Morphologic Demonstration of Cytoplasmic AS_{SAM}-Related Antigenic Substance (CAS_{SAM}) by an Immunoperoxidase Technique

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Murine senile amyloid protein identified in the senescence-accelerated mouse (SAM) was called AS_{SAM}, and the AS_{SAM}-related antigenic substance was detected in the cytoplasm of hepatocytes, columnar epithelia of the small intestine, and epithelia of the proximal convoluted tubules of the kidney, with the use of an immunoperoxidase method. This substance, called CAS_{SAM} (cytoplasmic AS_{SAM}-related antigenic substance), did not stain positively with Congo red nor fibril structure, as determined under an electron microscope. As the AS_{SAM} (senile amyloid) deposition increased with advancing age, CAS_{SAM} observed in hepatocytes and columnar epithelia decreased both in SAM-P and SAM-R strains. In the liver of the SAM-P strain in particular, the incidence and intensity in deposition of AS_{SAM} increased rapidly from 5 months of age; on the other hand, CAS_{SAM} observed in the hepatocytes decreased rapidly at about the same time. Cycloheximide-treated animals showed a significantly low concentration of SAS_{SAM} (serum AS_{SAM}-related antigenic substance) and also a low incidence and intensity of CAS_{SAM} observed in the cytoplasm of the hepatocytes and epithelia of the small intestine. In colchicine-treated animals, SAS_{SAM} concentration was slightly lower, and the severity of CAS_{SAM} observed in the cytoplasm was slightly higher, in the liver and kidney, as compared with control values. CAS_{SAM} is assumed to be synthesized in the cytoplasm of the cell and to be secreted alone or in the lipoprotein form into the serum. This CAS_{SAM} or lipoprotein including CAS_{SAM} is perhaps a constituent of SAS_{SAM} (CAS_{SAM} is assumed to include $apoSAS_{SAM}$) and the hepatocytes and intestinal mucosal epithelia are possible production sites of $apoSAS_{SAM}$. (Am J Pathol 1985, 121:455–465)

THE MURINE MODEL of accelerated senescence (senescence-accelerated mouse, or SAM), developed recently by Takeda et al,[1] is divided into two major groups: SAM-P (accelerated senescence-prone mouse), and SAM-R (accelerated senescence-resistant mouse). SAM-P and SAM-R strains include the substrains SAM-P/1, SAM-P/2, SAM-P/3, and SAM-P/4 and SAM-R/1, SAM-R/2, and SAM-R/3, respectively. The characteristics of aging in the SAM-P are earlier onset and irreversible advancement[2] of senescence, as manifested by clinical signs and gross lesions such as alterations of general behavior, loss of skin glossiness, increased skin coarseness, hair loss, periophthalmic lesions, cataract, increased lordokyphosis of the spine, and so on, after a normal process of development.

A quantitative evaluation of these aging processes was performed according to the Grading Score System[3] devised in our laboratory. Accelerated senescence observed in SAM-P is also revealed in an analysis of aging dynamics such as Gompertz function, growth curve, survivorship curve, and shortened life span.

The amyloidosis in this model is designated as a spontaneous age-associated one.[4–6] This amyloid protein is

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a unique fibril protein, has a molecular weight of 5200 daltons, and differs in amino acid composition from any other murine amyloidosis.[7,8] This unique fibril protein, called AS_{SAM},[9-10] differs biochemically, immunochemically, and immunohistochemically from other murine amyloid proteins such as secondary or experimentally induced amyloid (AA).[11-16]

While continuing to use the immunohistochemical examination now widely applied for detection of specific amyloid protein[17] (except for the extracellular space with amyloid deposition and which reacted with the anti-AS_{SAM} antibody), we also found the reactive substance in the cytoplasm of hepatic cells, epithelial cells of the small intestine, and renal convoluted tubular cells. This substance was not stained with Congo red, nor did it show birefringence under the polarizing microscope.

We also reported that a substance which reacts with antiserum against AS_{SAM} exists in normal mouse serum. This compound, termed SAS_{SAM} (serum AS_{SAM}-related antigenic substance), is a physiologic substance and circulates in blood mainly in the form of high-density lipoprotein$_2$ (HDL_2). Gel-filtration chromatography of delipidated HDL revealed that the protein part of SAS_{SAM} is a low-molecular-weight apoprotein of HDL (apoSAS_{SAM}).[18] The present study was undertaken to investigate morphologically the organ distribution of AS_{SAM}-related antigenic substance in the cytoplasm, the relationship between the incidence and intensity in the presence of this substance and those in extracellular deposition of AS_{SAM} with advancing age. Also discussed are the possible organs producing AS_{SAM}-related antigenic substance, on the basis of changes in morphology after colchicine and cycloheximide treatment.

Materials and Methods

Animals of SAM-P/1, a representative of SAM-P strains with characteristic accelerated senescence and a high incidence of senile amyloidosis, and SAM-R/2, a representative of SAM-R strains with normal aging and a low incidence of senile amyloidosis, from newborn to 19 months of age, were used for histologic and immunohistochemical examinations. All the organs were fixed with 10% neutralized formalin and embedded in paraffin. Paraffin sections were prepared for hematoxylin and eosin (H&E) staining (4 μ) and Congo red staining (6 μ). Other sections (4 μ) were prepared for immunohistochemical examination using the unlabeled antibody peroxidase-antiperoxidase (PAP) method of Sternberger et al,[19] as modified by Fujihara et al.[20] Antibody was used at 1:2500 to 1:5000 dilutions. The intensity of anti-AS_{SAM} antibody reactive substance in the intracytoplasm and the deposition of AS_{SAM} in extracellular spaces were graded at four levels, and the average for each group was obtained.

Preparation of Anti-AS_{SAM} Antibody and Its HRP Conjugated Fab'-fragment

The anti-AS_{SAM} antibody was prepared from immunized rabbits inoculated with the purified AS_{SAM} and Freund's complete adjuvant.[10]

From the anti-AS_{SAM} antibody, the Fab' fragments were prepared by digestion with pepsin and isolated with Sephadex G-100 (Pharmacia) gel filtration. Horseradish peroxidase (HRP; Sigma) was added to the eluted Fab' fragments solution and conjugated according to Wilson and Nakane.[21] This Fab' fragment was used for electron-immunohistochemical examination.

Tissue Specimens for Electron Microscopy

Animals (SAM-R/2) aged 14 to 19 months were anesthetized with ether and perfused with periodate lysine-paraformaldehyde (PLP) solution. Small pieces about 3×3×3 mm taken from the liver and intestine were fixed with PLP solution and shaken overnight at 4 C. After fixation, they were washed with 10%, 15%, 20% PBS-sucrose, and 20% PBS-sucrose plus 5% glycerin, with continuous shaking. Subsequently, the pieces were embedded in OCT compound (Lab-Tek Products), frozen with dry-ice methanol, and stored at −80 C.

Immunohistochemical Technique for Electron Microscopy

Six-micron-thick frozen sections were prepared on albumin-coated slides, completely dried for over 30 minutes, immersed in cold 10% PBS-sucrose, and incubated with 10% normal rabbit serum to protect against reactions of nonspecific substances, as well as for light microscopy. These sections were then washed with cold 10% PBS-sucrose and incubated peroxidase-labeled Fab' (1:2500 to 1:5000 dilutions) overnight in a moist chamber at 4 C, then washed with cold 10% PBS-sucrose and fixed with cold 1% glutaraldehyde in PBS, washed with cold PBS until the glutaraldehyde was completely removed, then immersed in incomplete Karnovsky's[22] DAB solution (without hydrogen peroxidase), which included 1% dimethyl sulfoxide, pH 7.6, and complete Karnovsky's solution. They were then washed with cold PBS and fixed with 2% osmium tetroxide dissolved in phosphate buffer, pH 7.4, dehydrated in a graded series of ethanol, and embedded in LUVEAC 812. After the LUVEAC block had become hard enough, the slides were warmed by a gas flame,

and the block was separated from the slides. Ultrathin sections were prepared on an ultramicrotome (LKB Type 480/A). Sections not stained with uranyl acetate and lead citrate were examined under an electron microscope (JEM-100C) with an accelerated voltage of 80 kV.

Control Test

Antiserum (1:250) was mixed with purified antigens (10 mg of antigens per milliliter of original serum) on a rotating mixer at 4 C for 24 hours and centrifuged at 12,000 rpm for 20 minutes. The supernatant and normal rabbit serum were used at the appropriate dilutions for control studies of the PAP reactions.

Antiserum (Fab'-HRP) (1:8 dilution in tris[hydroxymethyl]aminomethane-buffered saline with 1% normal goat sera) was incubated with purified antigens (0.25 mg of antigens/ml of original serum) for 1 hour at room temperature, mixed on a rotating mixer at 4 C for 24 hours, and then centrifuged at 12,000 rpm for 20 minutes. The supernatant was used at appropriate dilutions for the control studies involving electron-microscopic immunohistochemical examinations.

Colchicine and Cycloheximide Treatment

To determine which organs produce AS_{SAM}-related antigenic substance, we made use of colchicine, which disturbs the secretion of synthesized protein,[23] and cycloheximide, which inhibits protein synthesis. Sixteen (ICR) male mice aged 6 weeks were used in this experiment. All the animals were deprived of food for 15 hours and put in three groups. The control group included 4 animals treated with 1 ml of 0.85% saline, the colchicine group included 6 treated with 1 ml of 1 mg/ml saline, and the cycloheximide group included 6 treated with 1 ml of 25 mg/ml saline.

For another 7 hours after the single intraperitoneal injection of these drugs, the animals were killed, without being fed. AS_{SAM}-related antigenic substance concentration in the serum was examined by single radial immunodiffusion test.[24] Morphologic detection in the organs was performed with the PAP method described above.

Results are presented as means ± SE computed from the average measurements obtained from each mouse. Significant levels for comparisons between different measurements were determined with the use of the Student t test.

Results

Light Microscopy

On the H&E-stained sections, various sized vesicles, including amorphous substances which were weakly stained with eosin, were evident in the cytoplasm of the hepatocytes. There was no staining with Congo red, nor any birefringence, under the polarizing microscope. This substance reacted positively only with anti-AS_{SAM} antibody, by the immunoperoxidase method.

AS_{SAM}-related antigenic substance was observed in the cytoplasm of the hepatocytes, columnar cells of the

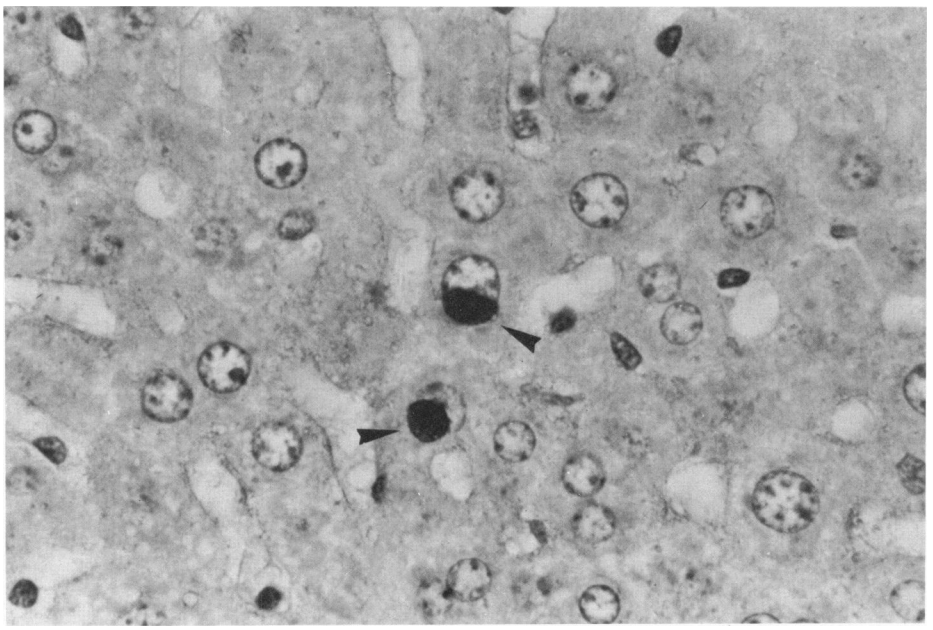

Figure 1—Light micrograph of the liver. AS_{SAM}-related antigenic substance is in large round or oval vesicles adjacent to the nucleus in the cytoplasm of the hepatocytes (*arrowheads*). (PAP-stained section, ×1770)

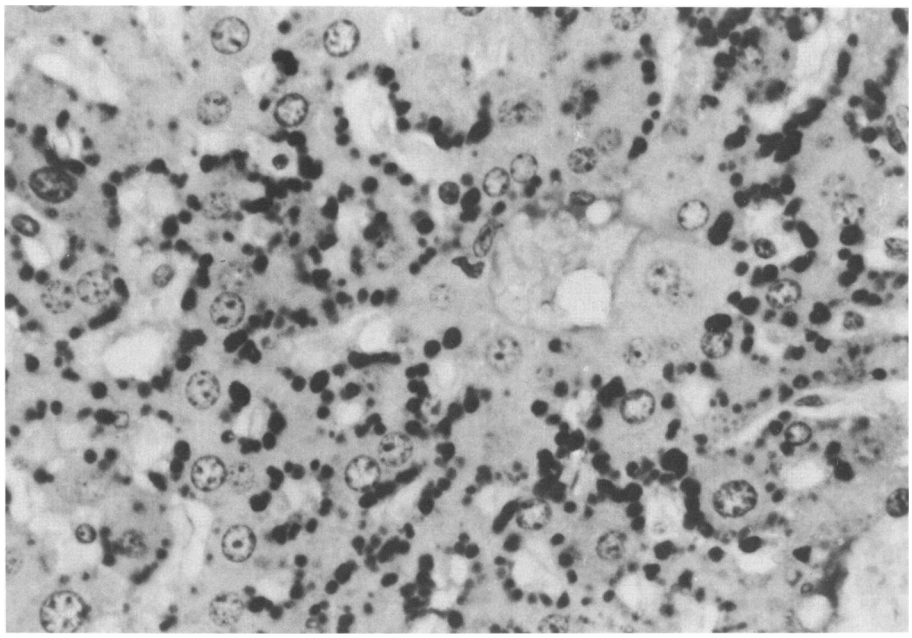

Figure 2—Light micrograph of the liver. AS_{SAM}-related antigenic substance in small vesicles in the cytoplasm of the hepatocytes forms a line facing the space of Disse. (PAP-stained section, ×1770)

small intestine, and epithelia of the proximal convoluted tubules of the kidney.

In the liver, this reactive substance in the hepatocytes was mainly observed around the central vein. Generally, the PAP-reacted brown substance in the cytoplasm of the hepatocytes was in the form of vesicles of various sizes. Several tiny vesicles presented diffusely in the cytoplasm, and a large one was sometimes the size of the nucleus of the cell. Because of the compression by this vesicle, the outline of the nucleus appeared irregular (Figure 1). At other areas, small vesicles including the AS_{SAM}-related antigenic substance in the cytoplasm formed a line facing the space of Disse (Figure 2).

Many columnar epithelia of the small intestine also presented the small vesicles, including the AS_{SAM}-related antigenic substance, which seemed to be small granules in the cytoplasm. Most of the vesicles were located more basally than the nucleus (Figure 3A and B). In the small intestine, several vesicles also formed a line in the intercolumnar epithelia. The large vesicles seen in the liver were rare in the cytoplasm of the intestinal epithelia.

Demonstration of the AS_{SAM}-related antigenic substance in the kidney proved to be difficult because of the small amount. Tiny granules were demonstrated in the cytoplasm of the cuboidal epithelia facing the lumen in the proximal convoluted tubules. The large vesicles seen in the liver were absent in the kidney.

Electron Microscopy

The brown substances observed in the cytoplasm of the hepatocytes under light microscopy were detected as an amorphous nonfibrillar substance under electron microscopy. This AS_{SAM}-related antigenic substance was present in vesicles of various sizes in the cytoplasm. These vesicles had a clearly outlined unit membrane. The small vesicles including the AS_{SAM}-related antigenic substance existed diffusely or facing the space of Disse in the cytoplasm, and the large vesicles sometimes compressed the nucleus, as seen under light microscopy (Figure 4). Fab′-HRP-reactive substance was also detected in the endoplasmic reticulum and Golgi apparatus (Figure 5).

In the columnar cells of the small intestine, the morphologic features of this Fab′-HRP-reactive substance were the same as seen in the hepatocytes (Figure 6).

Round or oval homogeneous amorphous lipid droplets sometimes existed, together with this Fab′-HRP reactive substance, in the hepatocytes (Figure 7) and columnar cells of the small intestine.

Relationship Between the Incidence and Intensity in Presence of AS_{SAM}-Related Antigenic Substance in the Cytoplasm and Those of AS_{SAM} Deposition With Advancing Age

The incidence of demonstration of the intracytoplasmic AS_{SAM}-related antigenic substance in the liver was over 80% in the SAM-P/1 up to 10 months of age, except the newborn; however, in the 15-month-old animals, the incidence was only 20%. In SAM-R/2, more than 60% was demonstrated in every stage up to 20 months of age, except the newborn. The small intestine also showed a high incidence at any month in both SAM-P/1 and SAM-R/2. In the latter, under 5

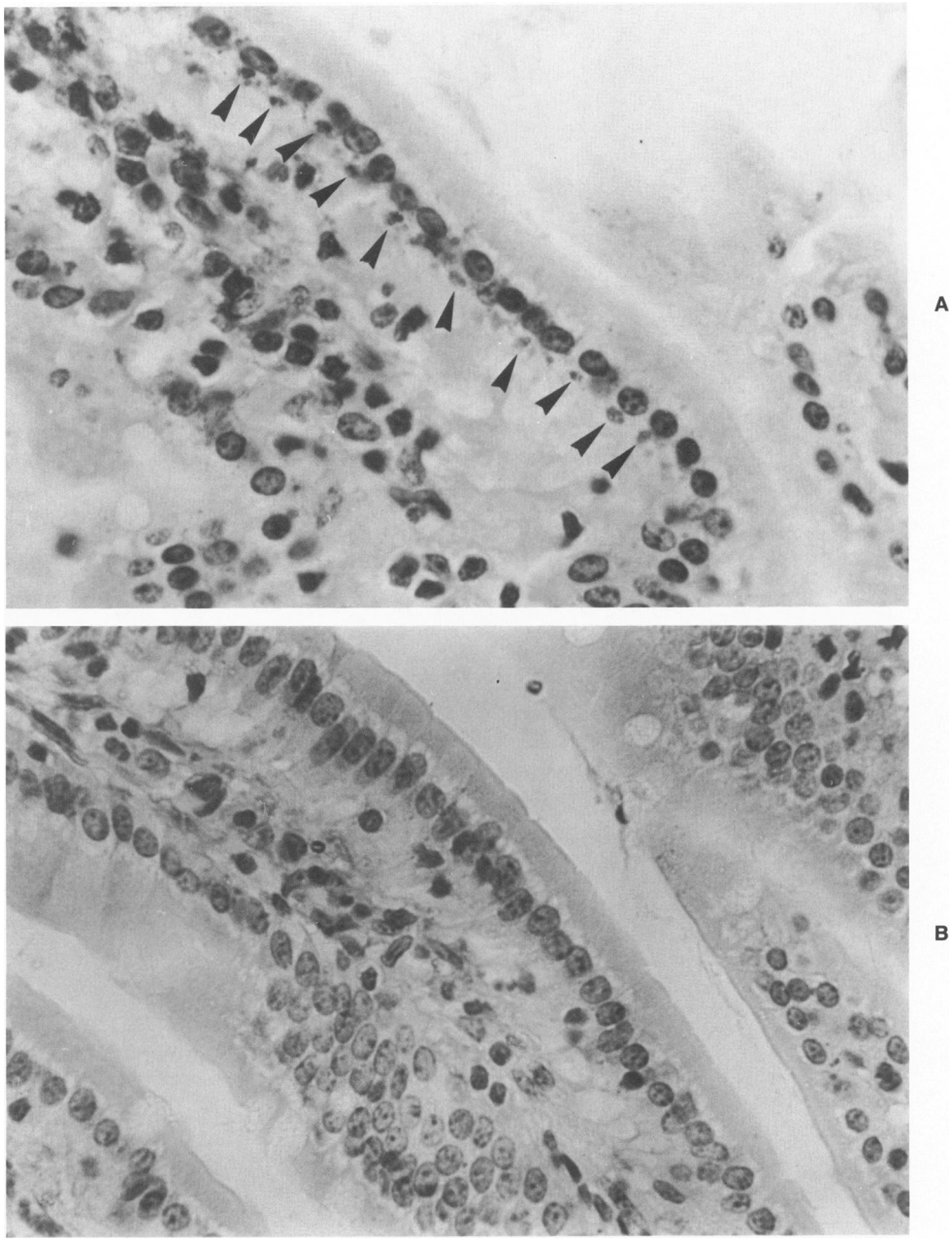

Figure 3A—Light micrograph of the small intestine. Small vesicles, including AS_{SAM}-related antigenic substances are present more basal than the nucleus in the cytoplasm of the columnar epithelium (*arrowheads*). (PAP-stained section, ×1770) **B**—Light micrograph of the small intestine. No reactive substances are observed in the cytoplasm of the columnar epithelium in the control preparation. (PAP-stained section, ×1770)

months of age, except the newborn, the incidence was 100% (Table 1).

Intracytoplasmic AS_{SAM}-related antigenic substance was intensively in hepatocytes in 5-month-old mice, in both SAM-P/1 and SAM-R/2, and then decreased rapidly in SAM-P/1 and gradually in SAM-R/2 with advancing age. The incidence and intensity in deposition of the substance observed in the extracellular space (Amyloid protein) increased rapidly from 5 months of age in SAM-P/1, and that of SAM-R/2 increased very slowly from 10 months of age (Figure 8).

Columnar epithelia of the small intestine contained much of the anti-AS_{SAM}-reactive substance in both SAM-P/1 and SAM-R/2 at 1 or 2 months of age, and thereafter the decrease was gradual. Amyloid deposition in the extracellular space, mostly in lamina propria, was seen from 4 to 5 months of age in SAM-P/1 and at 8–10 months of age in SAM-R/2 (Figure 9).

Demonstration of intracytoplasmic AS_{SAM}-related antigenic substance and extracellular amyloid fibril protein in the kidney showed a pattern similar to that seen in the liver in SAM-P/1. In SAM-R/2, though the intensity was not great in the epithelia of the convoluted tubules, the peak of the demonstration in the cells was

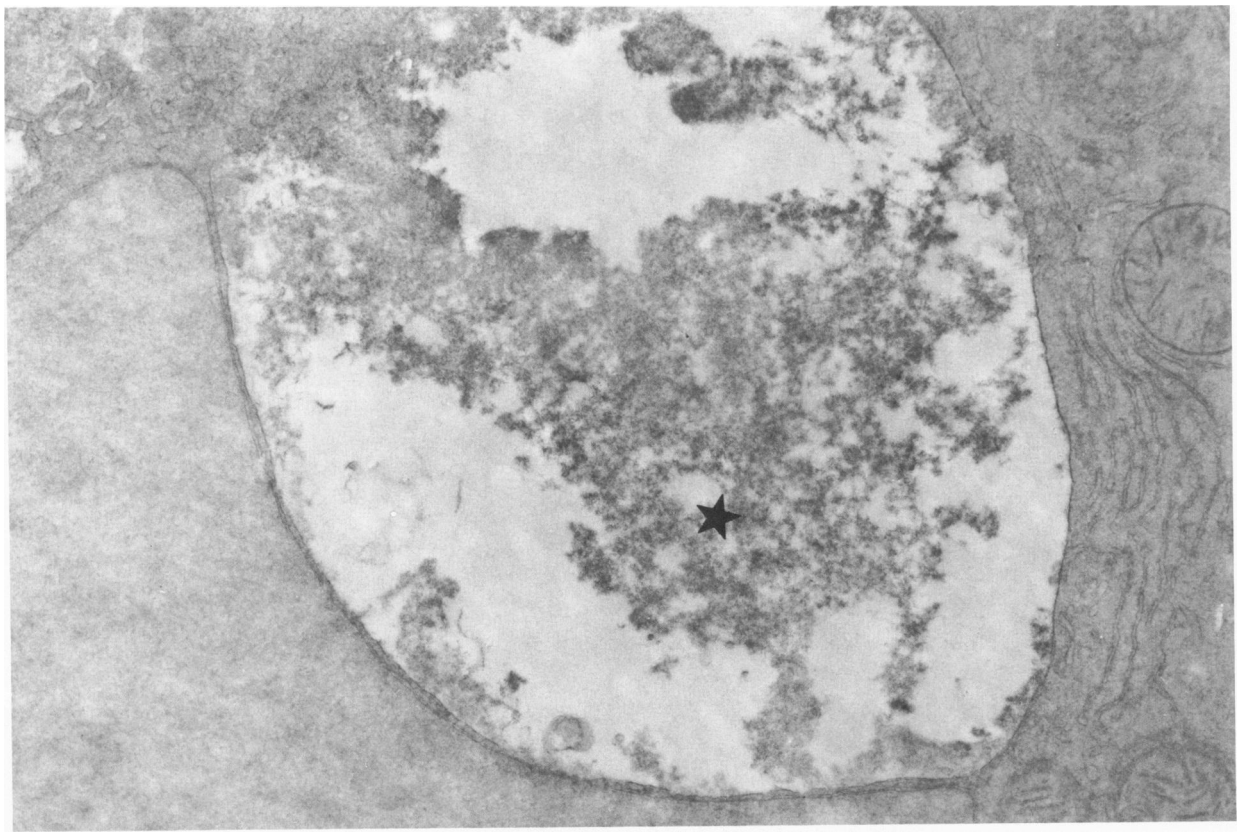

Figure 4— Immunoelectron micrograph of the hepatocyte. Fab′-HRP-reactive substance (*asterisk*) is present in the large vesicle, and the nucleus is compressed. The amorphous Fab′-HRP-reactive substance shows no fibril formation. (Fab′-HRP-stained section, ×16,900)

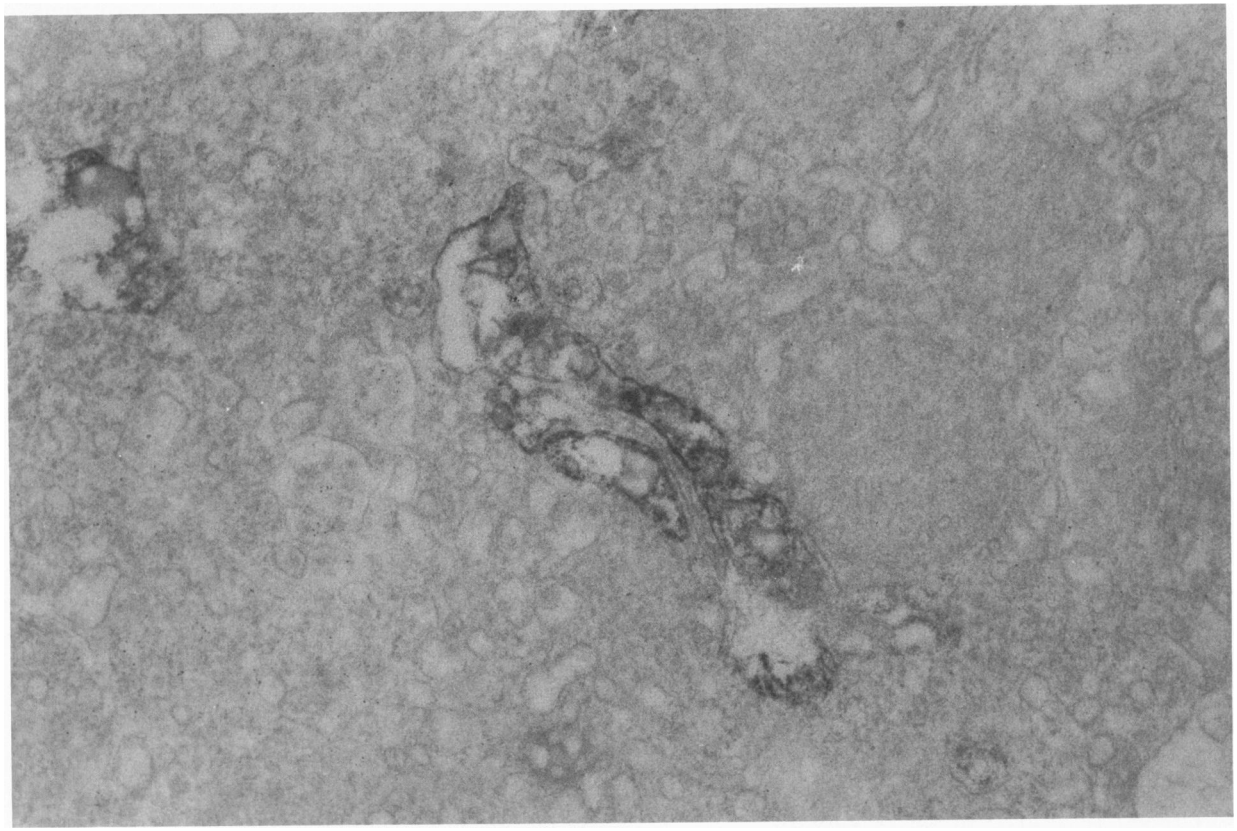

Figure 5—Immunoelectron micrograph of a hepatocyte. Electron-dense Fab′-HRP reactive substance is present in the Golgi apparatus. (Fab′-HRP-stained section, ×44,400)

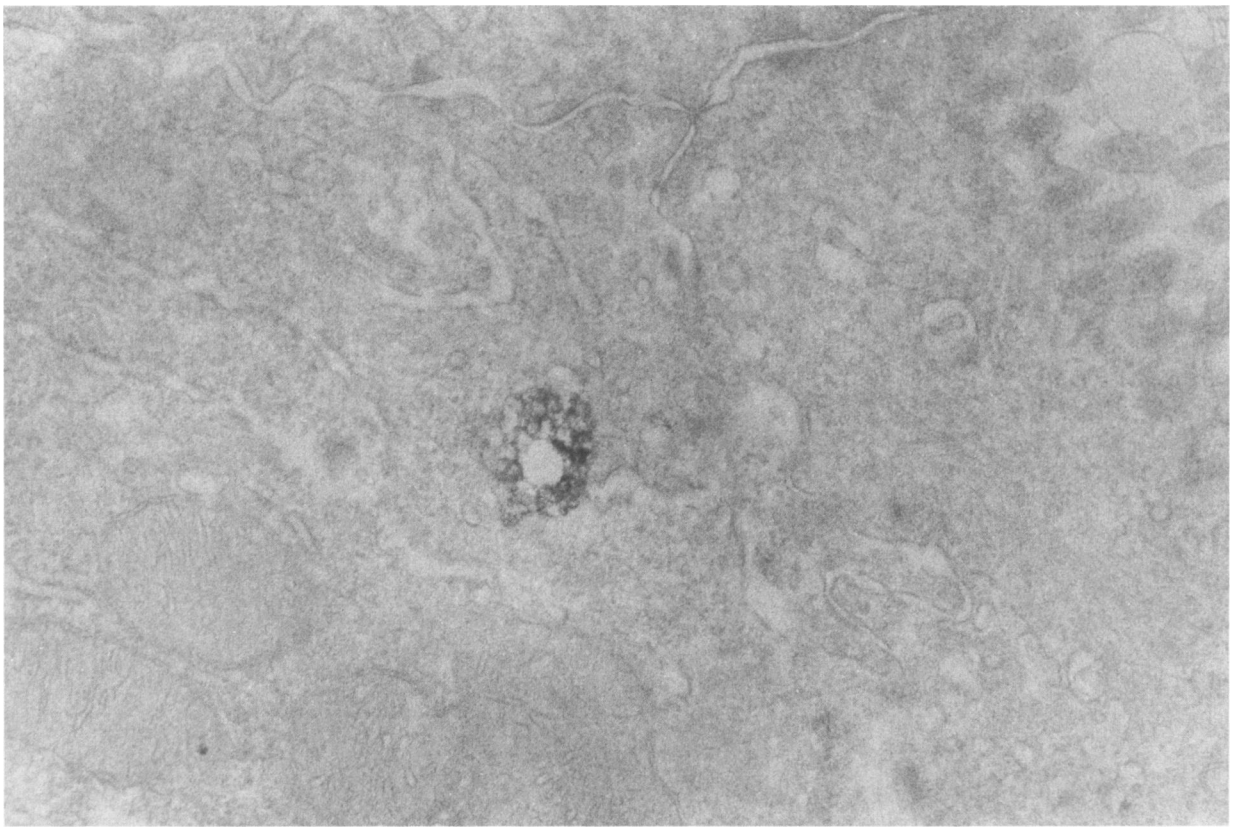

Figure 6—Immunoelectron micrograph of the columnar epithelia of the small intestine. Electron-dense Fab'-HRP-reactive substance is present in the cytoplasm. (Fab'-HRP-stained section, ×40,700)

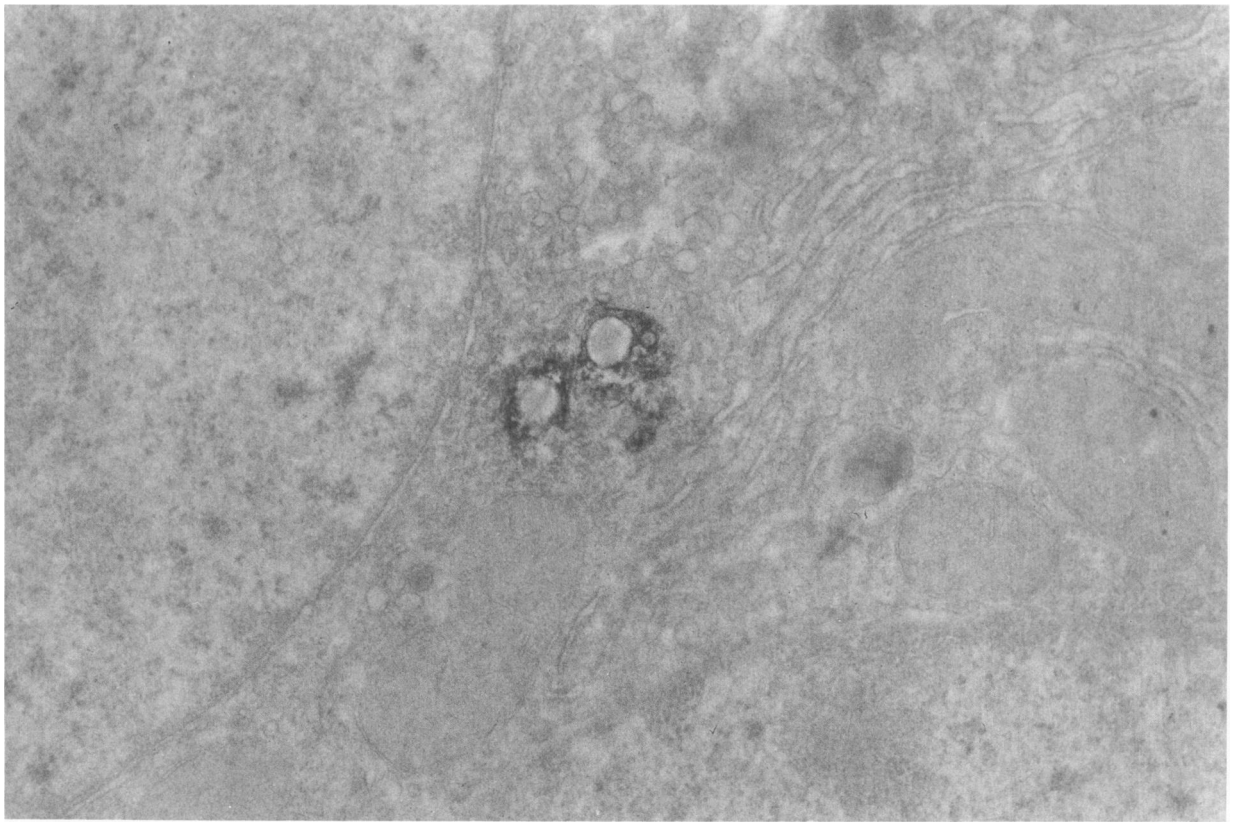

Figure 7—Immunoelectron micrograph of the hepatocytes. Round or oval homogeneous amorphous lipid droplets exist together with electron-dense Fab'-HRP-reactive substance in the cytoplasm. (Fab'-HRP-stained section, ×25,800)

Table 1—Incidence in the Presence of CAS_{SAM} with Advancing Age

Age (months)	Number of animals	Liver (%)	Intestine (%)	Kidney (%)
SAM-P/1				
Newborn	11	27	45	14
1–2	12	92	100	58
5	7	100	57	86
10	9	100	89	78
15	11	20	64	0
SAM-R/2				
Newborn	16	0	61	0
1–2	10	90	100	80
5	7	86	100	71
9–10	9	67	78	56
16–20	7	100	86	57

Animals were considered positive for AS_{SAM}-related antigenic substance when more than one respective cell of the organs (hepatocyte, columnar epithelium of the small intestine, renal convoluted tubular cell) contained brown substance in the cytoplasm, detected by the PAP method.

seen in mice at 5 months of age. Amyloid fibril protein was deposited mostly in mice over 10 months of age.

Organs Producing AS_{SAM}-Related Antigenic Substance

The experimental results on modification in synthesis of secretion of AS_{SAM}-related antigenic substance by colchicine or cycloheximide are shown in Table 2.

AS_{SAM}-related antigenic substance concentration in the serum (SAS_{SAM}) in the cycloheximide-treated group showed a significantly low level by single radial immunodiffusion test, as compared with the 0.85% NaCl-treated group (control). The colchicine-treated group also showed levels slightly lower than the control, but not to a significant degree. Morphologically, all the animals treated with NaCl and colchicine clearly had AS_{SAM}-related antigenic substance in the cytoplasm of the hepatocytes, but only 2 out of 6 animals treated with cycloheximide showed positive findings. The intensity of the AS_{SAM}-related antigenic substance in the hepatocytes showed statistically significant low levels in the cycloheximide-treated group, as compared with the control group. As for the small intestine and kidney, the cycloheximide-treated group also showed a low incidence and a slight degree of intensity in the presence of AS_{SAM}-related antigenic substance in the epithelia. The colchicine-treated group showed levels slightly higher in the liver and kidney and slightly lower in the intestine, as compared with findings in the control group.

Control Test

Antigen-antibody mixed solution and normal rabbit serum did not react with AS_{SAM}-related antigenic

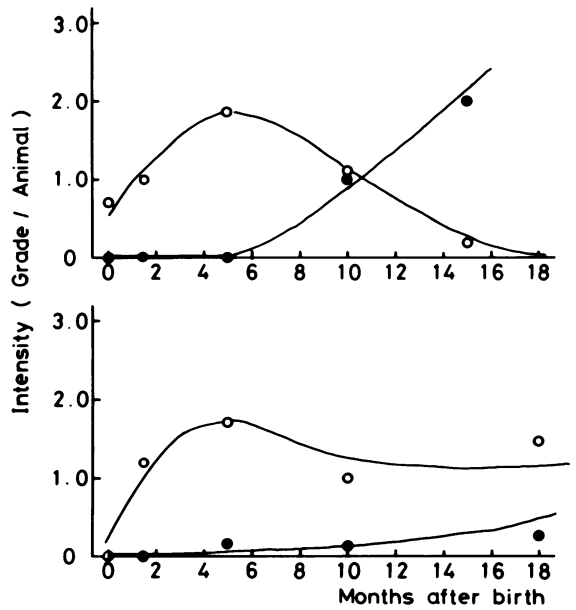

Figure 8—Intensity in deposition of AS_{SAM}-related antigenic substance in SAM-P/1 (*upper*) and SAM-R/2 (*lower*) in the liver; O——O, presence of AS_{SAM}-related antigenic substance in the intracytoplasm; ●——●, deposition of AS_{SAM}-related antigenic substance in the extracellular space (amyloid protein AS_{SAM}).

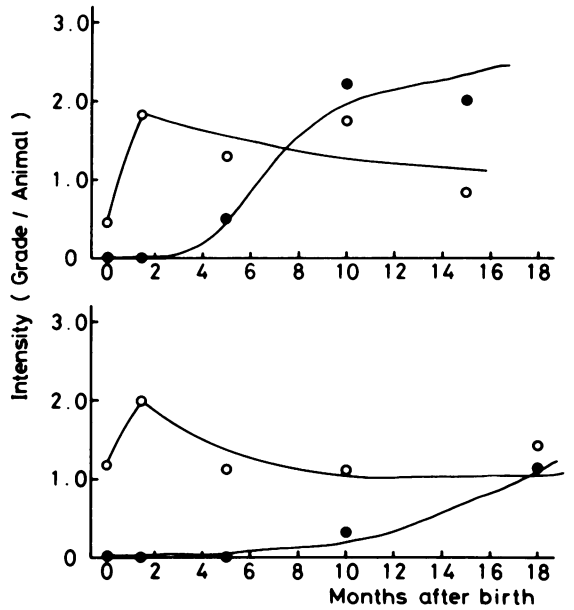

Figure 9—Intensity in deposition of AS_{SAM}-related antigenic substance in SAM-P/1 (*upper*) and SAM-R/2 (*lower*) in the small intestine; O——O, presence of AS_{SAM}-related antigenic substance in the intracytoplasm; ●——●, deposition AS_{SAM}-related antigenic substance in the extracellular space (amyloid protein AS_{SAM}).

Table 2—Effects of Colchicine and Cycloheximide Treatment on SAS_{SAM} and CAS_{SAM}*

Drug	Dose	Number of animals	SAS_{SAM} (ng/μg)	CAS_{SAM} Incidence			CAS_{SAM} Intensity		
				Liver	Intestine	Kidney	Liver	Intestine	Kidney
NaCl	0.85%	4	1558 ± 88	4	4	1	1.25 ± 0.25	1.25 ± 0.25	0.25 ± 0.25
Colchicine	1 mg/ml	6	1054 ± 89	6	6	1	1.33 ± 0.33	1.17 ± 0.17	0.33 ± 0.33
Cycloheximide	25 mg/ml	6	1153 ± 88†	2	5	1	0.33 ± 0.21†	0.83 ± 0.17	0.17 ± 0.17

Values are means ± SE.

* Animals deprived of food for 15 hours were given 1 ml of the drug intraperitoneally and killed in the fasting state 7 hours later.

† Significant differences ($P < 0.05$) in SAS_{SAM} concentration determined by a single radial immunodiffusion test and intensity in the presence of CAS_{SAM} demonstrated morphologically from the corresponding control (NaCl-treated) group. Intensity (see Materials and Methods) for each animal was graded at four levels, and the average for each group was obtained.

substance, as determined under light and electron microscopy.

Discussion

In murine amyloidosis, several types of amyloid protein have been reported: murine protein AA,[25,26] amyloid protein in SJL/J,[7] and senile amyloid protein AS_{SAM}, discovered in our laboratory.[9] The serum precursor of the former amyloid protein is assumed to be SAA, and this precursor is associated with HDL_3 lipoprotein.[27-29] Benson and Kleiner[30] measured the SAA level in the serum and hepatic cells by radioimmunoassay using casein-treated CBA/J mice, and suggested that the SAA was synthesized by hepatocytes and secreted rapidly by casein injection. They also demonstrated SAA morphologically in hepatocytes, using immunohistochemical staining. Most recently, Shirahama et al[31] reported the demonstration and intracellular pathways of anti-amyloid protein AA-reactive substance, and Takahashi et al[32] reported serum amyloid A synthesis in murine hepatocytes. In the present study, the substance observed in the cytoplasm of the hepatocytes, columnar epithelia of the small intestine, and epithelia of convoluted tubules of the kidney reacted with anti-AS_{SAM} antibody by the immunoperoxidase method but did not stain with Congo red or show birefringence and fibril formation with polarizing microscopy and electron microscopy, respectively. We named this substance CAS_{SAM} (cytoplasmic AS_{SAM}-related antigenic substance).

The colchicine-treated mice clearly showed CAS_{SAM} in the liver and the small intestine, while the cycloheximide-treated mice exhibited a low incidence and a slight intensity in its presence. Since colchicine disturbs the secretion of synthesized protein and cycloheximide inhibits protein synthesis in the cell, it can be assumed that both the colchicine- and cycloheximide-treated animals would have a decrease in SAS_{SAM} content in the serum. In particular, the finding that cycloheximide-treated animals showed a significant decrease of SAS_{SAM} concentration in the serum suggests that the CAS_{SAM} is synthesized in the hepatocytes and columnar epithelia in the small intestine, while CAS_{SAM} detected in the epithelia of proximal convoluted tubules seems to be a reabsorbed substance.

Morphologic features observed light and electron microscopically support this hypothesis. Under light microscope, small vesicles, including the CAS_{SAM} facing the space of Disse, seem to be the events just before the CAS_{SAM} secretion from the cells. Under the electron microscope, CAS_{SAM} in the hepatocytes is seen in the endoplasmic reticulum, Golgi apparatus, and large secretory vesicles. In the small intestine, CAS_{SAM} mainly locates in the basal area, as seen under the light microscope; whereas it was seen on both sides of the nucleus under the electron microscope. AS_{SAM}-related antigenic substance also existed in the intracellular space of the columnar epithelia in nonfibrillar structures. These findings observed in the liver and intestine confirm that CAS_{SAM} follows the common routes of synthesis, intracellular pathways, and secretion established for other proteins.[33]

Because CAS_{SAM} and SAS_{SAM} (serum AS_{SAM}-related antigenic substance) reacts with anti-AS_{SAM} antibody, they must have the same antigenic determinant. Furthermore, because SAS_{SAM} concentration in the serum and intensity in the presence of CAS_{SAM} in the intracytoplasm show expected changes with colchicine and cycloheximide treatment, CAS_{SAM} is assumed to be a precursor of SAS_{SAM}. The finding that SAS_{SAM} circulates in the form of HDL in the serum leads to the idea that CAS_{SAM} is also secreted in the form of lipoprotein. Whether the secreting form of CAS_{SAM} is

much the same as the form of SAS_{SAM} in the serum and whether there is a precise conversion from CAS_{SAM} to SAS_{SAM} and from SAS_{SAM} to amyloid protein AS_{SAM} are subjects of ongoing studies.

Higuchi et al suggested that AS_{SAM}-related antigenic substance existed as an apoprotein in the normal serum, regardless of the strain,[18] and this apoprotein is physiologically vital for mice. They also showed that SAS_{SAM} concentration in the serum gradually decreased with aging in SAM-P.[24] The incidence and intensity in the presence of CAS_{SAM} in the hepatocytes and columnar epithelia of the small intestine was high in young mice, in both the SAM-P and SAM-R, and decreases of CAS_{SAM} were observed in the liver and the small intestine with advancing age in SAM-P. All our observations, taken together, suggest that CAS_{SAM} is probably synthesized in the hepatocytes and the intestinal mucosal epithelia and is a precursor of SAS_{SAM}.

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