The Presence of Two Low Molecular Mass Proteins Immunologically Related to 14 Kilodalton Serum Amyloid A in the Lipoprotein Fraction and Their Decreased Serum Concentrations in Calves with Experimentally Induced Pneumonia

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ABSTRACT .  A 14 kilodalton (kDa) serum amyloid A (apoSAA) protein was purified from cow serum.  Rabbit antiserum to the 14 kDa apoSAA recognized, in addition to the 14 kDa protein, a 7.5–9.0 kDa protein and a protein having a molecular mass of less than 6.5 kDa (<6.5 kDa protein).  The possibility that the two proteins were contaminants was excluded by results showing that the two proteins detected in early stages of purification procedures were not found in the purified 14 kDa apoSAA fraction, as revealed by immunoblot analysis.  As in the 14 kDa apoSAA, the 7.5–9.0 kDa protein was localized in the high-density lipoprotein fraction, while the <6.5 kDa protein was in the low-density lipoprotein fraction.  In calves with pneumonia induced by inoculation to the lungs of Pasteurella haemolitica, the serum concentration of the 14 kDa apoSAA was increased, whereas those of the 7.5–9.0 kDa and the <6.5 kDa proteins were conversely decreased.  The time-course study indicated that the increase in concentration of the 14 kDa apoSAA and decrease in that of the <6.5 kDa protein occurred almost simultaneously.  These results suggest that the 14 kDa apoSAA and the immunologically related 7.5–9.0 kDa and <6.5 kDa proteins act as positive and negative acute phase reactants, respectively, and also that concentrations of the three proteins are regulated in concert in acute phase plasma. — KEY WORDS: acute phase protein, apolipoprotein, calf, pneumonia, serum amyloid A.


Serum amyloid A (apoSAA) is an acute phase protein of liver origin and is associated in plasma with the high-density lipoprotein (HDL) fraction [1].  Bovine apoSAA consists of 112 amino acid residues, and its complete sequence has been reported [18].  The physiologic relevance of apoSAA has not yet been elucidated, though its roles in cholesterol transport [10], immunosuppression [2], and platelet inactivation [30] have been suggested.  ApoSAA is a substrate for protein kinase C; however, the functional relevance of this phosphorylation is unknown [15].  As for its pathologic aspect, apoSAA is a precursor for tissue amyloid A (AA) proteins found in amyloid fibrils in amyloidosis [9, 18, 25].  We are now searching for sensitive indicators for early diagnosis of calf pneumonia, an economically important disorder of calves.  During the course of purification of apolipoprotein C-III (apoC-III; one of the candidate proteins for this purpose) from cow serum [26], we co-purified a 14 kilodalton (kDa) protein localized in the HDL fraction.  The present study was prompted by the finding that the 14 kDa protein was apoSAA.  The major results presented here are that anti-14 kDa apoSAA recognized, in addition to the 14 kDa protein, a 7.5–9.0 kDa protein in the HDL fraction and a protein having a molecular mass of less than 6.5 kDa (<6.5 kDa protein) in the low-density lipoprotein (LDL) fraction and, moreover, the serum concentration of the 14 kDa apoSAA was increased whereas those of the two low molecular mass proteins were conversely decreased in sera from calves with pneumonia induced by inoculation of Pasteurella haemolitica.

MATERIALS AND METHODS

Purification of apoSAA:  ApoSAA was purified from cow serum during the course of purification of apoC-III [26].  Briefly, the total lipoprotein fraction [d<1.21, containing chylomicrons (CM) to HDL] prepared from sera of Holstein cows during midlactation was treated with an equal volume of acetone to extract low molecular mass apolipoproteins such as apoC-III and apoSAA.  The supernatant obtained by centrifugation at 140 × g for 10 min was lyophilized and thereafter delipidated by mixing with 20 volumes of 2-propanol.  Apolipoproteins pelleted by centrifugation at 1,300 × g for 10 min, were dissolved in 6 M urea and 50 mM Tris-HCl (pH 8.6), and applied to a column (2.5 × 90 cm) of Sephadex G-75 (Pharmacia Biotech, Uppsala, Sweden).  Fractions rich in low molecular mass apolipoproteins were next applied to a DEAE-Sephacel (Pharmacia Biotech, Uppsala, Sweden) column (2 × 25 cm), and bound proteins were eluted by use of a linear gradient of NaCl (0–0.125 M, total 1 liter).  Fractions containing apoSAA were dialyzed against 10 mM ammonium hydrogen carbonate, using cellulose dialyzer tubing with a 3,500 molecular weight cutoff, after which the dialysate was...
Purified apoSAA (1 mg) was dissolved in 0.5 ml of phosphate-buffered saline (PBS) and emulsified in an equal volume of Freund’s complete adjuvant, and was injected into foot pads of rabbits. After 3 weeks, 1 mg of the protein emulsified in Freund’s incomplete adjuvant was administered as a booster. Blood samples were obtained by cardiac puncture 1 week after the booster injection.

**Protein blotting and amino acid sequencing:** After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gels were washed in a solution containing 48 mM Tris, 39 mM glycine and 20% methanol for 5 min with gentle agitation, and were electroblotted to a polyvinylidene difluoride (PVDF) membrane (ProBlott, Applied Biosystems, Foster City, Calif, U.S.A.) at 0.8 mA/cm² for 3 hr. The membrane was washed twice with Tris-(BioRad Laboratories, Hercules, Calif, U.S.A.) at 0.8 mA/cm² for 3 hr. After being rinsed with water, the membrane was stained with Coomassie Brilliant Blue for 2 min, and quickly destained with several changes of a solution containing 60% methanol and 10% acetic acid. The destained membrane was rinsed with water, and a band corresponding to the 14 kDa protein was excised. Sequence analysis of the protein sample was done using a protein sequencer (Model 490 Procise, Applied Biosystems, Foster City, Calif, U.S.A.).

**Immunoblot analysis:** SDS-PAGE gel was washed with 48 mM Tris, 39 mM glycine and 20% methanol, and proteins on gel was transferred to PVDF membranes (BioRad Laboratories, Hercules, Calif, U.S.A.) at 2 mA/cm² for 1 hr. The membrane was washed twice with Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5 and 0.5 M NaCl), and then incubated overnight at 4°C with 5% non-fat dry milk in TBS. After washing twice with TBS containing 0.05% Tween 20 (TTBS), the membrane was incubated with anti-bovine apoSAA, anti-human apoSAA (Calbiochem-Novabiochem International, La Jolla, Calif, U.S.A.), or anti-bovine apoC-III rabbit serum for 30 min at room temperature. The membrane was washed 3 times with TTBS, and thereafter incubated for 30 min at room temperature with goat anti-rabbit IgG serum conjugated with horseradish peroxidase (Cappel Products, Organon Teknika, West Chester, Pa, U.S.A.). After being washed 3 times with TTBS, bound peroxidase were detected by use of a chemiluminescence reagent (ECL, Amersham International, Little Chalfont, Buckinghamshire, UK). The relative amounts of immunoreactive proteins were determined by densitometry (CS-9300PC, Shimadzu, Kyoto, Japan). Appropriate amounts of acetone extract (see Fig. 3) were included in the same gel to obtain standard curves for 14 kDa apoSAA, 7.5–9.0 kDa and <6.5 kDa proteins.

**Induction of pneumonia:** Eight 1.5-month-old male Holstein calves weighing 65 to 80 kg were used. Pasteurella haemolytica (serotype 1, I29 strain; 5.5 × 10⁹ colony forming units) suspended in 0.1 ml of RPMI-1640 was diluted to 10 ml with PBS. Six calves were sedated with xylazine (40 mg/calf) and atropine (0.025 mg/kg of body weight), and a 10-ml aliquot of the bacterial suspension was administered to each calf, using a fiber-optic bronchoscope (Olympus SIF type 10, Olympus, Tokyo, Japan). Two other calves were similarly treated, received the vehicle alone, and used as controls. Clinical and pathologic changes induced by the bacterial inoculation were essentially as described in earlier reports [5, 23].

**Other methods:** SDS-PAGE using a Tricine (N-[tris (hydroxymethyl)methyl]glycine) buffer system was performed by the method developed by Schägger and von Jagow [19], with slight modification [26]. CM, very low-density lipoprotein (VLDL), LDL, HDL and the lipoprotein-deficient fraction (d>1.21) were prepared as described previously [21]. Cow apoC-III was purified and rabbit anti-apoC-III serum was prepared [26]. Protein concentration was determined by the method of Bradford [4].

**RESULTS**

ApoC-III from cow serum was purified using separation by ultracentrifugation of the total lipoprotein fraction, extraction from the lipoprotein fraction with acetone, delipidation by 2-propanol, Sephadex G-75 gel filtration and DEAE-Sephacel ion exchange chromatography. A typical elution profile of the ion exchange chromatography is depicted in Fig. 1. ApoC-III was eluted into peaks 3, 4 and 5. Peak 1 was eluted in the breakthrough fractions, and contained several protein species. Peak 2 was detected just after the gradient elution was started (eluted by approximately 0.01 M NaCl). Analysis by SDS-PAGE of the peak 2 fraction revealed the presence of a 14 kDa...
protein. Apparent contaminants were not found, at least by staining with Coomassie Brilliant Blue. The band of 14 kDa protein was subjected to N-terminal amino acid sequence analysis (Table 1). The analysis indicated that the 13 amino acid residues sequenced were identical with those of bovine apoSAA [18]. In immunoblot analysis, no band was detected when non-immune rabbit serum was used (figure not shown). The analysis using anti-bovine apoC-III revealed that the 14 kDa protein did not react with anti-apoC-III (Fig. 2A). Anti-14 kDa protein reacted with the 14 kDa protein, but not with 7.3 kDa apoC-III (Fig. 2B). Commercially available anti-human apoSAA immunostained the 14 kDa protein, although this antiserum cross-reacted with apoC-III and, moreover, with a high molecular mass protein (probably serum albumin; Fig. 2C). Thus, the 14 kDa protein was identified as apoSAA.

Immunoblot analysis of cow serum using anti-bovine apoSAA frequently showed the presence of <6.5 kDa protein (Fig. 3, lane 1). To assess the specificity of anti-apoSAA, fractions from the apoSAA purification steps were analyzed by immunoblotting. In addition to the 14 kDa protein, 7.5–9.0 kDa and <6.5 kDa proteins were detected in the total lipoprotein fractions (Fig. 3, lane 2). The two proteins immunologically related to the 14 kDa apoSAA were also found in the acetone extract (lane 4). The total lipoprotein fraction and acetone extract were separately dialyzed against 6 M urea and 10 mM Tris-HCl (pH 8.0), and thereafter immunoblotted. Long exposure to urea did not result in any changes of immunostaining patterns (compare lanes 2 and 3, and lanes 4 and 5), indicating that the two immunologically related proteins were not produced by chemical modifications (such as carbamylation) of the 14 kDa apoSAA. In addition to the two low molecular mass proteins, analysis of the pellets that were produced after the total lipoprotein fraction had been treated with acetone, showed the presence of a protein having a molecular mass higher than 26.6 kDa (lane 6). Delipidation of the acetone extract did not largely alter the immunoblot pattern (lane 7). By Sephadex G-75 chromatography, higher molecular mass proteins such as 24 kDa apolipoprotein A-I and 67 kDa serum albumin (lane 8; [26]) were effectively removed from proteins such as 24 kDa apolipoprotein A-I and 67 kDa serum albumin (lane 8; [26]) were effectively removed from the total lipoprotein fraction (Fig. 3, lane 2). The two proteins immunologically related to the 14 kDa apoSAA were also found in the acetone extract (lane 4). The total lipoprotein fraction and acetone extract were separately dialyzed against 6 M urea and 10 mM Tris-HCl (pH 8.0), and thereafter immunoblotted. Long exposure to urea did not result in any changes of immunostaining patterns (compare lanes 2 and 3, and lanes 4 and 5), indicating that the two immunologically related proteins were not produced by chemical modifications (such as carbamylation) of the 14 kDa apoSAA. In addition to the two low molecular mass proteins, analysis of the pellets that were produced after the total lipoprotein fraction had been treated with acetone, showed the presence of a protein having a molecular mass higher than 26.6 kDa (lane 6). Delipidation of the acetone extract did not largely alter the immunoblot pattern (lane 7).

By Sephadex G-75 chromatography, higher molecular mass proteins such as 24 kDa apolipoprotein A-I and 67 kDa serum albumin (lane 8; [26]) were effectively removed from the fraction containing apoSAA and apoC-III (lane 9). This chromatographic step was also successful to reduce the amounts of the 7.5–9.0 kDa and <6.5 kDa proteins. Finally, the 14 kDa apoSAA was recovered as a purified form by DEAE-Sephacel chromatography (lane 10).

Lipoprotein fractions were prepared from sera of calves before and after inoculation to the lung of Pasteurella haemolitica (Fig. 4). In calves before inoculation, the 14 kDa apoSAA was found to be localized in the HDL fraction (lane 4). The 7.5–9.0 kDa protein was also distributed in this fraction. The <6.5 kDa protein was not found in the HDL, but was detected in the LDL fraction (lane 3). None of the three proteins were found in CM, VLDL and the lipoprotein-deficient fraction. In serum, the <6.5 kDa protein was detected, but the 14 kDa apoSAA and 7.5–9.0 kDa protein were not (lane 6). By contrast, the 14 kDa apoSAA was clearly observed in sera of calves after inoculation, but the <6.5 kDa protein nearly completely disappeared (lane 12). In the HDL (lane 10), the density of the 14 kDa apoSAA was increased (1.9-fold that of lane 4), whereas that of the 7.5-9.0 kDa protein was conversely decreased (23% of lane 4). The <6.5 kDa protein was faintly detected in the LDL (lane 9). The three proteins were undetectable in the lipoprotein fractions other than HDL and LDL.

In the next series of experiment, serum concentration changes after bacterial inoculation of apoSAA and the <6.5 kDa protein were monitored in individual calves (Figs. 5A and 5B). Of the 2 control calves, C1 showed an increased concentration of 14 kDa apoSAA. In this calf, the concentration of the 14 kDa protein was distinctly increased at 1 day after vehicle administration. At the same time, the <6.5 kDa protein concentration was decreased. Concentrations of apoSAA and <6.5 kDa protein of the other control calf (C2) were relatively constant; the 14 kDa protein concentration was elevated at 7 and 10 days, and the <6.5 kDa protein concentration was concomitantly decreased at the same days. In calves to which Pasteurella haemolitica were inoculated, increases in concentrations of the 14 kDa protein and simultaneous decreases in those of the <6.5 kDa protein were observed in calves 1 and 2. In calf 3, the band for the <6.5 kDa protein was not distinctly detected. Results of the analysis of sera from three other inoculated calves were similar to those observed in calves 1 and 2 (figure not shown). The 7.5–9.0 kDa protein was not detected in any

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**Table 1. Comparison of amino terminal sequences of a 14-kDa protein and bovine apoSAA**

<table>
<thead>
<tr>
<th>14-kDa protein</th>
<th>QWMSFFGEAYEGA</th>
<th>Bovine apoSAA [18]</th>
<th>QWMSFFGEAYEGA</th>
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<tr>
<td>One letter amino acid code: A=alanine, E=glutamic acid, F=phenylalanine, G=glycine, M=methionine, Q=glutamine, S=serine, W=tryptophane, Y=tyrosine.</td>
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**Fig. 2. Immunoblot analysis of purified apoSAA.** A, anti-bovine apoC-III; B, anti-bovine apoSAA; C, anti-human apoSAA. Lanes are: 1, bovine apoC-III (0.586 µg); 2, bovine 14 kDa apoSAA (0.309 µg); and 3, cow serum (23.3 µg).
sera analyzed. The time-course study presented in Figs. 5A and 5B, in conjunction with data in Fig. 4, indicated that concentrations of the <6.5 kDa and the 7.5–9.0 kDa proteins were decreased when that of the 14 kDa apoSAA was increased.

DISCUSSION

The 14 kDa protein purified from cow serum was identified as apoSAA by the following two criteria: 1) N-terminal amino acid sequence of the 14 kDa protein was identical to the reported sequence of bovine apoSAA; and 2) the 14 kDa protein was immunostained by anti-human apoSAA. The distribution in the HDL fraction and elevation of its serum concentration in calves with pneumonia further supported this conclusion. Anti-14 kDa apoSAA additionally recognized the two low molecular mass proteins; 7.5–9.0 kDa protein in the HDL and <6.5 kDa protein in the LDL. The possibility that the two proteins were contaminants could be ruled out by the following two results: 1) the purified 14 kDa protein fraction did not contain these two proteins (Fig. 2B); and 2) the two proteins could be removed during the purification procedures (Fig. 3). It is also unlikely that the two low molecular mass

Fig. 3. Immunoblot analysis of apoSAA in fractions from purification steps. Lanes are: 1, serum (23.3 µg); 2, total lipoprotein fraction (5.95 µg) dissolved in 0.9% NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.01% sodium azide; 3, total lipoprotein fraction (6.18 µg) in 6 M urea and 10 mM Tris-HCl (pH 8.0); 4, acetone extract (10.7 µg) in the NaCl solution; 5, acetone extract (12.1 µg) in the urea solution; 6, pellet obtained after extraction with acetone (10.9 µg, dissolved in the urea solution); 7, delipidated fraction (3.47 µg); 8 and 9, peaks 1 (10.9 µg) and 2 (4.07 µg) from a Sephadex G-75 column; and 10, peak 2 from a DEAE-Sephacel column (0.309 µg).

Fig. 4. Immunoblot analysis of apoSAA in lipoprotein fractions from sera of calves before and after inoculation of Pasteurella haemolytica. Combined sera from six calves obtained at 0 hr and at 1 day after were used as control (1–6) and pneumonic sera (7–12), respectively. Lanes are: 1 and 7, CM; 2 and 8, VLDL; 3 and 9, LDL; 4 and 10, HDL; 5 and 11, lipoprotein-deficient fraction; and 6 and 12, serum. One ml of each lipoprotein fraction (CM to HDL) was prepared from 4 ml of serum, and each fraction (3.3 µl) was applied. Serum and lipoprotein-deficient fractions were diluted 20-fold, and a 6.7 µl aliquot of each dilution was subjected to SDS-PAGE. Lane 13 is 14 kDa apoSAA (0.309 µg).
proteins were produced by proteolytic degradation of the 14 kDa apoSAA during the purification procedures, because the <6.5 kDa protein was detected in fresh serum or plasma, and because an immunologically related protein having a molecular mass higher than the 14 kDa apoSAA (>26.6 kDa) was also found (Fig. 3, lane 6).

ApoSAA is the precursor for tissue AA. During secondary amyloidosis caused by inflammation, apoSAA is incorporated into amyloid fibrils formed in tissues (such as kidney). Intact or proteolytically-degraded apoSAA is incorporated into the fibrils [25]. In cows, the molecular mass of apoSAA from the HDL is 14 kDa and those of two AA proteins isolated from the kidney of the cow suffering from amyloidosis are 14 kDa and approximately 9 kDa [9]. These three proteins are immunologically related and have identical N-terminal sequences [9, 24]. The 7.5–9.0 kDa
protein in the present study appeared to be similar to the 9 kDa AA. Husebekk et al. [9] reported that the 9 kDa AA protein was not detected in the HDL from acute phase serum. We found that the concentration of the 7.5–9.0 kDa protein was decreased in the HDL from sera of calves after bacterial inoculation, compared with that from calves before inoculation. This result suggested that if the 9 kDa AA is present, it would be more easily detected in non-acute phase than in acute phase serum.

Concomitant with the induction of pneumonia, the serum concentration of the 14 kDa apoSAA was increased, whereas those of the 7.5–9.0 kDa and the <6.5 kDa proteins were decreased (Fig. 4). The time-course study indicated that changes of their concentrations occurred almost simultaneously and quickly; for example in calf 1, concentration changes of both the 14 kDa apoSAA and the <6.5 kDa protein were apparent as early as 2 hr after the inoculation (Figs. 5A and 5B). The synthesis by the liver of the 14 kDa apoSAA was thought to be accelerated, as reported in other species [17]. With the onset of increased synthesis of the 14 kDa apoSAA, the <6.5 kDa and 7.5–9.0 kDa proteins are assumed to be removed from circulation owing to incorporation of them into tissues. An alternative explanation for the decreased serum concentrations of the 7.5–9.0 kDa and <6.5 kDa proteins is that these proteins are degraded to smaller peptides by protease(s) whose activity is enhanced in acute phase plasma. Decreased synthesis (presumably by the liver) of these two proteins also arises as another possibility. Whatever the mechanism, the simultaneous change of their serum concentrations suggests that the metabolism of the 14 kDa apoSAA and the two immunologically related proteins are regulated in concert. Moreover, the quick regulation of plasma concentrations suggests that the three proteins may have essential roles in the early cascades of the acute phase reactions.

The altered serum concentrations of the 14 kDa apoSAA and the <6.5 kDa protein were similarly observed in sham-treated control calves. The alteration in controls was not unexpected because apoSAA is induced by mild stimuli. For example, the apoSAA concentration of monkeys is elevated by chair restraint [16]. In rabbits, intravenous injection of saline solution or serial blood sampling results in a marked increase of the serum apoSAA concentration [12]. The elevation of serum concentrations of acute phase proteins by stimuli other than inflammation is not restricted to apoSAA. The serum concentration of haptoglobin, another major acute-phase protein in bovines, is increased in cattle subjected to transportation exhaustion [11, 13], in cows with fatty liver [14, 20, 22, 27], in cows to which dexamethasone is administered [6, 28], in cows during nonfeeding [28] and in feedlot cattle [29]. The acute phase proteins are induced in response to a wide variety of stimuli, and the response does not distinguish the kind of stimulus.

Apart from the concept of acute-phase proteins, evaluation of the serum apoSAA concentration is useful to detect calves with various diseases, including pneumonia, because it rarely happens that calves are exposed to artificial stimuli such as bronchoscope insertion. Because the concentrations of the 14 kDa apoSAA and the <6.5 kDa protein were relatively constant in one of the control calves (C2 in Figs. 5A and 5B) at least during 4 days after inoculation, it is unlikely that their concentration changes observed after bacterial inoculation were totally attributable to the artificial stimuli. The bacterial infection and immediate inflammatory reaction are presumed to be largely responsible for their concentration changes. The serum apoSAA concentration has been evaluated by enzyme-linked immunosorbent assay using polyclonal rabbit antisemum to bovine or human apoSAA [3, 7, 8]. It became evident in the present study that serum concentrations of the immunologically related 7.5–9.0 kDa and <6.5 kDa proteins were decreased during pneumonia. The use of a monoclonal antibody to the 14 kDa apoSAA that does not recognize the two low molecular mass proteins is recommended to enhance the diagnostic value of apoSAA.

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REFERENCES


