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Automated Measurement of a Constitutive Isotype of Serum Amyloid A/SAA4 and Comparison With Other Apolipoproteins

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A constitutive isotype of serum amyloid A (SAA4) is present mostly in high density lipoprotein (HDL) and a little in other lipoproteins. In this study, we developed an automated method for measuring SAA4 concentration in serum by kinetic nephelometry of anti-SAA4 antibody-coated latex agglutination. Rabbit antibodies generated by immunization of recombinant SAA4 were found to have no apparent reactivity with acute phase SAA. The values determined by this method and by the previous enzyme immunoassay showed good agreement (r=0.862). Serum SAA4 values of 26 healthy adults ranged from 37–109 mg/L (mean; 62 mg/L). Their SAA4 concentrations were not significantly related with those of apolipoprotein A-I, A-II, B, C-II, C-III or E. Also, SAA4 did not correlate with cholesterol in preparation after removal of very low density lipoprotein and low density lipoprotein. These suggest the unique behavior of SAA4 in lipoprotein metabolism, while what contributes to variation of SAA4 levels in serum, especially in HDL, remains to be clarified. J. Clin. Lab. Anal. 11:363–368, 1997.

Key words: high density lipoprotein; recombinant protein; latex agglutination; nephelometry; acute phase

INTRODUCTION

Serum amyloid A (SAA) was first described as a serum component sharing immunoreactivity with amyloid A protein (AA), the major constituent of reactive amyloid deposits [1,2]. SAA is now believed to be a serum precursor of AA. There is a polymorphism in SAA protein; six genetically determined isotypes (SAAlα, SAA1β, SAAlγ, SAA2α, SAA2β, SAA4) have been identified [1,3,4,5]. These are classified into two groups based on the regulation of their synthesis. One group is the acute phase isotypes (A-SAA) including SAA1 and SAA2, the serum level of which is markedly increased during inflammation. A-SAA has been proposed to be involved in several inflammatory events such as leukocyte chemotaxis [6], induction of collagenase [7] and inhibition of platelet agglutination [8]. As an apolipoprotein of high density lipoprotein (HDL), A-SAA may maintain the reverse cholesterol transport system by the increasing cellular affinity of HDL [9]. The other isotype, SAA4, was identified as a constitutive apolipoprotein [4]. SAA4 does not increase serum level in inflammation [10] nor serve as amyloid fibril precursor [11].

As for the characteristics of SAA4 as a plasma apolipoprotein, findings are that SAA4 is distributed primarily to HDL, and a little to very low density lipoproteins (VLDL) and low density lipoproteins (LDL) [12,13], and that serum SAA4 levels are elevated in hypertriglyceridemia [13].

We have developed a direct binding enzyme-linked immunosorbent assay (ELISA) utilizing anti-SAA4 monoclonal antibody for measuring SAA4 in serum [10]. However, since diluted serum is directly bound to plastic wells in this assay, interaction between serum components other than SAA4 and labeled antibodies cannot be avoided. It remains unknown if SAA4 in different lipoprotein particles is measured equally. In this study, we established a latex agglutination nephelometric immunoassay for measuring SAA4, based on its application for A-SAA [14]. In addition, the relationship between SAA4 and other apolipoproteins was investigated.

MATERIALS AND METHODS

Preparation of Antibodies

Human SAA4 was expressed in E. coli and purified as previously described [11]. Lyophilized SAA4 was solubilized at 1.0 g/L in 0.1 M Tris-HCl, pH 8.0, containing 0.05% Tween-20. Of this solution, 1 mL was mixed with 1 mL of Freund complete adjuvant and immunized subcutaneously into a New Zealand white rabbit bi-weekly for 4 months. Antiserum was obtained 3 days after the last injection (1 mL) without adjuvant. Specificity of the antibodies was examined by immunoblotting against serum sample and purified A-SAA as previously described [10].

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Latex agglutination nephelometric immunoassay (LA assay)

Immunoglobulin fractions were obtained by treatment of the antiserum with 40% ammonium sulfate and used as anti-SAA4 antibodies. Antibodies and polystyrene latex particles (mean diameter, 0.1 μm), at concentrations of 5 g/L and 20 g/L, respectively, in 0.1M HEPES buffer, pH 7.4, were incubated for 1 hr at 37°C. After being washed by centrifugation, anti-SAA4-conjugated latex particles were suspended at 0.4% in 0.005M HEPES buffer and used for LA assay as the latex reagent. Using an automated nephelometry analyzer, LX-3000 (Eiken Chemical, Tokyo, Japan), 100 μL of latex reagents, 200 μL of HEPES buffer, and 35 μL of 1:10 diluted serum samples were incubated at 37°C. Light scattering was measured every 130 sec. Increase in light scattering at appropriate intervals was used for calculation of SAA4 concentration. The same standard serum as for the previous ELISA was adapted for constructing a calibration curve

Samples

Serum samples were obtained from 26 healthy adults (19 male, 7 female, ages ranged 20–65). Plasma was obtained from a man with hypertriglyceridemia and fractionated into chylomicrion, VLDL(d<1.006), LDL(1.006<d<1.063), and HDL(1.063<d<1.21) by ultracentrifugation [15]. Patient samples which were removed VLDL and LDL by phosphotungstate magnesium chloride treatment for analysis of HDL-cholesterol were randomly selected. After excluding samples from subjects which had apparent diseases, the 38 samples were dialyzed against saline and subjected to SAA4 analysis. Twenty-eight sera were collected from patients with several inflammatory disorders for comparison with acute phase SAA.

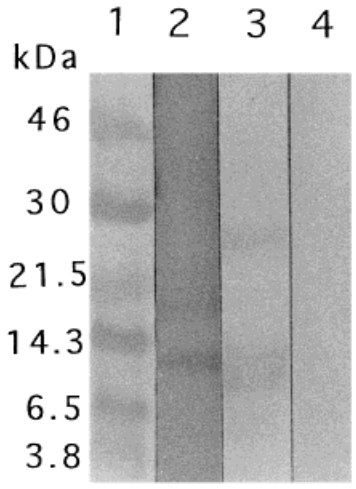

Fig. 1. Specificity of the antiserum generated against recombinant SAA4. A 1 mL sample of patient serum with 550 mg/L of acute phase SAA (lane 2 and 3) and 10 mg of purified acute phase SAA (lane 4) were subjected to SDS-PAGE followed by immunoblotting. Lane 2 and 4 were incubated with 1:200 diluted anti-SAA4 antiserum, while lane 3 was with 10 mg/L anti-acute phase SAA abtibodies [14]. Molecular weight markers were in lane 1.

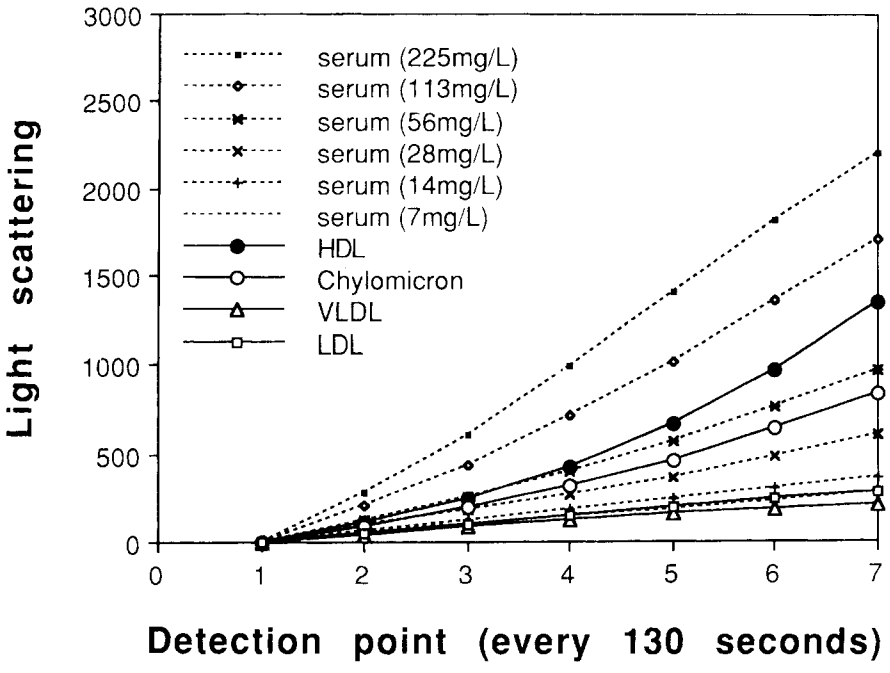

Fig. 2 Time course of latex agglutination. Serially diluted standard serum (concentration of SAA4 is indicated) and preparations of chylomicron (cholesterol, 360 mg/L; triglyceride, 1020 mg/L), VLDL (Cho, 1020 mg/L; TG, 533 mg/L), LDL (Cho, 6860 mg/L; TG, 1260 mg/L), HDL (Chol, 84 mg/L; TG, 14 mg/L) were subjected to the LA assay. Light scattering was detected every 130 sec. The latex reagents were added and incubation was started at detection point 1 in the figure.

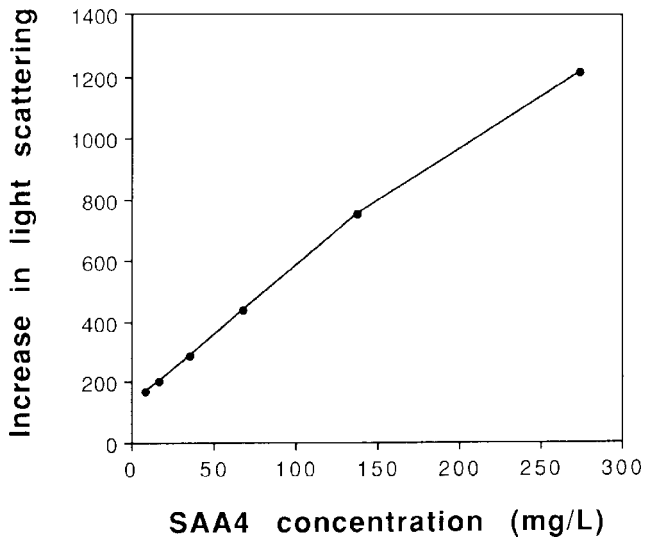

Fig. 3 Calibration curve of LA assay for SAA4. The curve was calculated by increase in light scattering generated by serially diluted standard serum.

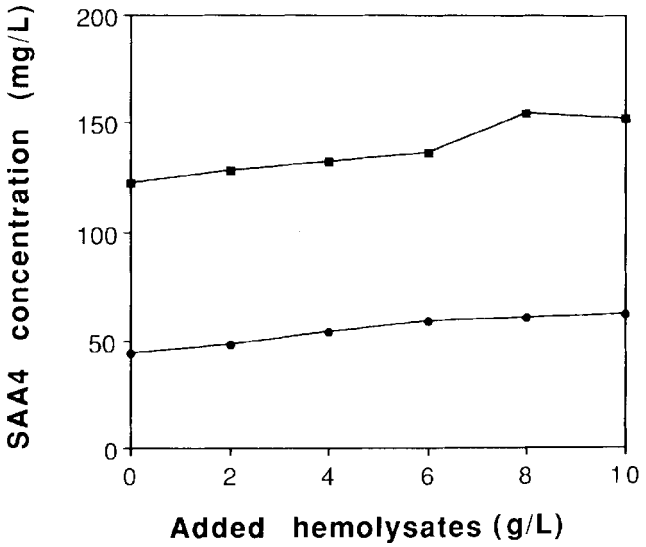

Fig. 4 Effect of hemolysates on the assay results of SAA4. Hemolysates are shown as concentrations of hemoglobin.

Others

SAA4 was quantitated also by ELISA as previously described [10,13]. Apolipoprotein A-I, A-II, B, C-II, C-III, E, and A-SAA were measured by turbidimetric immunoassay or latex agglutination nephelometric immunoassay (Eiken Chemical).

RESULT

Antibodies

The resulting antiserum did not yield a precipitation line against SAA4 in serum in agar double immunodiffusion. Therefore, immunoblotting was used to evaluate the specificity of the antiserum. The antiserum reacted with two components in serum corresponding to glycosylated and non-glycosylated forms of SAA4 but not with acute phase SAA (A-SAA) [Fig. 1].

Assay conditions of LA assay for SAA4

As shown in Fig. 2, time course of latex agglutination was almost parallel between standard serum, chylomicron, VLDL and LDL. HDL appeared to have a comparatively strong reactivity by prolonged incubation. Therefore, increase in light scattering in 390 seconds after addition of the latex reagents was used to generate a nearly linear calibration curve in the range of 8-275 mg/L of SAA4 (Fig. 3), which possibly includes the occurring serum level of SAA4.

Evaluation of SAA4 LA assay

The coefficient variation of within the assay (n=10) was 5.2% for serum with 53 mg/L of SAA4 and 3.5% for 162 mg/L. The recovery of SAA4 in standard serum added to the normal pooled sera resulted in values of 95% and 105%, when 55 mg/L and 137 mg/L of SAA4 was added, respectively. Interference studies indicated that 250 mg/L of bilirubin did not affect measurement, but hemolysates had a positive effect (20–40% increase under the presence of hemolysates with 10 g/L hemoglobin) (Fig. 4). Addition of 100 mg/L of A-SAA, which was purified from sera [14], had no positive effect on the assay results. SAA4 values determined by LA assay for 33 randomly selected subjects closely correlated to those by EIA (r=0.862, Fig. 5).

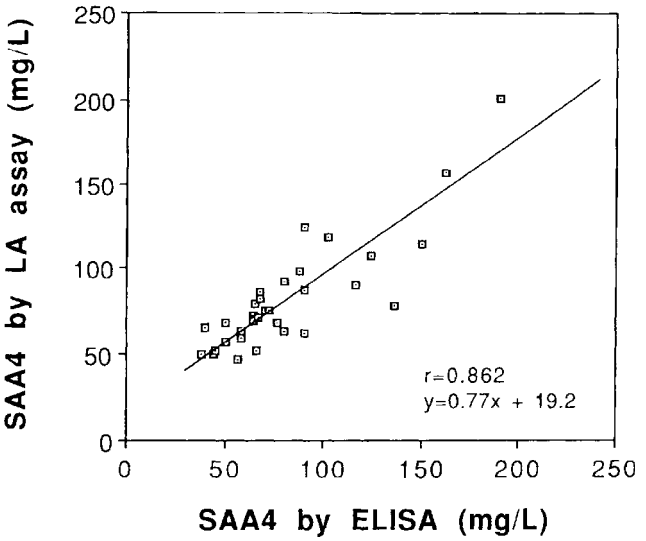

Fig. 5 Correlation of SAA4 values obtained by LA assay for 33 randomly selected subjects with those by enzyme-linked immunosorbent assay.

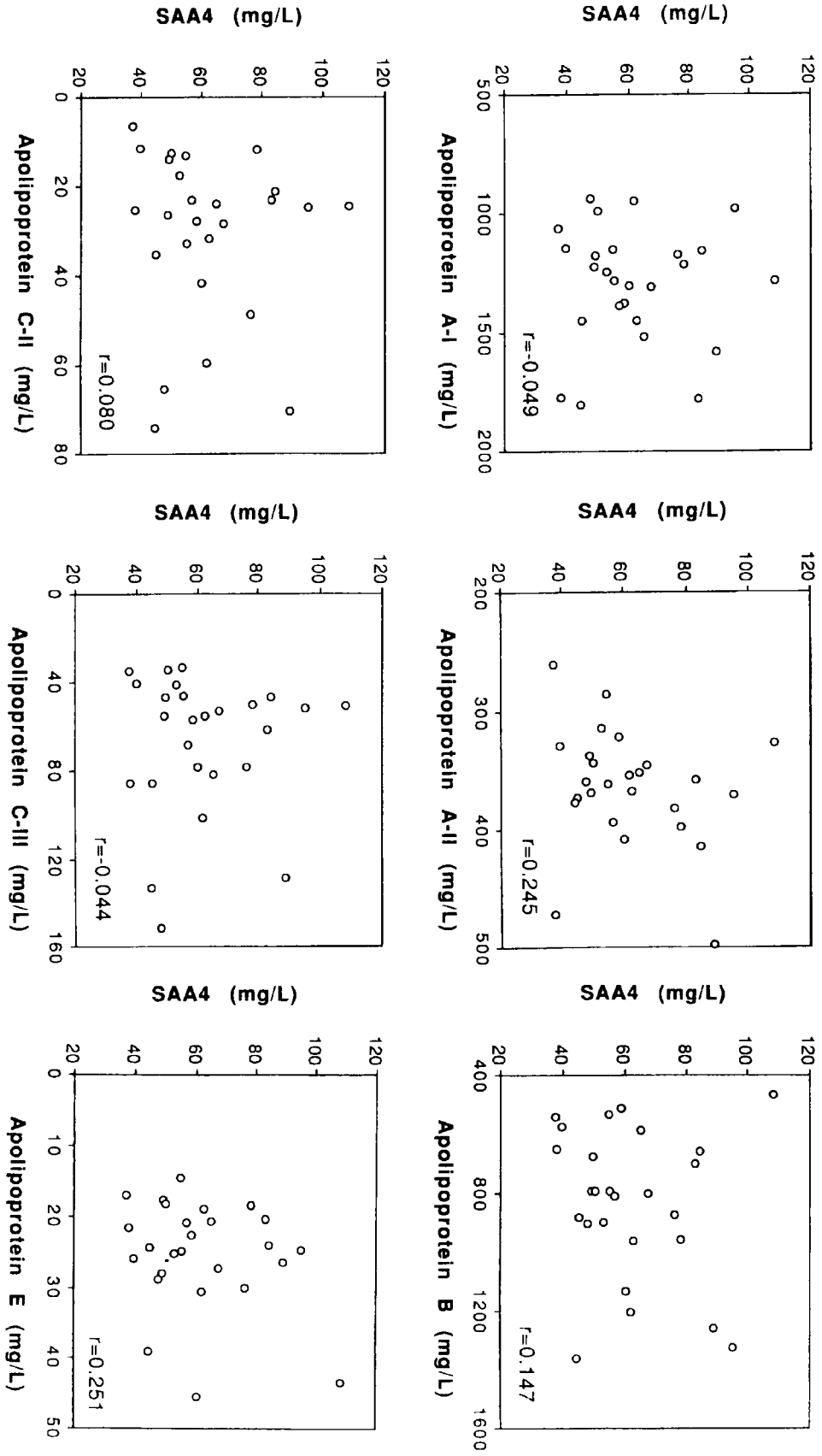

Fig. 6 Relation of SAA4 values to other apolipoproteins (A-I, A-II, B, C-II, C-III and E) in 26 healthy subjects.

SAA4 levels in healthy subjects and relationship to other parameters

SAA4 concentration of 26 healthy subjects ranged from 37–109 mg/L (mean±SD; 62±18.2 mg/L). No significant relationship in values between SAA4 and any apolipoprotein was found (Fig. 6). In preparations without VLDL and LDL, SAA4 levels were not correlate to cholesterol levels (Fig. 7). SAA4 levels had no relationship to A-SAA levels in inflammatory sera (Fig. 8).

DISCUSSION

In this study, recombinant proteins were used to generate antiserum, thus avoiding the requirement of a large amount of serum and laborious procedures to prepare SAA4. Furthermore, no contamination of serum-derived components in immunogens prevents the resulting antibodies from reacting with such components in practical assays. However, we observed positive interference of hemolysates in the assay. Although no component in the hemolysates was lightened by the present antiserum on immunoblotting (data not shown), it is possible that substances contaminated from E. coli components or culture media in the immunogens generated antibodies reactive to components in hemolysates. This should be further investigated.

The antiserum obtained did not react with A-SAA on immunoblotting. The LA assay also showed that addition of A-SAA (SAA1) had no positive influence. In contrast, the rabbit antibodies against A-SAA did not react with SAA4 (Fig. 1). This may be due to comparatively poor homology (approximately 50%) in primary structure between both isotypes [4].

SAA4 is present not only in HDL but also in VLDL and LDL [12,13]. The LA assay measured SAA4 of each lipoprotein fraction in almost the same fashion in the assay period, suggesting accuracy of this assay when dyslipidemic samples are measured. This study revealed that SAA4 was present also in chylomicron. Distribution of SAA into triglyceride-rich lipoproteins may be responsible in part for the poor relationship of this protein with other apolipoproteins. However, even in HDL-containing preparations without SAA4 in triglyceride-rich lipoproteins, SAA4 levels were not related to cholesterol levels, suggesting that variation of SAA4 in HDL may affect total serum levels of this protein. Though not significant, comparatively good relationships with apolipoprotein A-II and E were found. This may be due to the similarity of these apolipoproteins in their distribution among lipoprotein fractions. In inflammatory states, SAA4 behavior was indiscriminate, consistent with the previous findings [10,16].

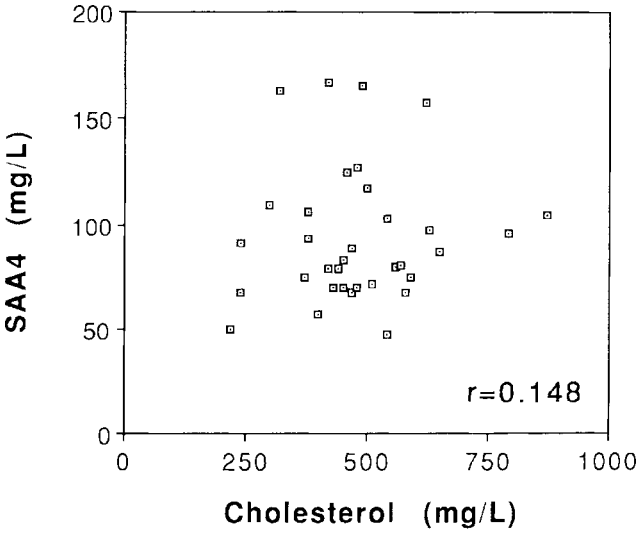

Fig. 7 Relationship in values between SAA4 and cholesterol in 38 preparations after removal of VLDL and LDL.

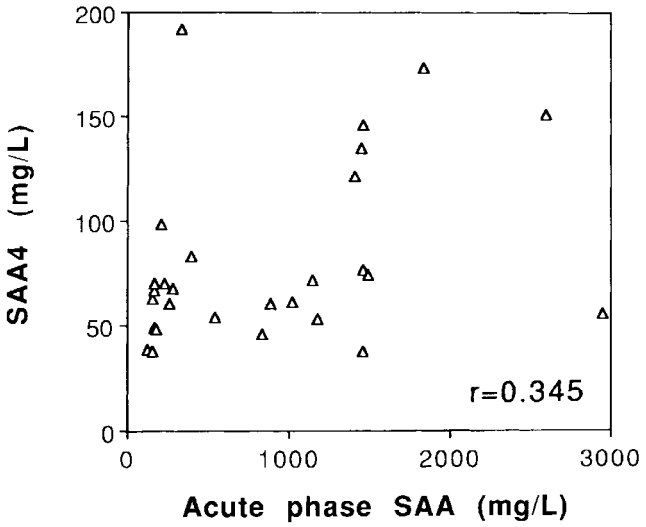

Fig. 8 Relationship in values between SAA4 and acute phase SAA in 28 inflammatory sera.

In conclusion, we established an automated method for measuring serum SAA4 levels. Comparison of SAA4 with other apolipoproteins could not determine a specific feature of this protein in lipoprotein metabolism. Further investigation including behavior of SAA4 in pathological conditions is necessary with this assay method.

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REFERENCES

1. Husby G, Marhaug G, Dowton B, Sletten K, Sipe JD: Serum amyloid A (SAA). biochemistry, genetics and the pathogenesis of AA amyloidosis. *Amyloid Int J Exp Clin invest* 1:119–137, 1994.

2. Malle E, de Beer FC: Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice. *Eur J Clin invest* 26:427–435, 1996.
3. Dwulet FE, Wallace DK, Benson MD: Amino acid structures of multiple forms of amyloid-related serum protein SAA from a single individual. *Biochemistry* 27:1677–1682, 1988.
4. Whitehead AS, de Beer MC, Rits M, Lelias JM, Lane WS, de Beer FC: identification of novel members of serum amyloid A protein superfamily as constitutive apolipoproteins of high density lipoprotein. *J Biol Chem* 267:3862–3867, 1992.
5. Baba S, Takahashi T, Kasama T, Fujie M, Shirasawa H: A novel polymorphism of human serum amyloid A protein, SAA1, is characterized by alanines at both residues 52 and 57. *Biochim Biophys* Acta 1180:195–200, 1992.
6. Badolato R, Wang JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, Kelvin DJ, Oppenheim JJ: Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J Exp Med* 180:203–210, 1994.
7. Mitchell TI, Coon CI, Brinckerhoff CE: Serum amyloid A (SAA3) produced by rabbit synovial fibroblasts treated with phorbol esters or interleukin 1 induces synthesis of collagenase and is neutralized with specific antiserum. *J Clin invest* 87:1177–1185, 1991.
8. Zimlichman S, Danon A, Nathan I, Moses G, Shaikin-Kestenbaum R: Serum amyloid A, an acute phase protein, inhibits platelet activation. *J Lab Clin invest* 116:180–186, 1990.
9. Kisilevsky R, Subrahmanyan L: Serum amyloid A changes high density lipoproteins cellular affinity. *Lab invest* 66:778–785, 1992.
10. Yamada T, Kluve-Beckerman B, Kuster WM, Liepnieks JJ, Benson MD: Measurement of serum amyloid A4 (SAA4). its constitutive presence in serum. *Amyloid Int J Exp Clin invest* 1:114–118, 1994.
11. Yamada T, Kluve-Beckerman B, Liepnieks JJ, Benson MD: Fibril formation from recombinant human serum amyloid A. *Biochim Biophys Acta* 1226:323–329, 1994
12. de Beer MC, Yuan T, Kindy MS, Asztalos BF, Roheim PS, de Beer FC: Characterization of constitutive human serum amyloid A protein (SAA4) as an apolipoprotein. *J Lipid Res* 36:526–534, 1995.
13. Yamada T, Miida T, Itoh Y, Kawai T, Benson MD: Characteriztion of serum amyloid A4 as a plasma lipoprotein. *Clin Chim Acta* 251:105–112, 1996.
14. Yamada T, Nomata Y, Sugita O, Okada M: A rapid method for measuring serum amyloid A protein by latex agglutination nephelometric immunoassay. *Ann Clin Biochem* 30:72–76, 1993.
15. Hatch FT, Lee RS: Practical methods for plasma lipoprotein analysis. *Adv Lipid Res* 6:2–68, 1968.
16. Kumon Y, Loose LD, Birbara CA, Sipe JD: Rheumatoid arthritis exhibits reduced acute phase and enhanced constitutive serum amyloid A protein in synovial fluid relative to serum. A comparison with C-reactive protein. *J Rheumatol* 24:14–19, 1997.