

Adaptation of Microarray Assay for Serum Amyloid A Analysis in Human Serum

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Abstract—Serum amyloid A is an inflammatory biomarker whose concentration changes during infectious and inflammatory diseases. SAA's tendency for aggregation and complex formation makes it difficult to determine its concentration in samples, especially when there is an increased level of it. Immunofluorescence SAA determination on a microarray was adapted for SAA quantification in human serum. Both the procedure and the diluent for the calibrator samples were chosen to obtain a dynamic range between 1 and 100 µg/mL. Mixtures of animal (rabbit, goat, mouse) sera with recombinant antigen diluted in certain concentrations were used for the calibrator samples. The method was tested using serum samples from 15 patients with rheumatoid arthritis or ankylosing spondylitis and 9 healthy donors. The results obtained on the microarray demonstrated a good correlation with the results determined by ELISA (Pearson's correlation coefficient is 0.93). The method developed could be a convenient tool for assessing SAA levels in a number of diseases, such as rheumatoid arthritis or infections of various etiologies, characterized by a significant increase in the level of this protein in the blood. The use of a microarray for the analysis allows the determination of the SAA concentration simultaneously with other inflammatory biomarkers.

Keywords: serum amyloid A, microarray, inflammatory biomarkers

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INTRODUCTION

Serum amyloid A (SAA) is an acute phase protein produced in various inflammatory processes. Proteins of the SAA family are synthesized primarily in the liver. As apolipoproteins, they usually exist in a bound form, forming complexes with lipoproteins, mainly high-density lipoproteins. Such binding stabilizes the alpha-helix in SAA and protects it from proteolysis and misfolding [1]. In addition to high density lipoproteins, SAA is able to interact with lipoproteins of other classes (low and very low density lipoproteins) [2], as well as with receptors involved in host defense or lipid metabolism (TLR, RAGE, SR-B1, CLA-1, LOX1, P2X7, and FPR2), basal membrane proteins (fibronectin, laminin), and blood plasma proteins (cystatin, fibrin) [3, 4].

The normal plasma SAA level in healthy adult donors does not exceed 10 mg/L, and the median SAA concentration in the European population is 3 mg/L [5]. The concentration of SAA in the blood increases with bacterial and viral infections. During the development of infectious inflammation, the level of SAA

and C-reactive protein (CRP) can increase by more than 1000 times, reaching a peak value 2–3 days after the onset of inflammation. It has been shown that a significant increase of SAA and CRP levels is more important for predicting the severity of COVID-19 than the levels of procalcitonin and leukocytes [6]. In addition, SAA content may vary depending on the stage or activity of lung cancer [7], in other oncopathology [8], in anterior uveitis [9], with periodic illness [10], and in most rheumatic diseases.

Despite the fact that for a number of diseases SAA is an effective and more sensitive marker than other markers of the acute phase such as CRP and erythrocyte sedimentation rate, the latter are currently preferred due to their availability and low cost. The introduction of SAA into routine clinical practice is hampered not only by the properties of the protein itself (its tendency for aggregation and complexation), but also by the difficulty of isolating and storing SAA at high concentrations, and the standardization of controls, calibrators, and antibodies used.

SAA is determined using both highly sensitive ELISA systems (detection limit up to 3 ng/mL) and immunonephelometric or immunoturbidimetric

Abbreviations: SAA, serum amyloid protein A; ELISA, enzyme-linked immunosorbent assay.

methods with a lower sensitivity (>3 mg/L) [11]. In some cases, when conducting ELISA it is necessary to predilute the sample, since at high SAA concentrations it is adsorbed on the surface of the plastic wells of the microtiter plate. In addition, these are monoplex methods, i.e., SAA is analyzed separately from other markers of inflammation.

Since SAA, like many other markers of inflammation, is not a highly specific protein, studies or analyses to determine the stage or activity of the disease may also include the determination of other markers of inflammation (for example, pro-inflammatory cytokines), the concentration of which in the samples may be comparable to the concentration of SAA (CRP) or much lower. Based on this, it is important to have a method that, on the one hand, allows the detection of SAA in plasma or serum samples without dilution, and on the other hand, does not exclude the use of this analysis in conjunction with the determination of other necessary antigens.

Earlier [12], we developed a method for the simultaneous determination of SAA and a number of other markers of inflammation in culture media using biological microarrays. In this work, the immunofluorescence method has been adapted for detecting SAA in the blood serum of patients on microarrays, and its efficiency is shown in comparison with the classical method, ELISA.

EXPERIMENTAL

Reagents and buffers. We used the following reagents: Tween-20, polyvinyl alcohol (PVA) 50 kDa, polyvinylpyrrolidone (PVP) 360 kDa, streptavidin-Cy5 conjugate (Streptavidin-Cy5) (GE Healthcare, USA), phosphate buffered saline pH 7.4 (PBS), PBS with 0.01% Tween-20 (PBST); dilution buffer (PBS, 0.14% PVA, 0.14% PVP), rabbit blood serum (Biosera, France), goat blood serum (Biosera), mouse blood serum (Biosera), monoclonal antibodies to SAA (clone SAA1, clone SAA15) and their conjugates with biotin (HyTest, Russia), and human SAA (HyTest).

Microarray manufacturing. Microarrays were prepared according to the method described earlier [12]. For immobilization, we used mouse monoclonal antibodies to human SAA, as well as human SAA antigen, the concentrations of which in the gel compositions were 1.4 and 0.3 mg/mL, respectively. Monoclonal antibodies and SAA antigen mixed with the gelling agents, as well as a protein-free gelling composition (empty gel), were applied using a Qarray pin robot (Genetix Limited, UK) on glass slides pre-activated with Bind-Silane. The copolymerization of the gel pads was carried out after application under a UV lamp with a maximum radiation of 350 nm in a nitrogen flow. After polymerization, microarrays were washed in PBST and blocked in 1% PVA. The presence of all

gel pads was monitored using a microscope in transmitted light.

Samples. The work analyzed 15 blood serum samples from patients with rheumatoid arthritis or ankylosing spondylitis, as well as 9 blood serum samples from healthy donors provided by the Moscow Loginov Clinical Scientific Center (MCSC). The study was approved by the local ethics committee of the MCSC.

Microarray sandwich analysis. For the sandwich analysis, 65 μ L of the sample (blood serum, SAA solution in dilution buffer, or a mixture of animal sera) was applied to the pads of the microarray and incubated at 37°C from 15 min to 20 h. After washing in PBST (20 min), the microarrays were rinsed with distilled water and dried. The pads were loaded with 65 μ L of a solution of biotinylated antibodies SAA1 or SAA15 (6 μ g/mL) and incubated for 1 h at 37°C. After washing in PBST (20 min), the microarrays were again rinsed with distilled water and dried. The pads of the microarray were loaded with 50 μ L of a streptavidin-Cy5 solution (5 μ g/mL) in dilution buffer and incubated for 30 min at 37°C. After incubation, microarrays were washed in PBST (30 min), rinsed with distilled water, dried, and images of the pads were obtained on a fluorescent microscope.

Analysis of fluorescent images of microarrays. Fluorescent images of microarrays were obtained using a laser-excited fluorescence microscope developed at the Engelhardt Institute of Molecular Biology (EIMB), Russian Academy of Sciences [13]. Fluorescent signals were analyzed using ImageAssay software (EIMB).

ELISA of blood serum samples. The SAA concentration in the samples was determined using SAA Human ELISA kit (Hycult Biotech, USA) according to the manufacturer's instructions.

Plotting calibration curves. To construct the calibration curves which were used to determine the concentration of SAA in the samples and recalculate the fluorescent signals from the pads containing anti-SAA antibodies, the calibration samples were additionally analyzed. A solution of recombinant human SAA in a mixture of animal sera (mouse, goat, rabbit) mixed in equal volumes was used as calibration samples. The SAA concentration in the solution varied from 0 (zero sample) to 100 μ g/mL.

RESULTS

In this work, microarray sandwich analysis was adapted for the detection of SAA in human serum. The microarray used for the analysis was designed earlier and contained, in addition to SAA, a number of other markers of inflammation. Since the concentration of SAA in the samples is relatively high (μ g/mL compared to ng/mL and pg/mL for other markers), during the manufacture of the microarrays, the concentration of anti-SAA for immobilization in pads was reduced, as well as the exposure time when obtaining

fluorescent images on the microscope. The quantitative assessment of SAA in the samples, along with the analysis on the microarrays of the samples themselves, involves the construction of a calibration curve in each of the experiments. Samples containing SAA in various concentrations in a mixture of animal (rabbit, mouse, goat) sera and in dilution buffer were tested as calibration samples.

Testing of Dilution Buffer for Calibration Samples

Initially, samples with microarrays were incubated for 20 h at 37°C, since earlier [12] we showed that in this way it is possible to obtain acceptable calibrations for a given antigen. The results of the analysis of the calibration samples based on the dilution buffer are shown in Fig. 1. It can be seen that in the case of such calibration samples, satisfactory results are obtained at an SAA concentration of no more than 10 µg/mL. At higher SAA concentrations, a significant increase in the fluorescent signal from pads that do not contain immobilized proteins (“empty gel”) is observed.

A sample containing SAA at a concentration of 10 µg/mL was analyzed using biotinylated antibodies (SAA15 or SAA1) on microarrays with immobilized SAA1 antibodies. When using either of the antibodies, fluorescent signals that were significantly different from the signals of the empty gel were observed (Fig. 2).

Testing of Calibration Samples Based on Animal Sera

In addition to the calibration samples based on the dilution buffer, the calibrators, which are a mixture of blood serum of animals with the addition of certain concentrations of recombinant human SAA, were also tested.

Analysis of samples containing animal serum during prolonged incubation with microarrays did not allow calibration curves to be obtained. Fluorescent signals from pads containing SAA at concentrations from 1 to 100 µg/mL were extremely similar to each other and to the fluorescent signal of an empty gel.

We also analyzed a number of parameters that can influence the result, such as the addition of detergent, temperature, and changes in incubation time. Changing the incubation temperature and adding various detergents to the sample did not significantly affect the results.

A significant reduction in the incubation time of the sample on the microarray made it possible to obtain a set of fluorescent signals for plotting calibration curves; with decreasing incubation time, an increase in fluorescence signals was observed. Fig. 3 shows the calibration curves obtained at different times of sample incubation on microarrays: 15 min, 30 min, 1 h, 2 h, and 3 h. The signal from the empty gel in all experiments did not exceed 0.25 arb. units

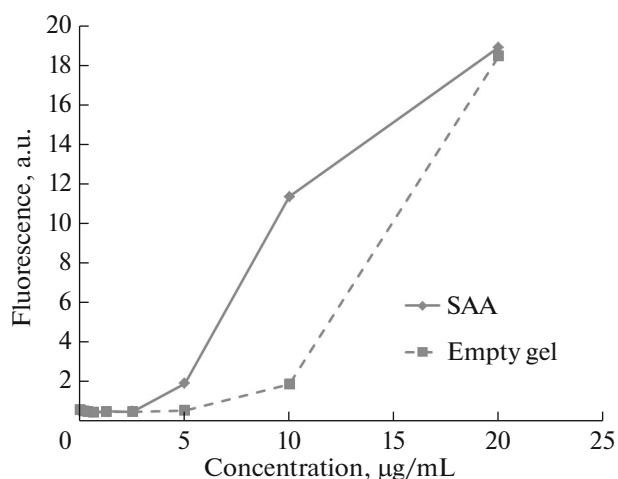


Fig. 1. Dependence of the intensity of the fluorescent signal on the concentration of SAA in the analysis of antigen dissolved in the dilution buffer. SAA, fluorescent signal from a pad with immobilized SAA; empty gel, the fluorescent signal from the pads with immobilized gel which does not contain proteins and characterizes a nonspecific interaction.

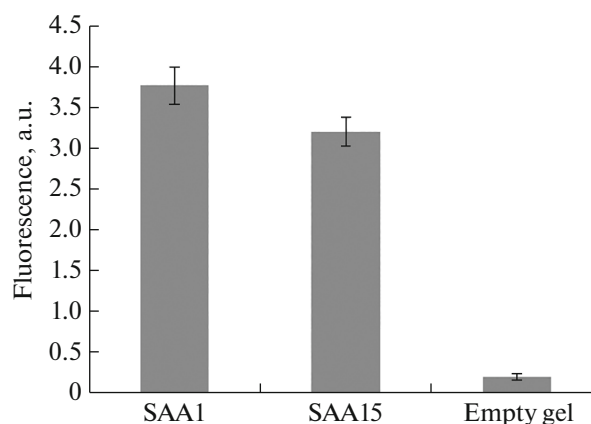


Fig. 2. Fluorescent signals from pads with immobilized antibodies to SAA (SAA1 and SAA15) and from empty gel after analysis of SAA solution (10 µg/mL).

An additional experiment was carried out to assess the stability of the calibrators. It was shown that when analyzing calibration samples immediately after preparation, the calibration curve does not differ (within the limits of statistical error) from the calibration curve obtained using calibrators 1 h after preparation.

Based on these results, the analysis of SAA in blood sera was carried out for a short period of time (30 min), and calibration curves constructed on the basis of calibrators with added serum were used to recalculate the fluorescent values.

To assess the effect of animal blood sera as a matrix, we performed a comparative analysis of two samples, which were a mixture of animal sera or

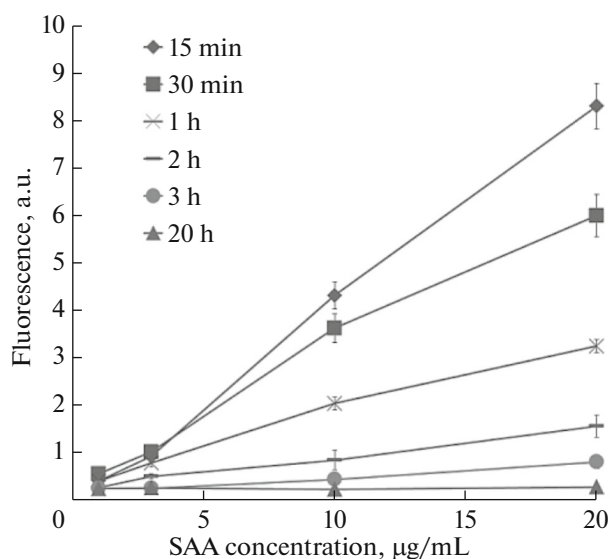


Fig. 3. Dependence of the fluorescent signal on the concentration of SAA in the analysis of antigen dissolved in a mixture of animal sera (mouse, goat, rabbit). Fluorescence values from pads containing anti-SAA antibodies are shown. The signal from the empty gel in all experiments did not exceed 0.25 arb. units.

human blood serum with a low SAA concentration (0.2 µg/mL according to ELISA results), to which recombinant human SAA was added at a concentration of 10 µg/mL. It was shown that the fluorescent signals in the analysis of SAA in a mixture of animal sera do not differ significantly from the fluorescent signals in the analysis of SAA in human serum, which indicates that the selected serum medium does not influence the analysis result.

Analysis of Sera Containing SAA on a Microarray

Analysis of blood sera containing SAA was carried out by short-term incubation of the sample with a microarray. A typical fluorescent image of the pads after a short-term incubation of serum is shown in Fig. 4. For comparison, there is also the typical appearance of the pads after prolonged incubation of the same serum and a sample containing SAA solution in dilution buffer. It can be seen that in contrast to the calibration sample, where the fluorescent signal and hence SAA are relatively uniformly distributed in the pad volume, in the case of SAA from serum a higher concentration of antigen is observed in the surface layer of the gel near the edges of the pad.

Comparison of Data Obtained on a Microarray with ELISA Results

The results of analysis of blood serum samples on microarrays were compared to the concentrations in the same samples measured using an ELISA test system.

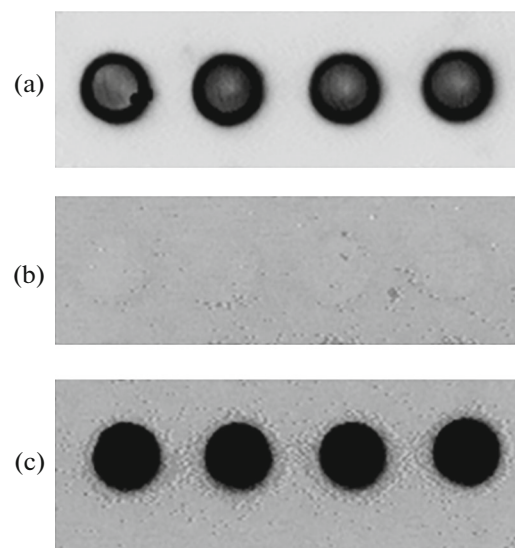


Fig. 4. Fluorescent image of pads with immobilized antibodies to SAA after analysis of analytes containing SAA. (a) Analysis of serum containing SAA, short-term incubation with the sample. (b) Analysis of serum containing SAA, incubation with the sample for 20 h. (c) Analysis of SAA solution in dilution buffer (PBS, 0.14% PVA, 0.14% PVP), incubation with the sample for 20 h.

The SAA concentration in the analysis of healthy donor samples on a microarray did not exceed 1 µg/mL. The SAA concentrations in the same samples, determined by ELISA, ranged from 0.23 to 1.2 µg/mL. The results of SAA determination in samples from patients with rheumatoid arthritis and ankylosing spondylitis are shown in Fig. 5. Pearson's correlation coefficient was 0.93.

DISCUSSION

In this work, the immunofluorescent multiplex method for the analysis of proinflammatory markers on a microarray was adapted for the quantitative determination of SAA in human serum.

Much attention was paid to testing calibration samples containing recombinant human SAA, since, unlike other inflammatory mediators, the classical buffer-containing calibrators that we used earlier were unsuitable: at concentrations above 10 µg/mL, there is a significant increase in the fluorescent signal from the negative control, i.e., pads that do not contain immobilized proteins (Fig. 1). The results can be explained by the physicochemical properties of SAA, in particular, its tendency for aggregation [1], as a result of which the antigen, at its high concentrations in a buffer solution that does not contain substances capable of stabilizing it, nonspecifically interacts with the surface of microarrays.

For immobilization on a microarray, we chose SAA1 monoclonal antibodies, which, on the one hand, are able to interact with only one antigenic

determinant, and on the other hand, when analyzing the calibrators, can form the following sandwich: SAA1 antibodies—SAA from solution—biotinylated SAA1 antibodies (Fig. 2). Since the SAA structure does not contain repeated amino acid sequences, the performance of the immobilized SAA1—detecting SAA1 pair, can apparently be explained by the partial oligomerization of SAA in solution. The oligomerization of SAA is generally not typical for the antigen present in the serum; therefore, the recombinant SAA in dilution solution containing no additional stabilizing proteins was not subsequently used to construct calibration curves.

Calibrators based on animal serum avoid the formation of oligomers, since SAA will naturally bind to the lipoproteins present in the serum. In addition, serum proteins help to reduce the nonspecific adhesion of SAA to pads.

Analysis of calibration samples at different incubation times showed that short incubation (15–30 min) gives more intense fluorescent signals than longer incubation (more than 1 h) (Fig. 3). In addition, a similar decrease in the fluorescent signal with increasing incubation time was observed in a human serum sample (Fig. 4b). It should be noted that the analysis of SAA in blood serum is characterized by an uneven distribution of the signal over the pad volume (Fig. 4b): a more intense glow, and hence a higher concentration of SAA available for binding to detecting antibodies, is observed in the surface layer of the gel. There are two reasons for the resulting picture: (1) limited permeability of gel pads for SAA as part of heterogeneous protein–lipid complexes; (2) steric hindrances arising from the large accumulation of protein–lipid complexes near the pad surface. The latter reason also leads to the fact that the antibodies cannot penetrate into the pad in sufficient quantities to bind to the SAA molecules located in the depth of the pad. In this case, the longer the incubation time with samples containing serum, the greater the screening of the pad surface by protein–lipid complexes, and therefore, the lower the fluorescent signal.

The method developed allows the determination of SAA in blood serum in the range from 1 to 100 $\mu\text{g/mL}$, which is convenient for assessing the level of this antigen in a number of diseases, such as rheumatoid arthritis or infections of various etiologies, characterized by a significant increase in the content of this antigen in the blood.

The method shows good agreement with the results obtained by classical ELISA (Pearson correlation coefficient 0.93), and only requires dilution of the sample if the concentration exceeds 100 $\mu\text{g/mL}$, while in the most highly sensitive ELISA for measuring SAA (up to tens of ng/mL), the sample must be diluted without exception. Thus, the use of a microarray does not allow one to completely abandon the dilution, but it allows one to reduce it.

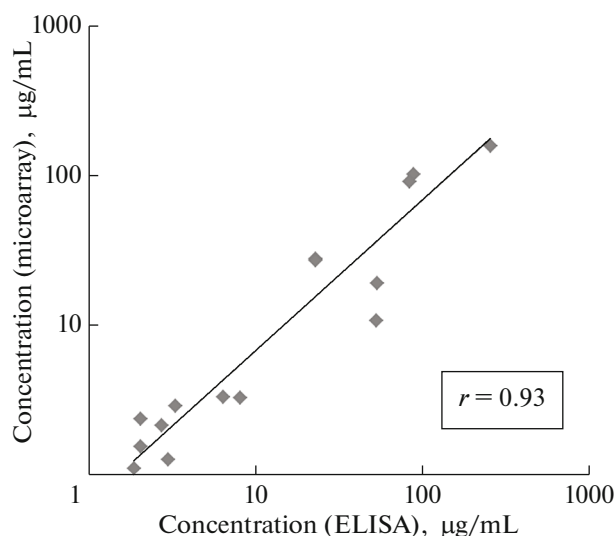


Fig. 5. Correlation graph of SAA levels obtained after analysis of samples on microarrays and after analysis of the same samples using the ELISA method. The Pearson correlation coefficient r is given. Values above 100 $\mu\text{g/mL}$ were calculated for the microarray by extrapolation.

In addition to ELISA, the SAA concentration is often determined using immunonephelometry, immunoturbidimetry, or immunofluorescence [14], as well as by immunochromatographic analysis [15, 16]. Comparison of the numerical values obtained in the determination of SAA by different methods given in the studies on the validation of various test systems shows a high correlation [17], comparable to that obtained by us in this study; however, in some studies, some concentration shifts are observed [14, 18]. At the same time, it is known that, for example, in rheumatoid arthritis, mean or median SAA values can be measured in ones [19], tens [20], or even hundreds [21] of $\mu\text{g/mL}$ (mg/L). In the population of healthy donors, there are also significant differences in the median and mean levels [22, 23].

The reason for such differences may not only be differences in the samples, but also the lack of standardization of calibration samples in various test systems, as well as differences in the principle of analysis. Consequently, for further validation and the possibility to apply the method in clinical practice, additional studies and comparison with other certified methods that are used to determine SAA, analysis of samples obtained from patients with various diseases, and an increase in the sample of healthy donors in order to determine the threshold concentrations (cutoff) for each of the pathologies are also needed.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare they have no conflict of interest.

All procedures performed in this work are in accordance with the ethical standards of the institutional committee on research ethics and the 1964 Declaration of Helsinki and its subsequent amendments or comparable standards of ethics. Written informed consent was obtained from all patients.

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