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Identification of a novel serum amyloid A protein in BALB/c mice

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Four serum amyloid A protein (*SAA*) genes and two *SAA* gene products, SAA_1 and SAA_2, were identified in BALB/c mice. Using analytical isoelectric focusing we have identified a quantitatively significant new member of the SAA family and designated it 'SAA_5'. This protein has characteristics never before described for any SAA molecule. In the highly conserved region between amino acids 33 and 44, identical in all SAAs from all species examined, SAA_5 had four amino acid substitutions. In addition, the induction of SAA_5 by lipopolysaccharide had different kinetics from that of the other mouse SAAs. Our data suggest that the mouse *SAA* gene family is more complex in composition and regulation than previously surmised.

INTRODUCTION

Serum amyloid A protein (SAA) is one of the major acute-phase reactants synthesized by the liver upon cytokine stimulation [1]. Its concentration can increase 1000-fold by increased transcription and mRNA stabilization [2]. It is an apolipoprotein of the high-density-lipoprotein (HDL) complex. During the acute-phase response, SAA can constitute as much as 80% of the total protein of HDL particles [3,4]. Given the phylogenetic conservation of SAA and the enormous surge in synthesis during inflammation, it is likely that the function of SAA is important and beneficial [5]. SAA is the precursor of amyloid A protein (AA), which aggregates into the fibrillar deposits of systemic amyloidosis [6]. Several isoforms of SAA have been identified in man [7,8], hamster [9,10] and mouse [3]. In the mouse, two distinct isotypes, SAA_1 and SAA_2, are present in nearly equal amounts on HDL [3]. Only mouse SAA_2 has been reported to give rise to amyloid A (AA) of amyloid deposits [10]. Four *SAA* genes have been characterized in BALB/c mice [12–15]. There is a pseudogene (SAA_4) lacking exons 1 and 2 and 25 bp in exon 3, resulting in an in-frame stop codon [13–15]. Two genes encode the SAA_1 and SAA_2 isotypes. Another gene encoding SAA_3 is transcribed in the liver co-ordinately with the SAA_1 and SAA_2 genes, but a corresponding protein has not been found [2,11]. On the basis of computer modelling, which predicted a pI of 10.35 for the protein product of the SAA_3 gene (corrected value of 9.2) [16], we used expanded basic electrofocusing gradients as well as a variety of anti-SAA antibodies to search for this protein in BALB/c mice. Although a SAA_3-gene protein product was not identified, at this basic pH range we did discover a distinct new member of the murine SAA family as an acute-phase apolipoprotein of BALB/c HDL. We designated this protein 'SAA_5', the product of a new gene: the SAA_5 gene.

MATERIALS AND METHODS

Female BALB/c mice, 2 months old, were obtained from Harlan Sprague–Dawley Laboratories (Indianapolis, IN, U.S.A.). An acute-phase response was elicited by intraperitoneal injection of 25 μg of lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4, Difco Laboratories, Detroit, MI, U.S.A.). Control animals received no LPS. For bulk preparation of HDL, EDTA-anticoagulated blood was collected 20 or 30 h later by cardiac puncture from metofane-anaesthetized animals. For a time course, EDTA-anticoagulated blood was collected from individual animals by cardiac puncture at various times indicated after LPS injection.

Preparation of HDL

To isolate HDL from plasma, the density was adjusted to 1.09 g/ml with solid KBr and the plasma was centrifuged for 5.3 h at 242000 *g* in a VTi80 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) at 10 °C. The infranatants containing HDL were collected, the density re-adjusted to 1.21 g/ml with solid KBr and re-centrifuged for 9.4 h at 242000 *g* in a VTi80 rotor at 10 °C. The pellicles containing HDL were extensively dialysed against 0.15 M-NaCl/0.01% (w/v) EDTA, pH 7.4 [4,8].

Electrofocusing

In order to expand the basic pH range of electrofocusing gels, ultrathin acrylamide gels were cast containing 20% (v/v) ampholines pH 3–10, 40% (v/v) ampholines pH 4–6.5 and 40% (v/v) ampholines pH 7–9 [8,16] (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.). Aliquots (200 μg) of mouse HDL, or 20 μl of mouse plasma collected during the time course, were freeze-dried and delipidated with 0.5 ml of chloroform/methanol (2:1, v/v) [17]. The delipidated proteins were resuspended in 1% (w/v) sodium decyl sulphate (Eastman Kodak Co., Rochester, NY, U.S A.)/7 M-urea/5% (v/v) 2-mercaptoethanol and electrofocused as described in [8,16].

Immunochemical analysis

Samples on electrofocused gels were pressure-blotted on to 0.2 μm-pore-size nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.) overnight at room temperature [8]. The membrane was wetted with 25 mM-Tris/HCl (pH 8.3)/192 mM-glycine/15% (v/v) methanol. After pressure-blotting the membrane was blocked for 16 h at 4 °C with 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS) containing 2% (w/v) BSA. Screening for SAA isoforms was performed with one of the following primary antibodies: rabbit

Abbreviations used: (S)AA, (serum) amyloid A protein; HDL, high-density lipoprotein; PVDF, poly(vinylidene difluoride); LPS, lipopolysaccharide; PBS, phosphate-buffered saline (0.137 M-NaCl/0.01M-sodium phosphate, pH 7.4).

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anti-mouse AA (gift from Dr. J. D. Sipe, Boston University School of Medicine, Boston, MA, U.S.A.) rabbit anti-human SAA (amino acid residues 95–104) (gift from Professor A. R. Steinmetz, University of Marburg, Marburg, Germany) or rabbit anti-mouse SAA_3 (gift from Dr. R. Kisilevsky, Queens University, Hamilton, Ontario, Canada). An alkaline phosphatase-conjugated goat anti-rabbit IgG was used as secondary antibody (A8025; lot no. 39F-88961, Sigma). The chromogenic substrates for alkaline phosphatase, 5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt and nitroblue tetrazolium chloride were applied according to the manufacturer's instructions (Bethesda Research Laboratories Life Technologies, MD, U.S.A.).

Electroblotting

Aliquots (200 μg) of acute-phase HDL from BALB/c mice were subjected to electrofocusing as described above. SAA_5 (pI 8.1 bands) was excised from Coomassie Blue-stained gels. Approx. 10–15 bands were pooled, boiled in SDS sample buffer, loaded into a single well and resolved in a second-dimension 5–20 %-(w/v)-acrylamide/SDS gel with a 3 % acrylamide stacking gel [18]. Subsequently SAA_5 was electroblotted [19] for 2.5 h at 200 mA on to poly(vinylidene difluoride) (PVDF) membranes (Millipore, Bedford, MA, U.S.A.) or on to nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.) using 25 mM-Tris/HCl (pH 8.3)/192 mM-glycine/10 % (v/v) methanol/0.05% SDS as transfer buffer. Electroblotted protein was identified by staining with Amido Black.

Proteinase cleavage of pI 8.1 protein *in situ*

For each proteinase cleavage *in situ*, SAA_5 from approx. 4 mg of acute-phase BALB/c HDL was electroblotted on to PVDF or nitrocellulose membrane and subjected to enzymic degradation as described in [20,21]. Briefly; the protein-binding sites were blocked with polyvinylpyrrolidone (M_r 24000) (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) and then the SAA_5 was digested with 1 μg of trypsin (Cooper Biomedical, Malvern, PA, U.S.A.) or 0.5 μg of Endoproteinase Lys-C or 0.04 μg of Endoproteinase Asp-N (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) for 15 h at 37 °C on a vertical rotating platform. The digestion buffers, formic acid and water washes were pooled and frozen at −20 °C or loaded immediately on a h.p.l.c. column.

Reverse-phase h.p.l.c. of peptides

Peptides generated by enzymic cleavage were desalted and separated by reverse-phase h.p.l.c. on a 4.6 mm × 250 mm Vydac (Hesperia, CA, U.S.A.) C_{18} column (TP silica). Elution was with a linear gradient of acetonitrile in 0.06 % trifluoroacetic acid at a flow rate of 1 ml/min. Absorbance was monitored at 214 nm. U.v.-absorbing peaks were collected and subjected to amino acid sequence analysis. A Hewlett–Packard 1050 h.p.l.c. system, consisting of a quartenary pump, autosampler, variable-wavelength detector and a model 3396 integrator, was used for all chromatography.

Amino acid sequencing

All protein-sequence analysis was performed at the Macromolecular Structure Analysis Facility at the University of Kentucky on an Applied Biosystems (Foster City, CA, U.S.A.) model-477A protein sequencer with on-line phenylthiohydantoin identification. For *N*-terminal sequencing, 3 mg of acute-phase BALB/c HDL was electrofocused as described above and SAA_5 electroblotted on to PVDF membrane. Direct sequencing of the protein blotted on to PVDF was performed by using a precycled Polybrene-coated glass-fibre disc and sequencer cycles modified for use with PVDF [22,23].

Quantification of SAA isoforms

Coomassie Blue-stained SAA isoforms were excised from electrofocused gels and the dye extracted with 25 % (v/v) pyridine. The dye was quantified by measuring the absorbance at 605 nm and comparing it with that of reference proteins of known protein concentration [24].

SDS/PAGE

For molecular-mass analysis, protein bands were excised from Coomassie Blue-stained electrofocused gels, boiled in SDS sample buffer and resolved by electrophoresis in a 5–20 %-acrylamide/SDS gel with a 3 % acrylamide stacking gel [18]. In order to optimize protein staining, several bands of a particular pI were pooled.

Glycosylation analysis

Acute-phase HDL from BALB/c mice (1 mg) was electrofocused in 200 μg aliquots. The Coomassie Blue-stained SAA_5 bands were excised, pooled and electroblotted on to 0.22 μm-pore-size nitrocellulose membrane (Schleicher and Schuell) as described above. The glycosylation state of SAA_5 was investigated by using a glycan detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

RESULTS

Analytical electrofocusing and immunochemical analysis of BALB/c HDL

The comparative isoelectric focusing of normal and acute-phase HDL from BALB/c mice showed the presence of a prominent acute-phase reactant at pI 8.1 (Fig. 1*a*). Minor acute-phase bands with pI values lower than that of SAA_2 were visible. Two of these (pI 6.15 and pI 5.9) reacted positively with rabbit anti-(mouse AA) antibody. Sequencing of the *N*- and *C*-termini

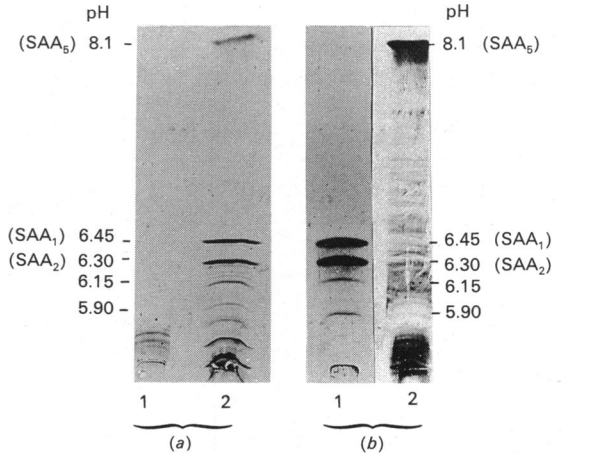

Fig. 1. (*a*) Coomassie stain of electrofocused BALB/c HDL and (*b*) immunochemical staining of electrofocused HDL from LPS-injected BALB/c mice

Lane 1, electrofocused HDL (200 μg) from control BALB/c mice; lane 2, electrofocused HDL (200 μg) from BALB/c mice killed 30 h after LPS injection showing SAA_1 (pI 6.45), SAA_2 (pI 6.3) and SAA_5 (pI 8.1). Minor bands at pI 6.15 and 5.9 are also present. (*b*) Lane 1, immunochemical staining with rabbit anti-(mouse AA) antibody as primary antibody, showing antibody binding to SAA_1 (pI 6.45) and SAA_2 (pI 6.3) only. Lane 2, immunochemical staining with rabbit anti-(human SAA) antibody (residues 95–104) as primary antibody, showing binding of antibody to SAA_5 (pI 8.1).

of these molecules identified them as intact SAAs closely resembling SAA_1 and SAA_2 (M. C. de Beer, C. M. Beach, S. I. Shedlofsky & F. C. de Beer, unpublished work).

A variety of antibodies was used to characterize the SAA isoforms. Rabbit anti-(mouse AA) antibody, which binds to SAA_1 (pI 6.45) and SAA_2 (pI 6.3) as well as minor proteins, did not react with SAA_5 (Fig. 1*b*). The SAA-like character of this protein was suggested by positive staining with a rabbit anti-(human SAA) (amino acid residues 95–104) antibody (Fig. 1*b*). An antibody raised against a synthetic peptide modelled on the first 12 amino acids of the putative mouse SAA_3 failed to react with any BALB/c apolipoprotein (results not shown).

LPS induction of mouse SAA: time course

The relative presence of SAA_1, SAA_2 and SAA_5 in blood collected from BALB/c mice at various times after LPS injection is shown in Fig. 2. This result is representative of three separate experiments. The lower part of the Figure represents immunochemical staining with rabbit anti-(mouse AA) antibody, and the upper half immunochemical staining with rabbit anti-(human SAA) antibody (amino acids 95–104). It is evident that, whereas SAA_1 and SAA_2 reached maximum concentrations 20 h after LPS administration, SAA_5 peaked at 30 h after LPS injection.

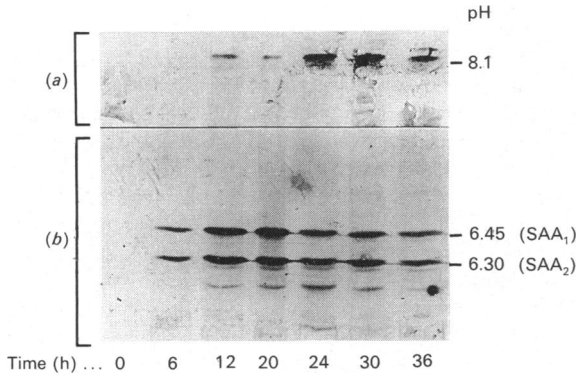

Fig. 2. Immunochemical staining of SAA isotypes in electrofocused plasma samples

BALB/c mice were killed at the times indicated after LPS injection. In (*a*), SAA_5 was identified with a rabbit anti-(human SAA) (residues 95–104) antibody. In (*b*), SAA_1 and SAA_2 were detected with a rabbit anti-(mouse AA) antibody.

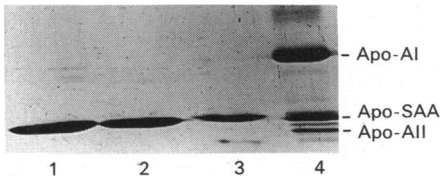

Fig. 3. Molecular masses of SAA isoforms

The Figure shows Coomassie Blue staining of SAA isotypes resolved by SDS/5–20%-PAGE, illustrating the size of molecules. Lane 1, SAA_1; lane 2, SAA_2; lane 3, SAA_5; and lane 4, BALB/c acute-phase HDL showing apo-AI, apo-SAA and apo-AII monomer.

Molecular-mass analysis

SAA_5 had a molecular mass of 14 kDa as determined by SDS/PAGE of excised electrofocused bands (Fig. 3). It was marginally larger than SAA_1 and SAA_2 (Fig. 3). The increased size was not the result of glycosylation (results not shown).

Quantification of SAA_5

SAA_5 was quantified in HDL prepared from the plasma of BALB/c mice killed 20 or 30 h after LPS injection. At 20 h, SAA_1 and SAA_2 each constituted approx. 40% and SAA_5 5% of total HDL SAA. Minor SAA isoforms made up the remaining 15% of HDL SAA. At 30 h SAA_1 and SAA_2 each constituted only approx. 35% and the SAA_5 15% of the total HDL SAA, the remaining 15% being contributed by minor SAA isoforms.

Amino acid sequence

N-Terminal sequencing, as well as sequencing of peptides generated by the digestion of SAA_5 with trypsin, Endoproteinase Lys-C and Endoproteinase Asp-N, provided the amino acid sequence of SAA_5 shown in Fig. 4.

The reverse-phase h.p.l.c. separation of peptides generated by trypsin digestion of SAA_5 yielded the profile in Fig. 5(*a*), peaks T1–T7 providing the sequences shown in Fig. 4. Peak T6 represented an incomplete digest, and in peak T7 the first amino acid, glutamic acid, was not assigned.

The Endoproteinase Lys-C digestion of SAA_5 provided only two prominent peaks after h.p.l.c. (Fig. 5*b*). These peaks provided sequences toward the *C*-terminal part of the molecule (Fig. 4).

Peptides from the Endoproteinase Asp-N digestion, separated by h.p.l.c., are shown in the chromatogram in Fig. 5(*c*). Sequences obtained from the five peaks are shown in Fig. 4. Peak A1 contained only a tripeptide, DNL (amino acid residues 23–25), which formed part of the peptide representing amino acids 23–32 (found in peak A2). The peptide contained in peak A5 (residues 82–92) represented a cleavage at the amino side of glutamic acid. The presence of the peptides found in A1 and A5 demonstrated incomplete specificity of the enzyme toward aspartic acid residues under the digestion conditions used.

In Fig. 6 the partial amino acid sequence of SAA_5 obtained by microsequencing strategies is compared with the sequences of BALB/c SAA_1, SAA_2 and the putative SAA_3 of Swiss mice. For comparison, the *N*-terminal sequence of the SAA-like protein identified by Brinkerhoff *et al.* [25] is included.

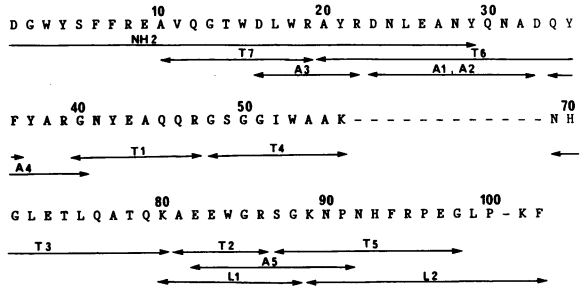

Fig. 4. Partial amino acid sequence of SAA_5

The amino acid sequence was obtained from *N*-terminal sequencing ('NH_2') and peptides generated by fragmentation with trypsin ('T'), Endoproteinase Lys-C ('L') and Endoproteinase Asp-N ('A'). Numbering is from the *N*-terminal end of the protein.

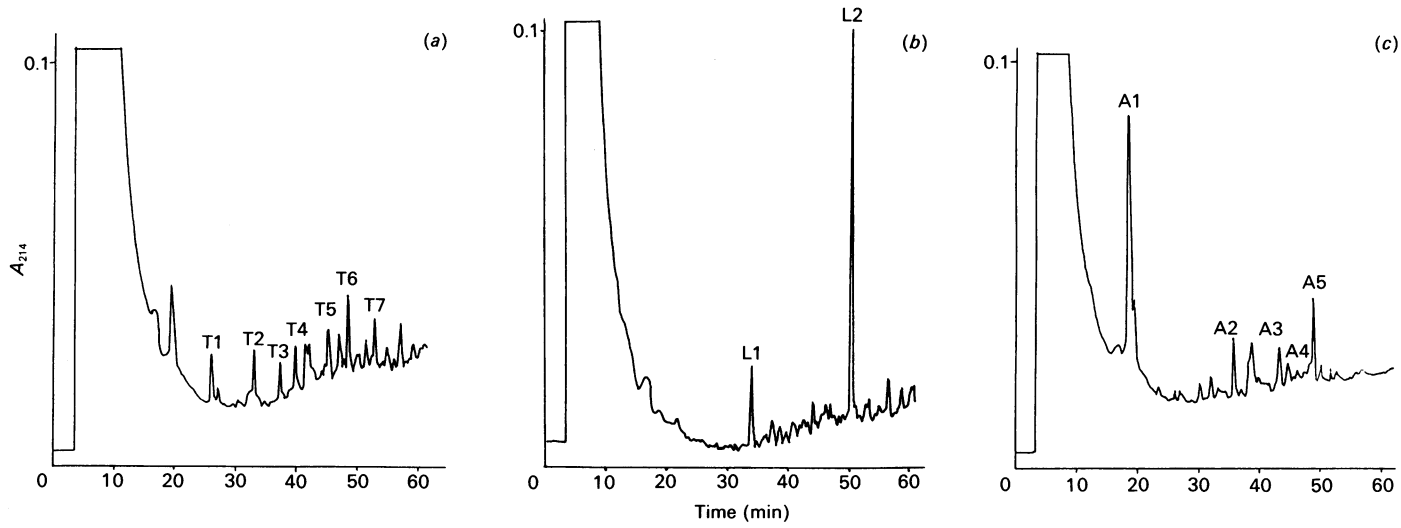

Fig. 5. Separation of peptides by reverse-phase h.p.l.c.

Peptides from the enzymic cleavage of BALB/c SAA_5 were separated by reverse-phase h.p.l.c. on a 4.6 mm × 250 mm Vydac C_{18} column with a linear gradient of acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 1 ml/min. Absorbance was monitored at 214 nm. (*a*) Peptides from tryptic digest of SAA_5 electroblotted on to nitrocellulose. (*b*) Peptides from the Endoproteinase Lys-C digestion of SAA_5 electroblotted on to PVDF membrane. (*c*) Peptides from the Endoproteinase Asp-N digestion of SAA_5 electroblotted on to PVDF membrane.

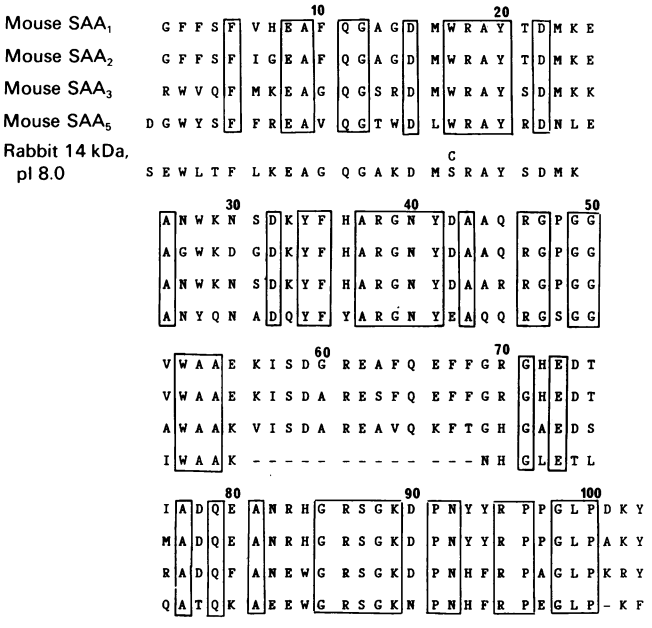

Fig. 6. Comparative amino acid sequences

The partial amino acid sequence of BALB/c SAA_5 is compared with the complete amino acid sequence of BALB/c SAA_1 and SAA_2 [11], SAA_3 from Swiss mice [13] as well as the *N*-terminal sequence of the 14 kDa rabbit protein isolated by Brinkerhoff *et al.* [25]. SAA_1 and SAA_2 sequences were deduced from clones from a BALB/c cDNA library. The SAA_3 sequence was derived from the clone (pRS 48) of a Swiss-mouse cDNA library. Numbering is according to that for mouse SAA_1 and SAA_2. Residues conserved between SAA_1, SAA_2, SAA_3 and SAA_5 are boxed.

DISCUSSION

Evidence for the existence of molecules related to, but distinct from, 'classical SAA' has recently been forthcoming in a number of species. Two SAA-like proteins produced by phorbol myristate acetate-stimulated rabbit synovial fibroblasts, which have the ability to induce autocrine collagenase, have been identified by Brinckerhoff *et al.*, and *N*-terminal sequencing produced 25 residues of sequence [25]. One of these SAA-like molecules, which has a molecular mass of 14 kDa and a pI of 8.0, shows 44% *N*-terminal similarity to our SAA-like protein. Sack & Talbot have described another member of the human SAA gene family, *gSAA*$_p$ which codes for a protein of 104 amino acids [26]. This SAA protein, which has yet to be identified, differs from the described human SAA proteins [16] in several usually conserved sequences. However, the sequence of its first 25 amino acids is virtually identical with that of the SAA-like protein described by Brinckerhoff *et al.* [25]. Furthermore, Ramadori *et al.* have raised antibodies against 12.5 kDa and 14.5 kDa proteins produced by mouse liver and peritoneal macrophages [27]. These antibodies did not bind to the 'classical' mouse SAAs, though various antisera against mouse AA bound to the 12.5 kDa and 14.5 kDa proteins [27]. Our findings provide definitive proof for the existence of another gene product (SAA_5) in BALB/c mice.

The pI of SAA_5 (8.1) is considerably more basic than that of SAA_1 (6.45) and SAA_2 (6.3). Basic proteins such as SAA_5 would not readily be detected in electrofocusing gradients used to date for the analysis of mouse SAA isoforms. Use of such gradients, as well as the slower kinetics of SAA_5 induction, may explain why this molecule had thus far escaped identification.

The pI of the SAA_3 protein is predicted to be 10.35, but when this value is corrected, a pI of 9.2 is obtained [16]. In electrofocused gels that had not been focused to completion no migrating proteins more basic than SAA_5 had been observed. Our findings therefore indicate that the SAA_3 protein is not an apolipoprotein of BALB/c HDL.

Microsequencing strategies were employed to obtain amino acid sequence data for subsequent definitive probe design and genetic analysis. These strategies were successful in providing 56 *N*-terminal and 35 *C*-terminal residues. The number of intervening residues is uncertain in that the SAA_5 protein had a molecular mass which was marginally higher than that of SAA_1 or

SAA_2. A conceivable explanation for the small increase in size might be the presence of an insert of several extra amino acids, as is the case in the cow, horse, cat and dog [28–30]. In these animals, eight or nine amino acids were found to be inserted in the region of amino acids 69–70, a section of SAA_5 that we have not sequenced.

SAA of various species differ most in exon 2 and 4, respectively coding for the *N*- and *C*-termini. Exon 3 has diverged less than exon 4 and, in particular, encodes a highly conserved region between amino acids 33–44 [13]. It is notable that SAA_5 differed in four of the 13 amino acids of this exon 3 region, which could imply a different function for this protein.

The first 56 amino acids of SAA_5 had 61, 57 and 57% similarity with SAA_1, SAA_2 and SAA_3 respectively. Amino acids 69–103 (part of exon 4) of SAA_5 had 63% similarity with SAA_3 and only 51% with SAA_1 and SAA_2. It is notable that 74% of the amino acids conserved among SAA_1, SAA_2 and SAA_3 were also conserved in SAA_5. Overall, on the basis of available sequence data, SAA_5 had 56% similarity to the SAA_1/SAA_2 subfamily and 59% similarity to SAA_3.

The mouse SAA-gene family has been most extensively studied in BALB/c mice and was said to consist of at least three genes and a pseudogene residing within a 79 kb region of chromosome 7 [13,15]. It was suggested that an ancestral *SAA* gene gave rise to the SAA_3 gene and a pre-$SAA_{1,2,4}$ gene and that the latter subsequently duplicated into the SAA_4 gene (pseudogene) and a pre-$SAA_{1,2}$ gene. Gene conversion of the latter resulted in the present SAA_1 and SAA_2 genes [13–15]. The most likely explanation for the existence of SAA_5 is that it has evolved, like the SAA_3 gene, through a distinct ancestral gene-duplication event. This is suggested by its sequence similarities with the SAA_1/SAA_2 and SAA_3 subfamilies, which characterize it as a new subfamily equally related to the previous two.

The fact that SAA_5 induction temporally differed from the other mouse SAAs is of interest in that it has not previously been shown in any other SAA from all species examined. Whether the induction of SAAs is mediated by different cytokines or whether there is differential control at the transcriptional or translational level, needs to be explored. Alternatively SAA_5 might have different associative kinetics with HDL. Our data could expand the potential of the mouse model to study cytokine-induced and differential acute-phase gene expression.

The discovery of a new SAA molecule in BALB/c mice implies that the mouse *SAA*-gene family is more complex than previously surmised. We view it as important that both molecular-biological and analytical-protein-chemical approaches be employed to elucidate the diversity of the *SAA*-gene family. The employment of too limited a repertoire of probes at the gene level could allow important members to go undetected.

Part of this work was submitted by M.C. de Beer to fulfill requirements for a Ph.D. degree at the University of Stellenbosch, Stellenbosch, South Africa.

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