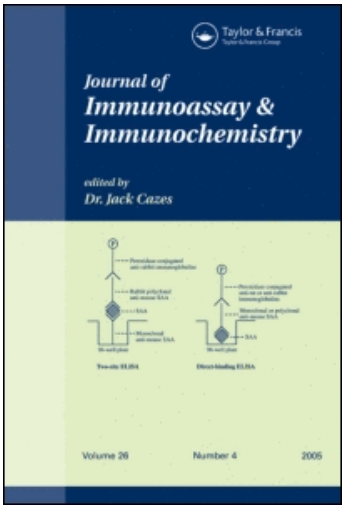

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MONOCLONAL ANTIBODY-BASED SENSITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR MURINE SERUM AMYLOID A

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ABSTRACT

Enzyme-linked immunosorbent assay (ELISA) methods for measuring murine serum amyloid A (SAA), a representative acute phase reactant, were developed utilizing a newly produced monoclonal antibody. Two site-ELISA, in which the monoclonal antibody was used as the captured antibody, was sensitive enough to determine the SAA concentration in mice at the steady state. Direct binding ELISA, in which the sample SAA bound to the plastic wells was detected by the antibody, was simple and suitable for measuring the elevated SAA, but could not analyze the resting level of SAA because of the need for high dilution in plasma samples. Plasma SAA concentrations were measured in ten ICR mice on the day of purchase and at the end of seven days of ordinary rearing. The SAA concentration of one animal decreased from 1.6 to 0.5 mg/l during a week, while the others had no obvious changes. The plasma SAA of the ten animals after one week of rearing ranged from 0.3 to 0.8 mg/l with a mean of 0.47. These mice, two days after 10 μg lipopolysaccharide were given, had increased SAA values up to a mean of 300 mg/l, though with variations between animals. (KEY WORDS : serum amyloid A, mouse, enzyme-linked immunosorbent assay, inflammation, monoclonal antibody).

INTRODUCTION

Mice are the most frequently being used animals in the *in vivo* experiment-based studies in the medical field. Recent progress in genetic engineering has accelerated the utilization of this animal. In the process of various experiments often the stress or inflammatory status needs to be assessed; i.e., evaluating rearing conditions or examining the effect of anti-inflammatory drugs. Among several approaches detecting these changes, the most practical and reliable way was a quantification of acute phase reactants, the series of plasma proteins which were produced mainly in the liver under stimulation of inflammation-related cytokines (1, 2). In mice, serum amyloid A (SAA) and serum amyloid P component proved useful for this purpose. The former was a serum precursor of protein AA, the chief component in the reactive amyloid deposits and an apolipoprotein primarily present in high density lipoprotein (3). The latter was similar to C-reactive protein in its tertiary structure and in its binding of calcium ion (4). In this study, assay methods for murine SAA were developed.

We selected the enzyme-linked immunosorbent assay (ELISA) as a practical method. A monoclonal antibody to murine SAA was generated and utilized for the assay because of the future convenience. Our primary requirement for the new method was detection sensitivity, which could determine the resting levels of SAA and which would not require a large volume of plasma for the assay. To our knowledge, none of the previously described ELISA methods satisfied the requirements of this study (5, 6).

Thus, we first established a monoclonal antibody to murine SAA and constructed a two site (sandwich type) -ELISA. In addition, a direct binding ELISA, in which diluted samples were directly adsorbed into the plastic well and were followed by reaction with antibodies, was also developed. To evaluate these

methods, we analyzed SAA concentrations in plasma from mice in the steady state and after treatment with inflammatory stimuli.

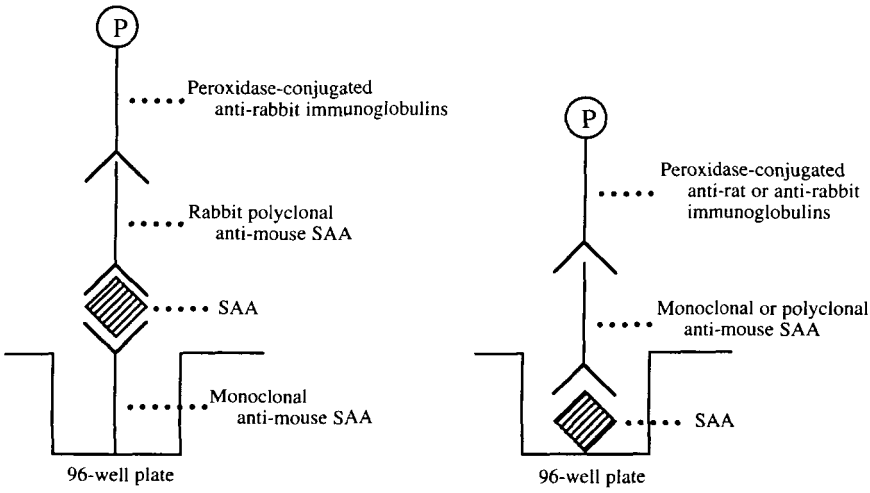
MATERIALS AND METHODS

Monoclonal Antibody

Rat-mouse heterohybridomas producing anti-murine SAA antibodies were established in the same way as were the anti-human SAA antibodies (7). Recombinant mouse SAA1 (former SAA2) (8), which was shared by type I mouse (BALB/C, CBA/J, etc), was expressed in E. coli and purified as previously described (9). Nine-week-old female WKAH/HKm rats (Japan SLC Inc, Hamamatsu, Japan) were injected subcutaneously with 1 mg of the SAA protein emulsified in Freund's complete adjuvant. After four weeks 0.5 mg of SAA without adjuvant was given. Three days after the last injection the spleen cells were obtained. Fusing these cells to a murine myeloma SP_2 cell, screening of antibody-producing hybridoma, and cloning of them were carried out as previously described (7). Ascites were generated in a nude mouse from the desired clones, fractionated to immunoglobulins, and used as the monoclonal antibodies. Rabbit polyclonal antibodies to murine SAA were prepared by immunizing rabbits with the recombinant SAA1 proteins (9).

Immunoblotting

Nine-week-old ICR female mice (Japan SLC) were injected daily with azocasein as previously described (10). Some animals were sacrificed at day-3 to obtain acute phase sera, and others were sacrificed at day-14 to obtain amyloid-laden spleen. Amyloid fibrils were prepared from the spleen as previously described (11). These were subjected to a tricine sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane.



a. Two site-ELISA b. Direct binding ELISA

FIGURE 1. Schemes of two-site ELISA (a) and direct binding ELISA (b) for measuring murine SAA.

Subsequently the reaction of the antibodies with the SAA or degraded SAA on the membrane was examined immunoenzymatically (12).

Two site enzyme-linked immunosorbent assay (Figure 1a)

The selected monoclonal antibody, 2 mg/l in 0.05M carbonate buffer, pH 9.6, coated a 96-well microtiter plate overnight at 4°C. For the reaction of process a volume of 100 µl was maintained throughout the experiment. The plates were blocked with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 3 hours at 37°C. Plasma samples, diluted 1:100 or more in 1% BSA-PBS containing 0.05% Tween 20 (T), were added to the plates (*step 1*). After a 2-hour incubation period followed by washing with PBS-T, the plates were incubated with 2 mg/l polyclonal anti-SAA in 1% BSA-PBS-T for 2 hours at 37°C (*step 2*). Then,

the plates were reacted with 1:1000 diluted peroxidase-conjugated anti-rabbit immunoglobulins (Dako, Carpinteria, CA, USA) for 1 hour at 37°C followed by color development (*step 3*). The primary standard of the assay was a pool of acute phase HDL, which was prepared by ultracentrifugation from acute phase sera. Its SAA content was determined by SDS-PAGE as previously described (13). The pool of acute phase sera with 1,000 mg/l of SAA was diluted to 10 mg/l of SAA with PBS and used as the working standard for each assay .

Direct binding enzyme-linked immunosorbent assay (Figure 1b)

Based on the method, by which human SAA was measured (14), murine plasma samples were diluted 1:1000 or more by 0.05M carbonate buffer, pH 9.6, containing 3M potassium bromide and coated 96-well plates overnight at 37°C. Following procedures noted above, the plates were blocked, then subjected to steps 2 and 3 with alternative use of the monoclonal anti-mouse SAA (1:1000 diluted) in step 2 and peroxidase-conjugated anti-rat immunoglobulins (Dako) in step 3.

Mouse experiment

Ten ICR female mice, each 7 weeks old, were purchased from the provider (SLC), separated into two groups (5 in a cage), and reared in a normal way. Plasma samples were obtained by bleeding into capillary tubes from eye lids on the same day of purchase and 7 days later. Then, each mouse was given intraperitoneally 10 μg lipopolysaccharide (LPS) (from E. coli 055:B5, Difco, Detroit, MI, USA). Two days later plasma was withdrawn. Plasma samples were kept at 4°C until the measurement of SAA concentration .

RESULTS

Monoclonal antibodies

Twenty wells containing hybridomas were positive on the initial screening. They were divided into three or more groups, based on the reactivity with the

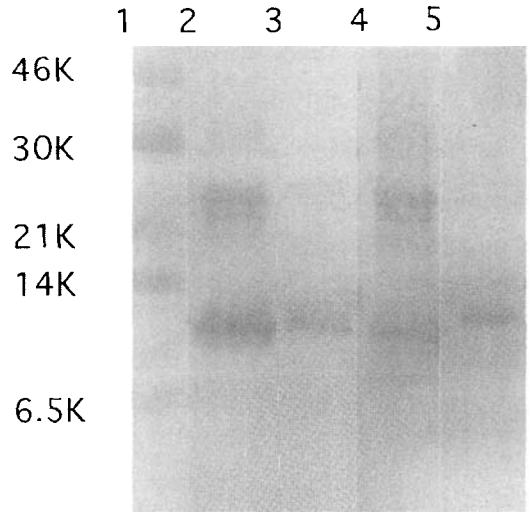


FIGURE 2. Immunoblotting for evaluating anti-murine SAA antibodies. Acute phase sera, 0.5 μl (lane 2,4) and amyloid fibrils, 0.5 mg (lane 3,5) were subjected to immunoblotting. The monoclonal antibody (M10) was reacted with lane 2 and3, and the polyclonal antibodies with lane 4 and 5. Lane 1 indicates the molecular weight markers.

degraded SAA proteins on the immunoblotting. A group, which was found to react with intact SAA and minimally degraded SAA (Figure 2), was successfully cloned (named M-10) by limiting dilution and used for the ELISA. The other two groups, one reacting only with intact SAA and the other reacting with intact SAA and largely degraded SAA the latter the same as polyclonal antibodies in Figure 2, were considerably unstable when subjected to limiting dilution procedures. Currently, cloning of the two has not been successful.

ELISA for murine SAA

The calibration curve of the two-site ELISA (t-ELISA) is shown in Figure 3a. The practical and measurable range of SAA is approximately 1 to 20 μg/l.

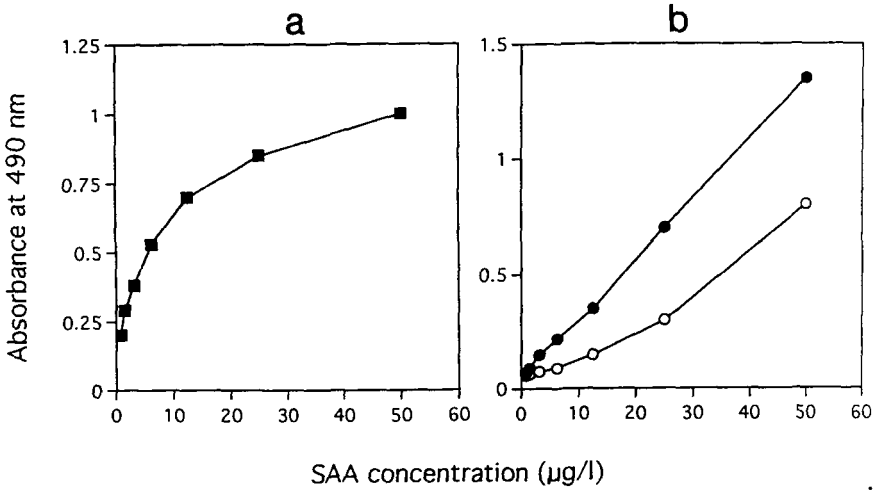


FIGURE 3. Calibration curve for SAA assay. Standard serum (1,000 mg/L) was serially diluted and subjected to the two site-ELISA (a), or the direct binding ELISA (b) with the use of the monoclonal antibody (open circle) or the polyclonal antibodies (closed circle).

When plasma is diluted 1:100 diluted, 0.1 to 2 mg/l of SAA can be quantified. When the plasma sample with 0.95 mg/l of SAA was measured, the coefficient variation (CV) was 5.6% and 8.9%, for within run (n=10) and day to day run (n=4), respectively. Recovery of the assay standard, when added to the normal pooled sera, was 95%.

The calibration curve of the direct binding ELISA (d-ELISA) is shown in Figure 3b. By the use of either the monoclonal or the polyclonal anti-SAA antibody as the first antibody, SAA could be measured in the range of 1 to 50 μg/l, wider than that by t-ELISA. The calibration curve of d-ELISA, when the polyclonal antibody was used, was nonlinear in the range of low SAA concentration. When the plasma sample with 12 mg/l of SAA was measured by the monoclonal antibody-

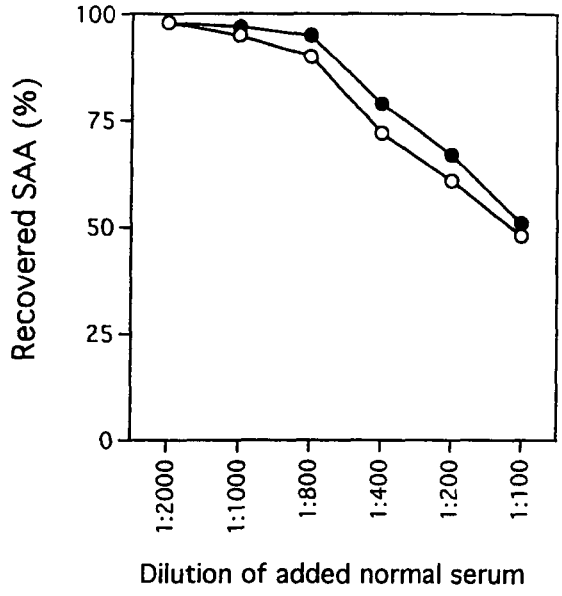


FIGURE 4. Recovery of added SAA to normal plasma in direct binding ELISA. Standard serum was added to severally diluted normal sera and SAA concentrations were measured by the direct binding ELISA with the use of the monoclonal antibody (open circle) or the polyclonal antibodies (closed circle).

based d-ELISA, the CV was 3.6% and 7.7%, for within run (n=10) and day to day run (n=4), respectively. To assess the appropriate dilution fold of plasma samples, the standard serum was measured after it was added (final SAA concentration; 12.5 μg/l) to serially diluted samples of normal plasma. When added to less than 1:80 diluted normal plasma, recovery of SAA became poor in d-ELISA (Figure 4). This means that plasma samples should be diluted more than 1:1000 before coating the plates. Thus, when 1:1000 diluted plasma was used, the assay range results in 1 to

FIGURE 5. SAA concentrations in ten mice on the day of purchase and after seven days of rearing. Each symbol indicates an individual animal.

50 mg/l of SAA. The values obtained by the monoclonal antibody-based d-ELISA showed a good agreement with those obtained by t-ELISA ($r=0.89$, $n=20$)

SAA concentrations in mouse plasma

SAA concentrations in non-inflamed and inflamed mice were measured by t-ELISA and dm-ELISA, respectively. As shown in Figure 5, one out of ten animals had a relatively higher SAA (1.6 mg/l) than others on the day of purchase. However, seven days later the value decreased to a level similar to those of the other animals. SAA concentrations of the other nine animals showed no obvious changes in this period. Those of the ten animals at the end of week-1 was 0.47± 0.17 mg/l (mean±SD). Two days after injection by LPS, the mean elevation of SAA concentrations reached 300 mg/l. There was a variation in SAA concentrations (59 mg/l at the least and 980 mg/l at the most) between animals as shown in Figure 6.

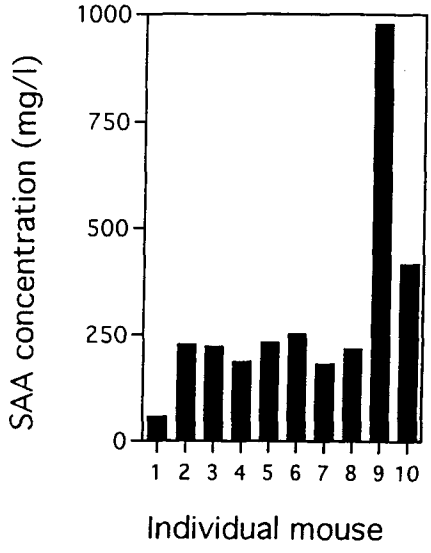


FIGURE 6. SAA concentrations in ten mice two days after the administration of lipopolysaccharide.

DISCUSSION

The monoclonal antibody established in this study was found to react with intact SAA and with minimally degraded SAA, but not with the common form of AA proteins, corresponding to the amino-terminal 76 residues of SAA. This suggested that the reacting epitope of the antibody was somewhere from the residue 76 to the carboxyl-terminus. Successful utilization of the antibody in the ELISA suggested that the epitope was exposed in HDL particles.

Two ELISA methods were established. Direct binding ELISA was simple to perform. Also more advantageous than the two-site ELISA would be wider and more linear calibration curve. An irregular calibration curve was obtained by the use of polyclonal antibodies, but not by the monoclonal antibody. While the reason

was unknown, probably polyvalent binding between polyclonal antibodies and antigens on the plastic surface was influenced by the density of the antigen. The hydrophobic nature of SAA, which allowed the protein preferentially to bind to the plastic surface, permited this ELISA method (14). Theoretically, the more plasma samples were diluted, the less other plasma components interfered with the binding of SAA to the plastics. Thus, this method was suitable for inflamed plasma, which needed high dilution. The required volume of plasma is 1-2 μl, which could minimize the amount needed in blood drawing. However, this study demonstrated that lower diluted plasma could not be subjected to the method because of the interference problem. This made impossible the analysis of SAA concentrations in non-inflamed mice.

The two-site ELISA was sensitive enough to determine SAA concentrations of mice at the steady state. Some investigators believed that animals experience stress when transferred from the providers to laboratory facilities. They proposed waiting for a period of time, perhaps weeks, before initiating experiments, thereby allowing animals to adjust to their new environment. In this study one animal showed a slight elevation of plasma SAA the day of purchase, followed by a decrease after seven days. Although there was no way to know whether the mouse was loaded by stress on the day of purchase, the change in the SAA concentration suggested that it was. Thus, this ELISA method can be utilized to detect a slight inflammation or stress in mice.

Treatment of mice with LPS yielded a clear elevation in SAA concentrations. There was a considerable variation in SAA values between animals. We sometimes experienced that ICR mice do not behave uniformly when induced amyloidosis. Variation in SAA response to the stimuli among individual animals may be responsible for this.

In conclusion, this study developed two ELISA methods for murine SAA. Each should be chosen according to the SAA concentration to be analyzed.

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