Immunocytochemical Study of Hepatocyte Synthesis of Amyloid AA

Demonstration of Usual Site of Synthesis and Intracellular Pathways but Unusual Retention on the Surface Membrane

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For determination of the intracellular site of synthesis and the pathways followed by amyloid protein AA, immunocytochemical localization of the anti-AA reactive substance was investigated in the livers of CBA/J mice in an acute-phase response evoked by a single subcutaneous injection of 0.5 ml of 10% casein. In the cytoplasm of the hepatocytes, the positive reaction was localized on and/or in the rough endoplasmic reticulum and the single membrane-bound vesicles, vacuoles and lamellae including the Golgi apparatus, confirming that amyloid protein AA follows the common routes of synthesis and secretion established for other proteins. The anti-AA–reactive substance was also localized on the free surface of the hepatocyte membrane, including the microvilli. The latter reaction appeared as early as but lasted at least several hours longer than its cytoplasmic counterpart, suggesting that a certain retention period exists before the release of the AA-reactive substance from the cellular surface to the free blood plasma. (Am J Pathol 1985, 118:108–115)

RECENT STUDIES have established the concept that SAA, the putative precursor of amyloid fibril protein AA and an acute-phase reactant,[1-8] is biosynthesized primarily in the liver and more specifically in the hepatocytes.[8-13] While these studies have elaborated considerable detail of the immunologic, biochemical, and molecular biology of SAA synthesis, credible morphologic evidence concerning the intracellular site of synthesis and pathways of this protein has so far been lacking. In this regard, we recently reported our immunohistochemical observations, which have strongly suggested that participation of the hepatocyte population in AA synthesis is not universal but that heterogeneity exists.[14]

The purpose of this communication is to report our electron-microscopic immunocytochemical findings, which have clearly localized the reaction against anti-AA on and/or in the rough endoplasmic reticulum (RER), the single membrane-bound vesicles, vacuoles and lamellae including the Golgi apparatus, and the surface membrane of the hepatocytes of the mice in acute-phase reaction.

Materials and Methods

Each of 8 week-old female CBA/J mice (Jackson Laboratories, Bar Harbor, ME) received a single subcutaneous injection of 0.5 ml of 10% casein solution. Groups of 3 mice were sacrified at 0, 2, 4, 6, 8, 12, 16, 24, or 48 hours after the casein injection. Three mice were also sacrificed after receiving 30 daily casein injections[15] and then a 2-week pause.

At sacrifice, each mouse was anesthetized with an intraperitoneal injection of chloral hydrate (0.36 g/kg),

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and was perfused via the abdominal vena cava with 25 ml of phosphate-buffered physiologic saline (PBS) followed by 50 ml of 4.0% paraformaldehyde–0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.6. Two tissue blocks not exceeding 3 mm in thickness were excised from each liver and were further immersed in the same fixative for 2 hours at room temperature. They were rinsed in 0.1 M phosphate buffer, pH 7.6, and exposed to 1% sodium borohydrate in the same buffer for 2 hours at room temperature.[16]

One of the two tissue blocks was processed for the preembedding immunocytochemical procedure. It was immersed in 5% glycerol–10% sucrose in the phosphate buffer for 2 hours and then in 10% glycerol–20% sucrose overnight for purposes of cryoprotection. The tissue was then frozen at liquid nitrogen temperature and thawed. After being rinsed in 10% glycerol–20% sucrose, in 5% glycerol–10% sucrose, and in the phosphate buffer, 50 μ tissue slices were cut from the block on a Sorvall TC-2 tissue sectioner and were processed for immunocytochemical demonstration of anti-AA–reactive substance with the use of an antibody bridging method: either the peroxidase–antiperoxidase technique[17,18] or the avidin–biotin–peroxidase method.[19] After the immunocytochemical staining, the tissue slices were thoroughly rinsed in the phosphate buffer, postfixed with 2% osmium tetroxide in the same phosphate buffer for 1 hour at room temperature, dehydrated in a series of graded ethanols, and embedded in Araldite 502. Thin sections were mounted on a bare copper grid and examined in a Siemens Elmiskop I without further staining.

Another tissue block was cut into smaller pieces (less than 1 cu mm). Aliquots of the small tissue blocks were postfixed in 2% osmium tetroxide in the phosphate buffer for 2 hours at room temperature and were then embedded, along with another aliquot which did not receive postfixation, in Araldite 502 by a routine procedure. Immunocytochemical reaction against anti-AA was carried out on the thin sections mounted on a bare nickel grid with the use of the peroxidase–antiperoxidase technique[17,18] or the avidin–biotin–peroxidase method.[19] The sections were then examined, with optional staining with 4% osmium tetroxide, in a Siemens Elmiskop I electron microscope.

Procedural controls were run by replacing the primary antiserum (rabbit anti-mouse AA) with normal rabbit serum or the primary antiserum absorbed with mouse serum with a high SAA level. Tissues from normal (0 hour) and amyloidotic (30 daily casein injections) mouse livers were included in each processing as baseline and positive controls.

Antiserum to mouse AA was prepared in rabbits as previously described.[20] Peroxidase-antiperoxidase complex, goat anti-rabbit IgG, normal goat serum, and normal rabbit serum were obtained from Cappel Laboratories (Cochranville, Pa) and avidin, biotinylated horseradish peroxidase, and biotinylated goat anti-rabbit IgG from Vector Laboratories (Burlingame, Calif).

Results

In the preparations of the livers from the mice that had received 30 daily casein injections and a 2-week pause, and treated for immunocytochemical demonstration of anti-AA reaction, the electron-dense reaction products were densely localized on the amyloid fibrils (Figures 1 and 2).

The hepatic tissue sections from the untreated mice (at 0 hour of the casein injection scheme) did not show any appreciable reaction against anti-AA.

A general survey of the present preparations from the mice in acute phase response (2–48 hours after the casein injection) corresponded well with the findings of our previous immunohistochemical studies,[14] in that 1) there were two major localizations of the immunocytochemical reaction against anti-AA, ie, in the hepatocyte cytoplasm and on the surface membrane; 2) only certain hepatocytes that were randomly spread in the lobules showed the cytoplasmic reaction; 3) the cytoplasmic reaction to the anti-AA started to be recognized at 2–4 hours after the casein injection, peaked in intensity and the number of cells involved at 6–8 hours, and became minimal or unrecognizable at 24–48 hours; and 4) the reaction on the surface membrane appeared as early as the cytoplasmic reaction, was heaviest at 12–16 hours, and still remained at 24–48 hours.

In the cytoplasm of the hepatocytes, the reaction products were localized on the membrane and the attached ribosomes of the RER, and in the lumens of the RER, the smooth endoplasmic reticulum (SER), the Golgi apparatus and the single membrane-bound vesicles and vacuoles. No significant reaction products were detected in and/or on other cell organelles, namely, the nucleus, mitochondria, etc. (Figures 3–5).

About the RER, the reaction products were localized mainly on the attached ribosomes and the membrane, but also were observed on occasions as if they were attaching to and extruding from the membrane into the cisternae. The cisternae of the RER were seen very rarely to be completely filled with the reaction products (Figure 6). The SER containing the reaction products was also occasionally identified. The Golgi apparatus was often easily identifiable, for it was heavily deposited with the reaction products (Figure 7).

Single membrane-bound vesicles and vacuoles also displayed positive reaction to anti-AA. The contents

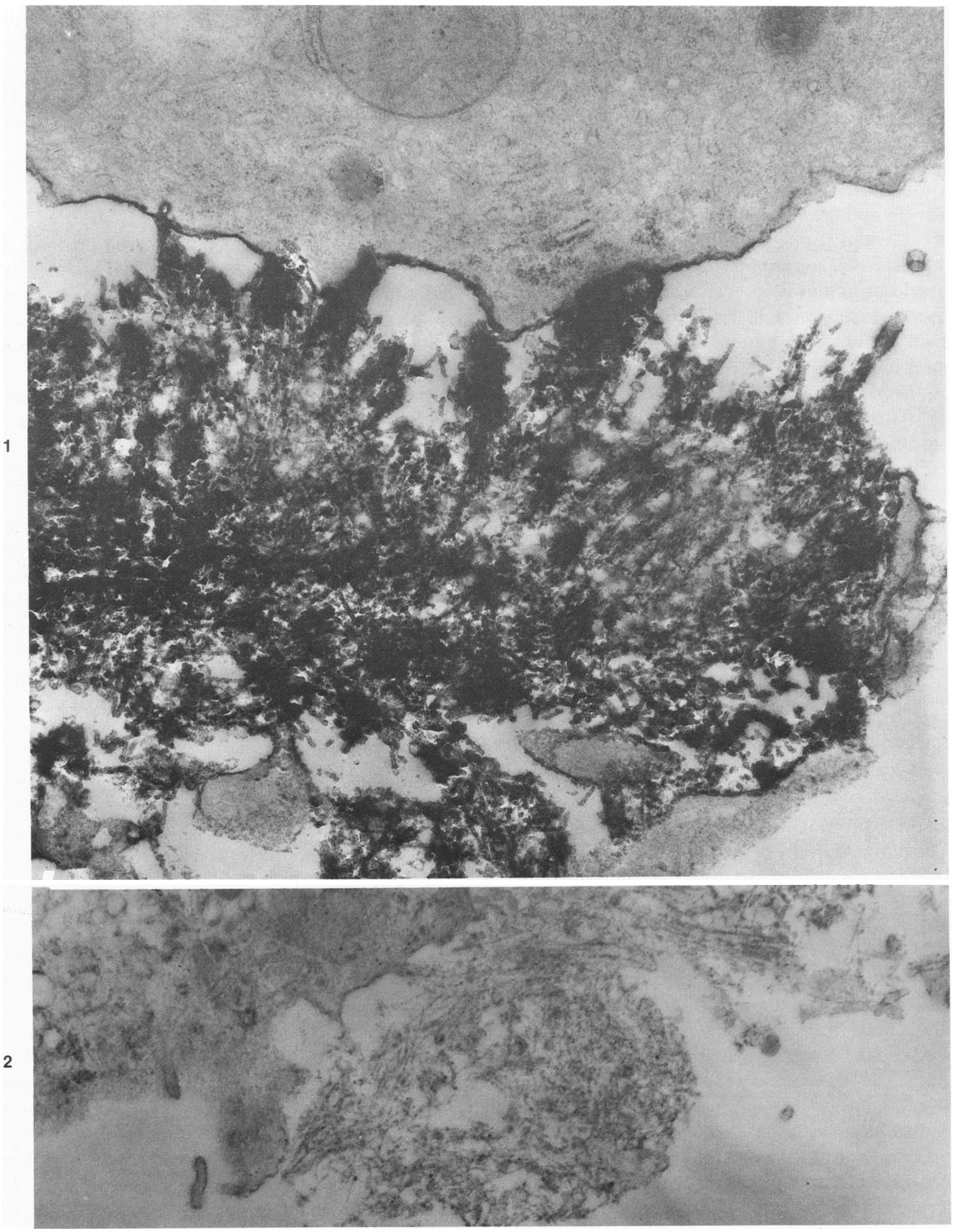


Figure 1 – Electron micrograph of the liver of CBA/J mouse after receiving 30 daily casein injections and a 2-week pause. Immunocytochemical preparation reacted against anti-mouse AA. Electron-dense reaction products deposit heavily on the amyloid fibrils located in the space of Disse. A portion of a hepatocyte (*top*) and several cross-sections of the sinusoidal endothelium (*bottom*) can also be seen. The portions of the plasma membrane facing the space of Disse are also coated with substance that shows a positive reaction. (× 25,000) **Figure 2** – Electron micrograph of a section from the same liver as Figure 1. Immunocytochemical control preparation where the primary antiserum (rabbit anti-mouse AA) was replaced by normal rabbit serum. No significant deposition of the reaction product is recognized either on the amyloid fibrils (*center*) or on the cell (*upper left*). (× 25,000)

with the positive reaction often filled completely the lumens of smaller vesicles but only partially larger vesicles and vacuoles. In the latter case, the contents with the positive reaction were localized close to the membrane, as if forming a thin rim along the inside of the membrane (Figures 8 and 9).

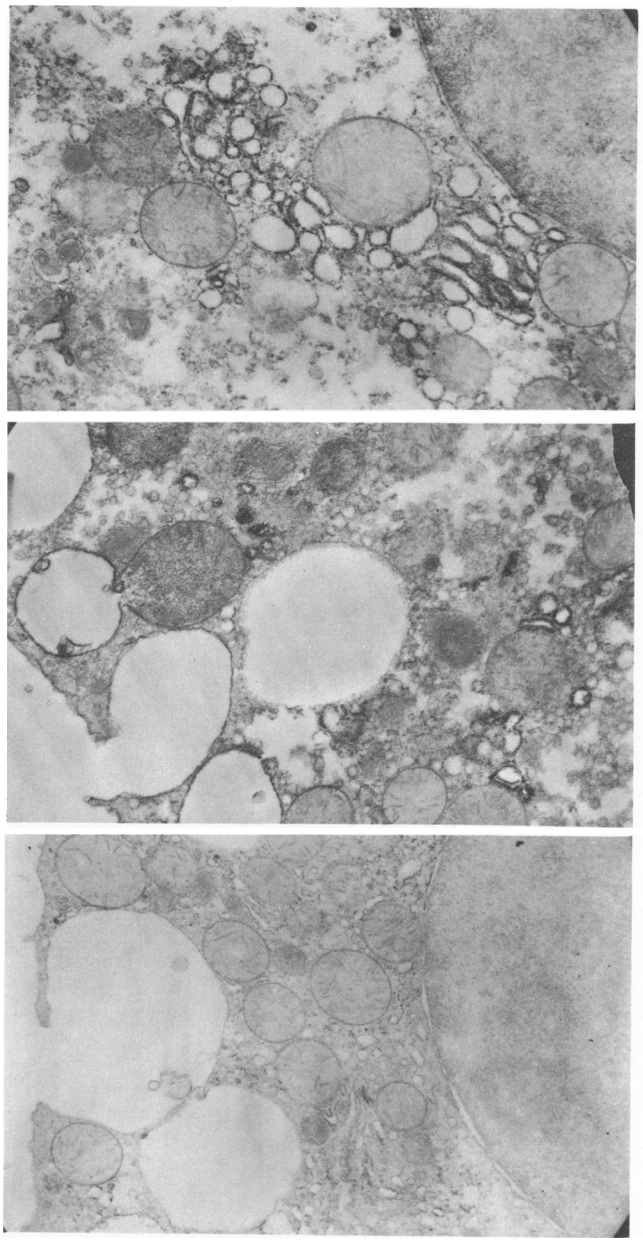
The free surface of the plasma membrane, including the microvilli, facing the sinusoid was often observed as if being coated with the reaction products (Figure 10).

The control preparations where the primary antiserum had been replaced with normal rabbit serum or the primary antiserum absorbed with SAA-rich mouse serum did not show any significant deposition of the reaction products (Figures 2 and 5).

Discussion

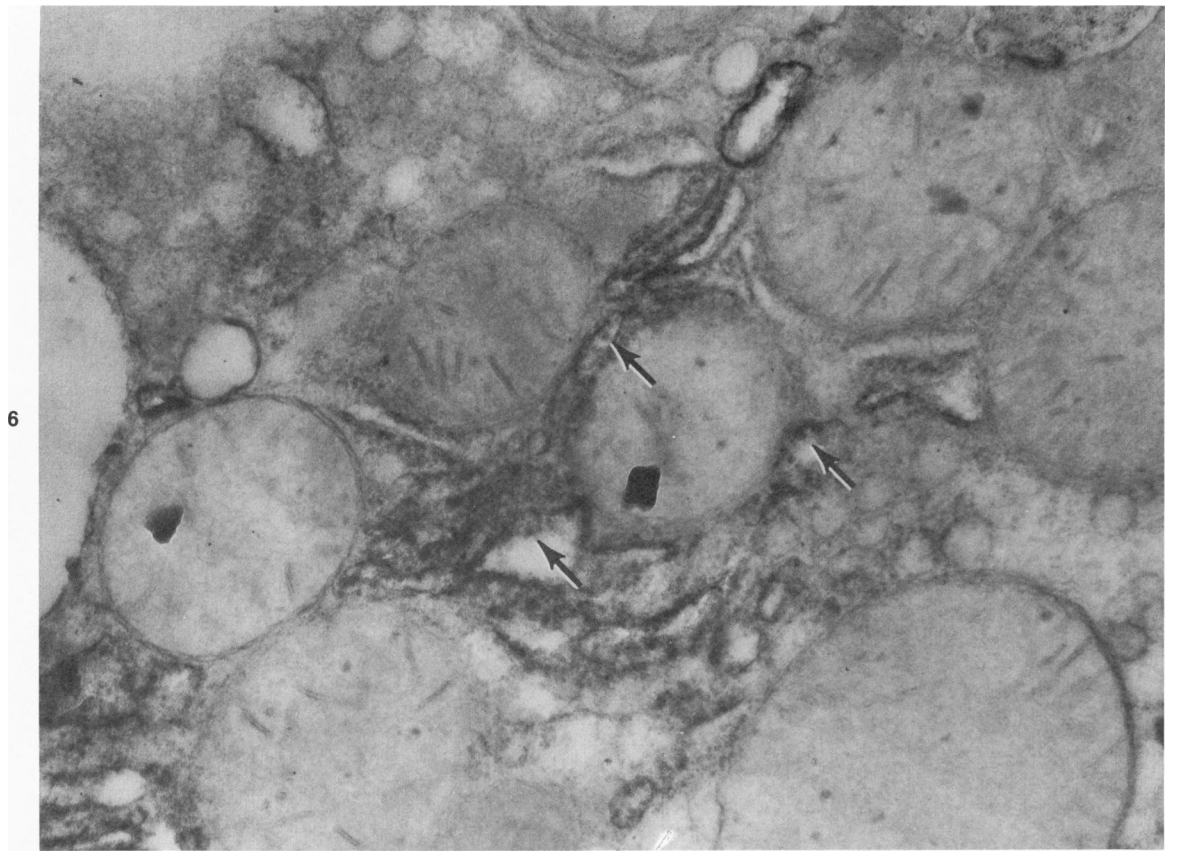
The present study has localized the immunocytochemical reaction against anti-AA on and/or in the RER, the single membrane-bound vesicles, vacuoles and lamellae (including the Golgi apparatus), and the surface membrane of the hepatocytes of the mice in acute-phase response.

A currently widely accepted concept regarding the pathogenesis of AA-amyloidosis[21] is, in short, as follows. An acute phase stimulus evokes production and release of a mediator, a peptide product of monunuclear phagocytic cells called interleukin 1.[8,11,22,23] This mediator actively stimulates the biosynthesis of protein AA primarily by the hepatocytes. At this stage, AA (a molecular weight of about 8500) is synthesized and transported through the intracellular channel in the form of its precursor, apoSAA (a molecular weight of about 12,000). ApoSAA then becomes associated with lipoprotein in the intravascular compartment and transforms into a state naturally found in the serum, SAA (a molecular weight of 160,000–200,000). Later, SAA is processed to form AA and is deposited as amyloid fibrils at the destination site. The mechanism involved in this latter process has not yet been clearly determined. It is, however, suspected that a specific enzymatic cleavage or a limited degradation, and therefore an impaired reticuloendothelial function, may be involved in this process.[21,24–26] This concept fits well the long-standing "two-phase theory of amyloidogenesis" of Teilum.[27] The first phase, synthesis and secretion of SAA, can be created rather simply and quickly by an acute-phase stimulus; whereas the second phase, transformation of SAA to AA and formation of amyloid fibrils, seems to require a certain latency period. For example, with our daily casein injection scheme in the mouse, a single casein injection can evoke high serum levels of SAA that start at 2–4 hours, reach a plateau at 12–18 hours, and last for 24–48 hours after the injection.[2,3,6] On the other hand, it usually requires 10–15 injections for any amyloid deposition to become recognizable in any tissue.[15]



Figures 3–5—Lower power electron micrographs of hepatocytes of a CBA/J mouse, 6 hours after a subcutaneous casein injection. Immunoperoxidase preparation against anti-mouse AA (**Figures 3** and **4**) and a control where primary antiserum was replaced with normal rabbit serum (**Figure 5**). The electron-dense reaction products are localized on and/or in the RER (abundant in the central cytoplasm shown in **Figure 3**) and the single membrane bound vesicles and vacuoles (prominent in the peripheral cytoplasm shown in **Figure 4**). No significant reaction is observed in the control preparation (**Figure 5**). (All ×9700)

For these reasons and those discussed previously, the use of anti-mouse AA rather than anti-mouse apoSAA was considered to be advantageous for the present study,



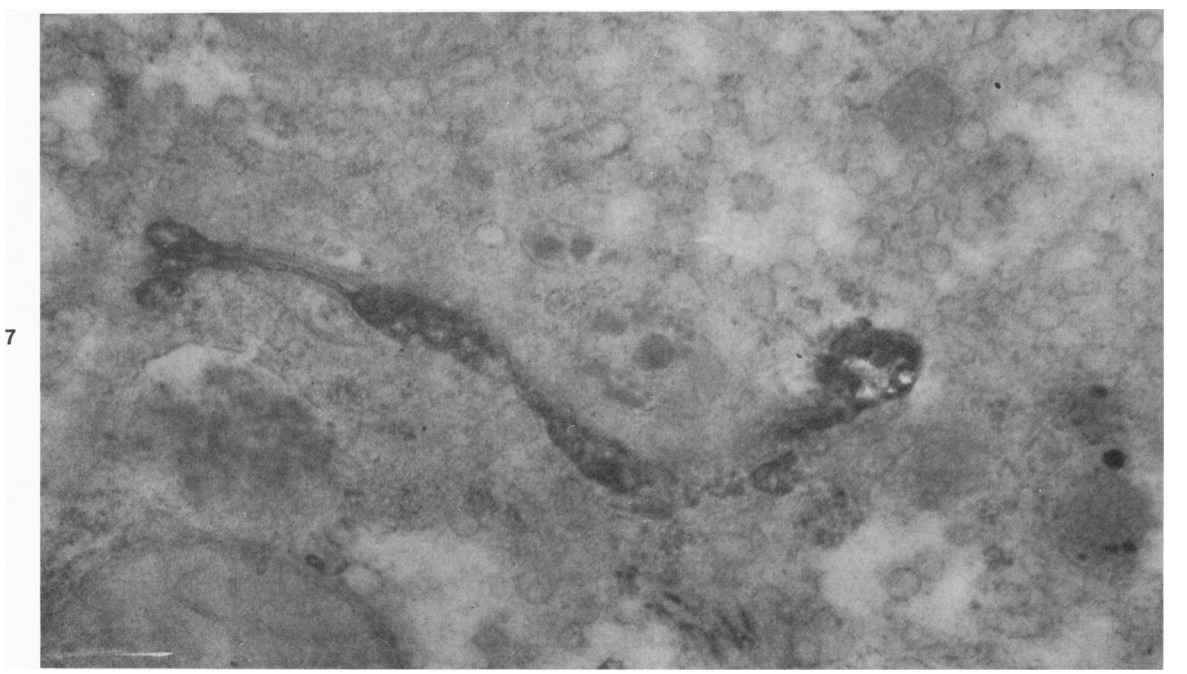
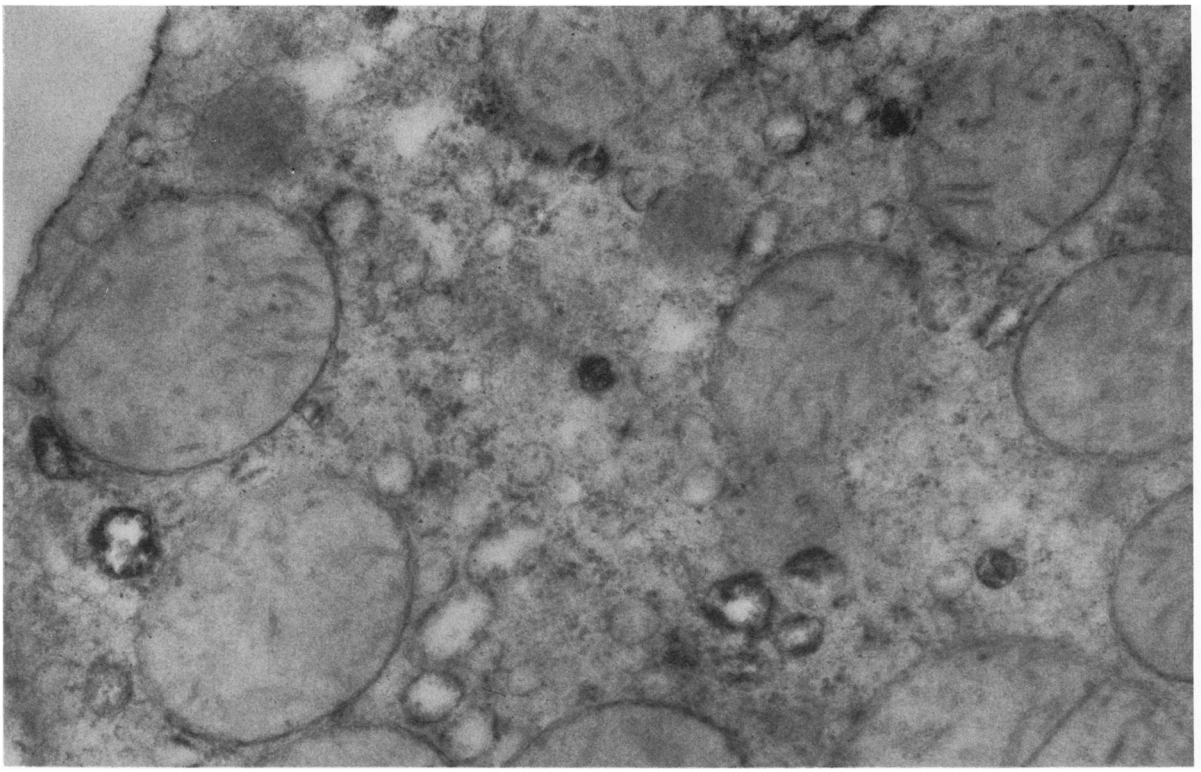


Figure 6—Higher power electron micrograph of an RER-rich portion of a hepatocyte of a CBA/J mouse sacrificed 6 hours after receiving a casein injection. Immunoperoxidase staining against anti-AA. The reaction products are localized mainly on the attached ribosomes and the membrane of the RER, and occasionally in the cisternae (*arrows*), as if attaching to and extruding from the membrane. (×40,000) **Figure 7**—Same preparation as Figure 6, showing an area containing a Golgi apparatus which demonstrates heavy deposition of the reaction products. (×40,000)



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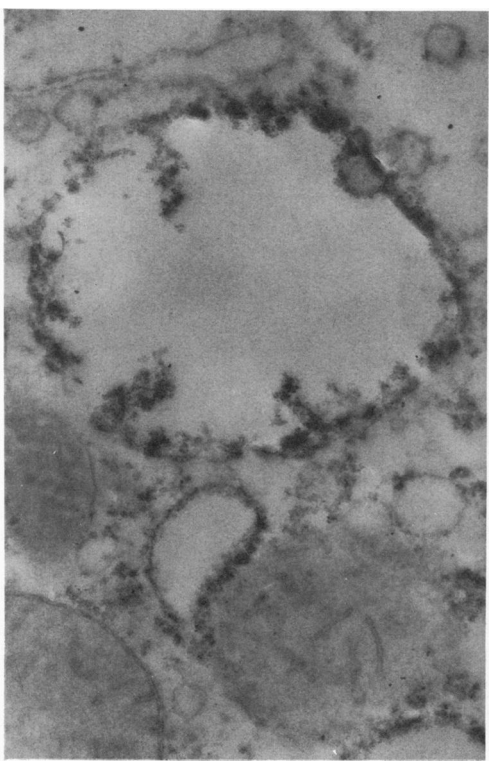

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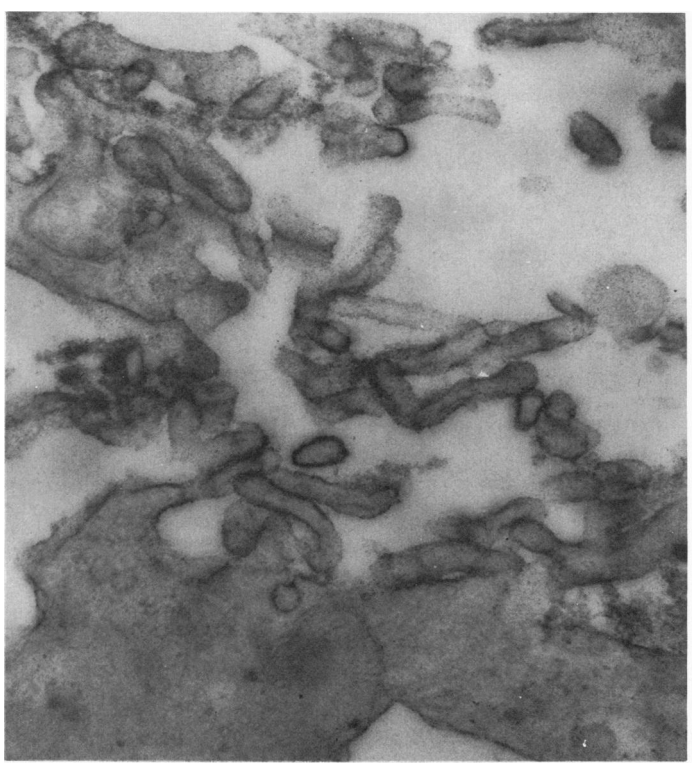

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Figure 8—Higher power electron micrograph of peripheral cytoplasm of a hepatocyte of a CBA/J mouse sacrificed 6 hours after a casein injection. Immunoperoxidase preparation against anti-AA. The contents of cytoplasmic vesicles that show positive reaction often fill completely the smaller vesicles and only partially the larger vesicles, leaving empty space in the center. The reaction products also cover thinly the sinusoidal surface of the plasma membrane (*upper left-hand corner*). (× 40,000) **Figure 9**—Similar preparation as Figure 8, showing a single membrane-bound vacuole and the surrounding area. The reaction products are deposited on the content that is attaching to the membrane in a thin layer. (× 40,000) **Figure 10**—Electron micrograph showing a portion of the sinusoidal surface of a hepatocyte of a CBA/J mouse sacrificed 16 hours after a casein injection. Immunoperoxidase staining against anti-AA. The sinusoidal surface of the membrane of the hepatocyte (*bottom*) and the microvilli are coated with the anti-AA reactive substance. (× 40,000)

and the present observations that were carried out with anti-AA at the early stage of the acute phase reaction may reasonably be attributed to the first phase of amyloidogenesis, ie, synthesis and secretion of the AA-precursor.

The localization of the anti-AA reaction in and/or on the RER, the Golgi apparatus, the vesicles, and the vacuoles is not unexpected. This finding confirms that amyloid protein AA follows the common routes of synthesis and intracellular pathways established for other proteins,[28] including C-reactive protein (CRP), another acute-phase reactant which has been very well studied and often compared with SAA,[8,11,29-32] while different hepatocyte populations may be involved in CRP and AA biosynthesis.[14,29,30] Moreover, in the present system of SAA synthesis, a similar effect of colchicine, ie, intracellular retention of synthesized protein, was observed at the ultrastructural level (T. Shirahama and A.S. Cohen, unpublished observations) as reported with CRP synthesis.[29]

The accumulation of the AA-reactive substance on the free surface of the hepatocyte plasma membrane may require a different interpretation. This appeared as early as but peaked and disappeared at least several hours later than its cytoplasmic counterpart. This observation appears to suggest simply that the AA-related substance which had been newly synthesized and transported to the cell surface was retained there for a considerable period of time. Moreover, the substance may have been bound to the membrane with a certain firmness, so that, at least, it was not removed by perfusion performed at sacrifice. This phenomenon may be of interest in relation to the mechanism of the release of the AA-precursor as well as interpretation of the data from chemical and immunologic studies.

This retention can be explained in several ways. First, apoSAA is known to be rather hydrophobic. Therefore, it is possible that newly synthesized apoSAA travels the intracellular channels of secretion, attaching closely to the membrane, and is brought to the cell surface still remaining to be attached to the membrane. In order to float in the plasma freely, it may need to be associated with lipoprotein, and therefore the several-hour lapse mentioned above may represent the waiting time for the association. Other possibilities would be 1) that the anti-AA reaction on the hepatocyte surface represents the sites of special SAA receptors, 2) that, even without special receptors, the plasma membrane has the ability (physical and/or chemical) to attract SAA, and 3) that it is an artifactual localization and simply represents the SAA concentration in the plasma.

Although many things remain yet to be clarified before any more definitive evaluation can be made as to exactly what kind and how much influence this phenomenon (retention of the AA-related substance on the surface membrane) has on the chemical and immunologic studies, it seems to be clear that it has some effect. For example, when SAA is assayed separately for the cellular components and the media in hepatocyte cultures in order to determine the degree of its synthesis and release, the AA-reactive substance being retained on the cell surface may be mistakenly counted as "cellular" even though it has already been secreted into the extracellular space. At any rate, this present observation should make us more aware of these possibilities.

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