects.** The frequency distribution of SAA values in sera from 105 normal healthy adults overlapped but was higher than that of CRP (Fig. 2). Nevertheless, 82% of SAA results

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**Table 1. Linear regression of IMx against RID SAA values over different ranges.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Slope (95% confidence limits)</th>
<th>Intercept (95% confidence limits)</th>
<th>( r ) (95% confidence limits)</th>
<th>Residual SD (SEE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA ≤100 mg/L by RID, ( n = 106 )</td>
<td>0.96 (0.086–1.05)</td>
<td>(-5 ) (−10 to 0)</td>
<td>0.90 (0.85–0.93)</td>
<td>13.6</td>
</tr>
<tr>
<td>SAA ≤200 mg/L by RID, ( n = 173 )</td>
<td>1.04 (0.99–1.10)</td>
<td>(-9 ) (−14 to 3)</td>
<td>0.95 (0.93–0.96)</td>
<td>20.0</td>
</tr>
<tr>
<td>SAA ≤300 mg/L by RID, ( n = 188 )</td>
<td>1.07 (1.01–1.13)</td>
<td>(-10 ) (−17 to 3)</td>
<td>0.94 (0.92–0.95)</td>
<td>26.7</td>
</tr>
<tr>
<td>SAA ≤380 mg/L by RID, ( n = 200 )</td>
<td>1.25 (1.12–1.29)</td>
<td>(-28 ) (−36 to −20)</td>
<td>0.97 (0.96–0.98)</td>
<td>38.6</td>
</tr>
</tbody>
</table>
were <5 mg/L and 96% were <10 mg/L. The mean (SD) was 3.7(3.6) mg/L, and the median (range) was 3.0(0.7–26.4) mg/L.

**SAA concentration in disease.** The range of SAA concentrations observed in chronic active inflammatory disease is exemplified by the results in rheumatoid arthritis, shown in Fig. 1. Similar values were seen in patients with infections, recipients of renal allografts during rejection episodes, postoperative cases, and in unstable angina and after myocardial infarction (submitted for publication). The highest value observed was 2200 mg/L.

**SAA concentration in AA amyloidosis.** The concentrations of SAA varied among patients with AA amyloidosis (Fig. 3), depending on the activity of their underlying inflammatory disease. However, it is interesting that the correlation coefficients between SAA and CRP values in these patients ranged from 0.01 to 0.99, with a median of 0.57 and mean (SD) of 0.54 (0.36). Representative examples are shown in Fig. 3.

**Discussion**

SAA in acute-phase serum is almost totally associated with HDL (23). Isolated acute-phase HDL, providing SAA in the same physiological, biophysical, chemical, and immunoreactive form, is therefore a valid reagent for standardization of serum SAA assays. This contrasts with the use by others of pure, denatured SAA or even AA for standardization, and probably accounts to some extent for the wide range of apparent mass values of

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**Fig. 3.** Serial monthly values of serum SAA (open bars) and CRP (solid bars) in patients with AA amyloidosis complicating chronic inflammatory diseases. Each panel represents a different patient; the correlation coefficient (r) between SAA and CRP for each patient is: 0.10 (A), 0.57 (B), 0.18 (C), 0.94 (D), and 0.26 (E).
SAA that have been reported in the literature. Although the present calibrator/control material prepared from SAA concentrate, and indeed all the assay reagents, are extremely stable, especially when stored at 4°C, heat has a marked effect on immunoreactivity of SAA in serum. Incubation at ≥37°C for a few hours produces increased apparent SAA concentrations, and careful sample handling is therefore mandatory for reliable SAA results.

There is growing interest in both research and clinical applications of SAA measurement, and a wide variety of immunoassays for SAA have been reported (24–30). However, the method used most extensively for clinical studies has been RID (6, 21). It is simple and has the advantage of direct visualization of the SAA-anti-SAA interaction, and we therefore used it as the comparison method both for quantification of the standard and for correlation studies. Precise and reproducible results were obtained and fully validated the IMx assay. Availability of the forthcoming WHO International Reference Standard for SAA Immunoassay will help to ensure uniformity in the results from different centers using different methods, whereas the large number of IMx instruments in diagnostic laboratories worldwide should facilitate provision of SAA measurements in research and routine clinical practice.

The normal range of SAA in healthy subjects reported here provides a useful reference range. The rare individuals with concentrations >10 mg/L, 5 of 105, were presumably mounting an acute-phase response to some intercurrent subclinical process, and unless they were developing a chronic condition should have lower values if examined a few days or weeks later.

Clinical studies in large groups of patients with a variety of disorders confirmed the rapid production and exceptionally wide dynamic range of the SAA response. AA amyloidosis is of particular interest because of the recent clear evidence that control of the underlying chronic inflammatory process retards progression and can permit regression of the amyloid deposits (15, 31). Hitherto, only measurements of CRP have been readily available outside research laboratories, but it is the production of SAA, the precursor of AA fibrils, that is critical. We show here that, despite excellent correlation in some cases, CRP concentrations in others do not move in parallel with the SAA response (Fig. 3), as has previously been reported for many inflammatory conditions (32).

When both CRP and SAA concentrations are only slightly increased, there is little correlation between them (Fig. 3A). This is not important, but in other situations the discrepancy may be of considerable clinical significance. For example, SAA may be extremely high and vary greatly while CRP remains rather constant (Fig. 3B); however, SAA may not be increased despite persistent increase of CRP (Fig. 3C), which could lead to inappropriate antiinflammatory therapy if only CRP were being monitored. Finally, there may just be a poor correlation between the behavior of the two proteins (Fig. 3E), confirming that specific assay of SAA is necessary. The variable relation between the CRP and SAA responses does not seem to be related to the underlying inflammatory disease or to treatment, but further study is required.

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References

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