

Mapping of Antigenic Determinants of Purified, Lipid-Free Human Serum Amyloid A Proteins

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Serum amyloid A (SAA) is the major apolipoprotein of high-density lipoproteins (HDL) present during the acute-phase reaction. To map specific epitopes on purified, lipid-free SAA, sequence-specific antibodies raised against synthetic peptides corresponding to amino acid residues 1–17, 14–30, 27–44, 40–63, 59–72, 68–84, 79–94 and 89–104 of human SAA₁ were studied. Using the indirect sandwich dissociation-enhanced lanthanide fluorescence immunoassay, antibodies raised against epitopes comprising residues 1–17, 14–30, 40–63 and 79–94 failed to recognize the corresponding domains on isolated human SAA₁/SAA₂ or a mixture of both isoforms, indicating that these epitopes are masked, apparently because of specific folding and/or self-aggregation (dimerization). The accessible antigenic determinants of isolated SAA are epitopes comprising residues 31–39, 64–78 and 95–104. The present findings indicate that: (i) the same epitopes are exposed, irrespective whether SAA is HDL-associated or in its lipid-free form and that (ii) monomeric and dimeric SAA co-exist to a similar extent in the lipid-free form, irrespective of whether conditions are non-denaturing, denaturing, acidic or basic. From our studies it is proposed that isolated, purified SAA may serve as a reliable standard for quantification of HDL-associated SAA and for mimicking the interaction of acute-phase HDL particles with peripheral tissues *in vitro*.

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INTRODUCTION

The serum amyloid A (SAA) family of proteins is encoded by multiple genes that display allelic variation and a high degree of homology in mammals [1,2]. In humans, SAA₁ and SAA₂ isoforms (104 amino acids, 12 kDa), the products of two almost identical genes [3], are extremely sensitive positive acute-phase proteins. Triggered by inflammation after stimulation of hepatocytes by lymphokine-mediated processes [4], the concentration of SAA may increase to levels 1000-fold greater than that found in the non-inflammatory state, thus representing an ideal marker for clinical practice [5]. Besides its role as an acute-phase reactant, SAA is considered to be the precursor protein of secondary reactive amyloidosis [6], where the N-terminal portion is incorporated into the bulk of amyloid fibrils [7]. However, an association with high-density lipoproteins (HDL) and subsequent modulation of the metabolic properties of its physiological carrier (HDL) appears to be the principal role of SAA [8]. Displacement of apolipoprotein A-I (apo A-I) [9], the major

protein component of native HDL, by SAA may significantly impede the protective function of HDL in 'reverse cholesterol transport'. In comparison with native HDL, SAA-enriched (acute-phase) HDL has reduced binding properties to hepatocytes [10]. In parallel, SAA-enriched HDL shows an increased affinity to macrophages, resulting in a significant HDL-mediated cholesterol/cholesterol ester delivery towards these cells. Consequently, monocyte-derived macrophages may develop into cholesterol-enriched foam cells, which are the hallmark of fatty streaks and the earliest recognizable lesion of atherosclerosis [11].

The association of SAA with its physiological carrier suggests that certain epitopes are buried in the HDL particle while others mediate the binding of acute-phase HDL to peripheral cells [12]. As isolated SAA protein is often used both as a standard for calibration purposes of SAA-associated HDL particles and as a protein mimicking the properties of acute-phase HDL, we attempted to identify *in vitro* the accessible antigenic determinants on purified, lipid-free SAA.

MATERIALS AND METHODS

Isolation of human SAA₁. SAA was prepared directly from human plasma (obtained from patients with rheumatoid arthritis or patients undergoing plasmapheresis for therapeutic purposes) by hydrophobic interaction chromatography on Octylsepharose CL 4B followed by gel permeation-fast protein liquid chromatography on Superdex™ 75 prep grade, as described previously [13]. Subsequently, SAA₁ was purified from remaining SAA₂ isoforms by preparative isoelectric focusing using the Rotofor system (Bio-Rad, Richmond, CA) and buffers containing 6 M urea, 10% (wt/vol) glycerol and 6.4% (wt/vol) carrier ampholytes.

Polyclonal antisera. Polyclonal antisera to purified SAA₁ and sequence-specific antibodies to SAA₁ (corresponding to amino acid residues 1–17, 14–30, 27–44, 40–63, 59–72, 68–84, 79–94 and 89–104 of human SAA₁) were raised in rabbits [13]. The immunogen was the synthetic peptide coupled via its N-terminal residue to N-maleimido-butyl-N-hydroxysuccinimide ester-activated keyhole-limpet haemocyanin. Affinity-purified immunoglobulin Gs (IgGs), specific for the various SAA₁ epitopes, were purified by immunoaffinity chromatography on SAA coupled to cyanogen bromide (CNBr)-activated Sepharose 4B or epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) under denaturing conditions (pH 9.5–10.5). The rabbit anti-SAA₁ peptide antisera (raised against amino acid residues 1–17, 14–30, 27–44, 40–63, 59–72, 68–84, 79–94 and 89–104 of human SAA₁) and the corresponding affinity-purified IgGs reacted immunochemically with both SAA₁ and the corresponding peptides only.

Immunoblotting. Immunochemical detection of SAA transferred to nitrocellulose after SDS-polyacrylamide gel electrophoresis (PAGE) under denaturing conditions or after non-denaturing PAGE [14] was performed by incubation with polyclonal or sequence-specific antisera (IgG) (diluted 1:500) followed by incubation with peroxidase-conjugated goat-antirabbit IgG (diluted 1:5000). To visualize bound antibody, ECL-Western-blotting detection reagents and Hyperfilm-ECL (Amersham, Buchler Braunschweig, Germany) were used.

Epitope mapping. Epitope mapping of isolated SAA₁, SAA₂ and a mixture of both isoforms was performed by an indirect enzyme immunoassay, the dissociation-enhanced lanthanide fluorescence immunoassay (DELFI), utilizing Eu³⁺ as a highly fluorescent compound [12]. The coating of microtitre plates with affinity-purified polyclonal antihuman SAA sheep IgGs, blocking and sample application and all the incubation conditions using primary (Eu³⁺-labelled) and secondary antibodies have been described previously [12]. The labelling of polyclonal goat anti-rabbit IgG and polyclonal antihuman SAA IgGs was performed with the Eu³⁺ chelate of N¹-(p-isothiocyanatobenzyl)-diethylenetriamine-N¹,N²,N³,N³-tetraacetic acid. Measurement of time-resolved immunofluorescence (excitation wavelength: 320 nm; emission wavelength: 615 nm) was performed on a 1234 DELFIA fluorimeter (Wallac, Oy, Sweden) [12].

HPLC analysis. Separation of SAA₁ and SAA₂ isoforms and corresponding des-Arg forms (lacking the N-terminal arginine) was performed on a Shimadzu (Korneuburg, Austria) LC-10 AT with a Sil-10AXL auto-injector (equipped with an cooling option and a SPD-10 A dual channel detector) using a Vydac (Hesperia, CA, USA) 218TPP5215 RP-18 column (300 Å, 5 µm, 150 × 4 mm). Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile (ultra gradient grade). The gradient was stepped from 10% solvent B to 60% B over 30 min. The flow rate was 1.0 ml/min. Detection was performed by means of a dual channel detector at 215 nm and 280 nm, respectively.

Mass spectrometry. Mass spectrometry analyses were performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (MALDI II, linear time-of-flight device; Kratos Ltd, Manchester, UK). The matrix solution contained α-cyano-4-hydroxycinnamic acid in 50% (vol/vol) water/acetonitrile. Horse myoglobin was used as the internal standard.

RESULTS AND DISCUSSION

The isolated SAA fraction (purified by hydrophobic interaction chromatography and gel filtration) used in the present study had a molecular mass of 12 kDa and was immunochemically reactive towards different polyclonal antibodies in Western blotting experiments.

The observation that isolated, purified SAA may be as suitable a standard for calibration purposes as SAA in its native state in the HDL particle, led to suggestions that similar antigenic determinants are accessible on the surface of both SAA species. We therefore tested the ability of different polyclonal antibodies raised against specific SAA peptides to recognize the corresponding epitopes of purified SAA in the DELFIA assay system. The results of these experiments are shown in Fig. 1. In contrast to isoelectric focusing and immunoblotting, where SAA is denatured and thus recognized by all the sequence-specific antisera raised against amino acid residues 1–17, 14–30, 27–44, 40–63, 59–72, 68–84, 79–94 and 89–104 of human SAA₁, it is evident that only a few epitopes of purified SAA are exposed and recognized by sequence-specific antibodies (Fig. 1, A and B). Affinity-purified IgGs raised against epitopes comprising residues 27–44, 59–72, 68–84 and 89–104 of human SAA protein are suitable for quantification of SAA but IgGs raised against epitopes comprising residues 1–17, 14–30, 40–63 and 79–94 failed to recognize the corresponding epitopes on pure SAA protein in the DELFIA assay system. The same results were obtained when sequence-specific antisera without further purification were used as detection antibodies in the indirect DELFIA assay.

As in humans the predominant SAA isoform of SAA-enriched HDL is SAA₁ (up to 87%) [9], the purified lipid-free SAA fraction described above in the Materials and methods was subjected to HPLC analysis, resulting in four peaks (Fig. 2) with retention times at 20.6, 21.1, 21.6 and 22.1 min. Analysis of these four fractions by MALDI-TOF mass spectrometry revealed an [MH]⁺ ion at m/z of 11633 (peak 1), an [MH]⁺ ion at m/z of 11687 (peak 2), an [MH]⁺ ion at m/z of 11480 (peak 3) and an [MH]⁺ ion at m/z of 11533 (peak 4). Peaks 1 and 3 thus correspond to SAA_{2α} and SAA_{2α} des-Arg (lacking the N-terminal arginine), while peaks 2 and 4 correspond to SAA₁ and SAA₁ des-Arg (lacking the N-terminal arginine), respectively. The percentage area was 24.3% (peak 1), 47.3% (peak 2), 10.4% (peak 3) and 17.9% (peak 4), indicating that SAA₁ and its corresponding des-Arg isoform represent the major SAA isoprotein in the samples investigated. Using these isolated, purified SAA isoforms in the indirect DELFIA assay, the same epitopes of both proteins (SAA₁ and SAA₂) as shown above for the mixture (containing both SAA₁ and SAA₂, Fig. 1), were found to be buried (epitopes comprising

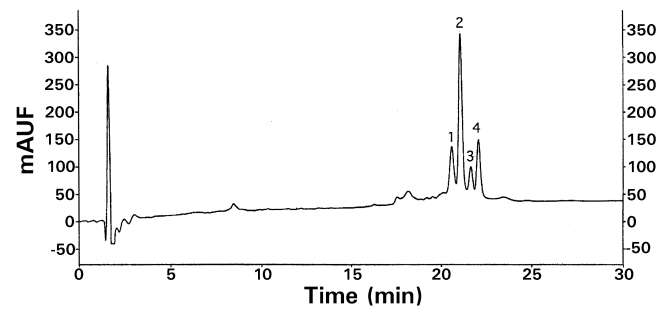
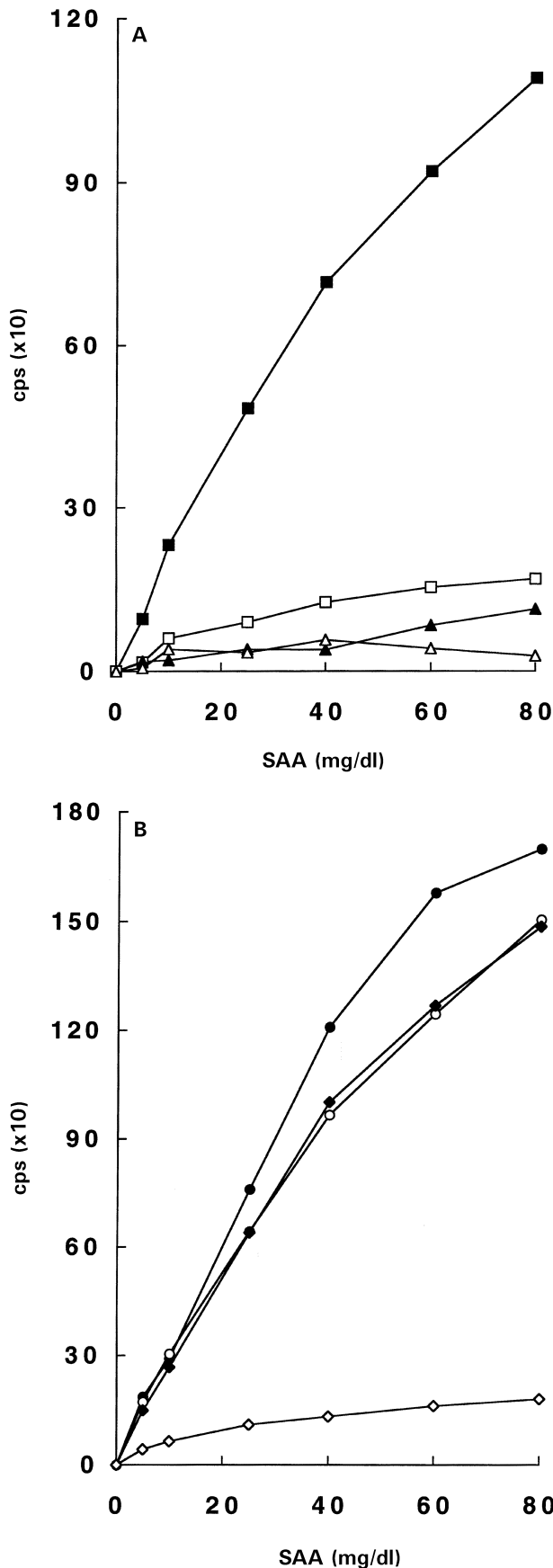


Fig. 2. Separation of SAA₁/SAA₂ and corresponding des-Arg isoforms by HPLC analysis (detection was performed at 215 nm) as described in the Materials and methods. mAU (milli absorbance units full scale).

residues 1–30, 40–63 and 79–94) or located on the surface of the protein (27–44, 59–72, 68–84 and 89–104).

As certain epitopes of SAA were not recognized by the antibodies, specific folding or self-aggregation [15] of purified isolated SAA may be postulated as a cause. After staining gels with Coomassie Brilliant Blue, the observed molecular masses in SDS-PAGE of either isolated, purified SAA or the mixture of both SAA₁ and SAA₂ revealed, in principle, the presence of monomeric and dimeric SAA proteins. Western blotting experiments using all the different sequence-specific antisera raised against epitopes comprising residues 1–17, 14–30, 27–44, 40–63, 59–72, 68–84, 79–94 and 89–104 of human SAA₁ confirmed the presence of major immunoreactive bands with molecular masses of 12 kDa (monomer) and 24 kDa (dimer) under physiological pH and basic conditions in the presence or absence of denaturing agents, i.e. SDS and mercaptoethanol (Fig. 3, lane A). Even at acid pH under complete denaturing conditions (5 M guanidine/HCl), immunoreactive bands with molecular masses of 12 kDa and 24 kDa were present, although to a lesser extent (Fig. 3, lane B). In some SAA preparations, an immunochemically reactive double band of 24 kDa as well as faint bands of about 37 kDa (trimeric SAA, Fig. 1) and 50 kDa (tetrameric SAA) were observed. The same monomer–dimer behaviour was observed when SAA was separated by non-denaturing PAGE prior to transfer to nitrocellulose. When polyclonal antisera

Fig. 1. Indirect sandwich DELFIA assay using affinity-purified sequence-specific SAA peptide antibodies raised against different epitopes of human SAA₁. Plates were coated (2 µg/well) with affinity-purified polyclonal antihuman SAA IgGs raised in sheep. Purified, isolated SAA was diluted 1:2000 with assay buffer to various defined concentrations and assayed in triplicate. Affinity-purified sequence-specific peptide IgGs (0.4 µg/well) and Eu³⁺-labelled goat-antirabbit IgGs (0.2 µg/well) were used as detection antibodies. Measurement of time-resolved fluorescence was performed on a DELFIA 1234 fluorimeter at 615 nm. Incubation procedures and measurements are as described previously [12]. Data are presented as mean ± SD for triplicate determinations of each concentration assayed. The following sequence-specific antibodies raised against the various epitopes of human SAA₁ were used. A: 1–17 (□); 14–30 (△); 27–44 (■); 40–63 (▲). B: 59–72 (◆); 68–84 (○); 79–94 (◇); 89–104 (●).

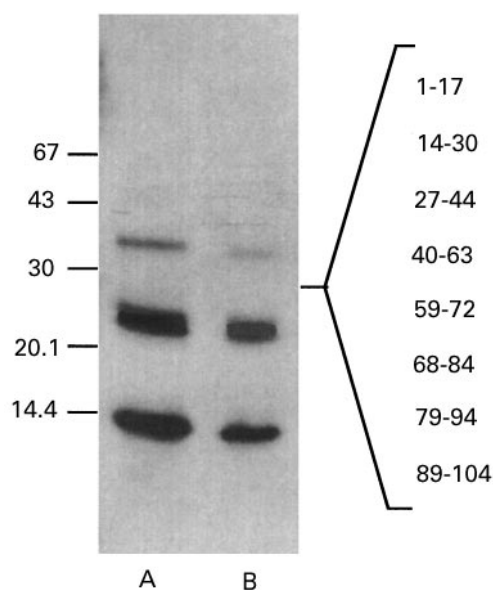


Fig. 3. Immunoblot experiments for the presence of different molecular masses of human SAA. Antigens (total SAA proteins, 10 µg/lane (lanes A and B) were separated on 10–20% SDS polyacrylamide gradient gels in the presence of mercaptoethanol (lane A) and under complete denaturing conditions (5 M guanidine/HCl, lane B). Proteins were transferred to nitrocellulose and detected using all the different sequence-specific antibodies (diluted 1:1000), raised against synthetic peptides corresponding to various amino acid sequences of human SAA₁, as indicated. Immunoreactive bands were visualized with peroxidase-conjugated goat-antirabbit IgG (diluted 1:2000) using the ECL-Western blotting detection system. An immunoblot using an antiserum raised against SAA epitope 27–44 as a primary antibody is shown as a representative example.

raised against human apo C, A-I and apo E were used as primary antibodies to SAA proteins fixed to nitrocellulose, no immunoreactive bands with molecular masses of 8 kDa, 26 kDa, and 34 kDa were observed, thus confirming the purity of the SAA fraction used.

In summary, we demonstrated that all antisera raised against epitopes comprising residues 1–17, 14–30, 40–63 and 79–94 failed to recognize the corresponding epitopes on purified SAA and the different SAA isoforms in the indirect DELFIA assay. This strongly indicates that the N-terminal portion (residues 1–30) and epitopes comprising residues 40–63 and 79–94 of SAA are apparently masked by self-folding of the protein. From Fig. 3 it is evident that SAA, owing to its amphipathic nature, may exist as a dimer with close association of those epitopes representing inaccessible domains (apparently β -turns) for some sequence-specific antibodies. In contrast, epitopes comprising residues 27–44, 59–72, 68–84 and 89–104 represent accessible antigenic domains for sequence-specific antibodies. Here, we demonstrated that both antigenic determinants and concealed epitopes (apparently the hydrophobic parts) of purified SAA and its isolated isoforms are identical to the epitopes present on acute-phase HDL-associated SAA [12]. We therefore propose

that isolated lipid-free SAA (i) serves as a reliable standard for calibration purposes and (ii) is a suitable apolipoprotein for mimicking the interaction of proinflammatory, acute-phase HDL particles with peripheral cells. The self-aggregation of SAA may occur at acidic and basic conditions to a similar extent as that reported for other apolipoproteins, i.e. apo A-I and apo A-II [16], based on hydrophobicity analysis by Chou & Fasman calculation [17]. It appears reasonable to assume that dimer association of SAA is mediated by hydrophobic domains located at the N-terminal portion and epitopes comprising residues 40–63 and 79–94.

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