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Ultrastructural evidence for intracellular formation of amyloid fibrils in macrophages*

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Summary. Early amyloid deposition in the spleen was studied by immunoelectron microscopy following the administration of rapid amyloid-inducing agents to mice.

Two days after the injection of an amyloid-enhancing factor and casein solution, a small amount of amyloid material was observed at the border of the white pulp and the marginal zone (perifollicular area) and also within the white pulp. At this stage, amyloid fibrils were seen mainly in an extracellular distribution along the cytoplasmic processes of reticular cells and also in the cytoplasmic invaginations of macrophages. By immunoelectron microscopy, gold particles labelled fibrillar structures in lysosome-derived organelles in some macrophages as well as dense bodies consisted of a homogeneous, granular matrix not having any recognizable fibrillar structures. Similar immunolabelled organelles were also observed in the amyloid resorption stage, although, at that stage, they commonly contained other phagocytized materials as well.

From these findings, we suggest that at least some amyloid fibrils are polymerized in the cytoplasm of the macrophages by the proteolytic cleavage of previously pinocytized serum amyloid A protein (SAA).

Key words: Amyloid – Spleen – Immunoelectron microscopy – Intralysosomal formation

Introduction

Since Smetana (1927) first reported on the intimate relationship between reticuloendothelial (RE) cells and amyloid deposition, it has been widely accepted that RE cells play an important role in the formation of amyloid fibrils (Gueft and Ghidoni 1963; Ranløv and Wanstrup 1967; Zucker-Franklin and Franklin 1970; Kazimierczak 1972; Shirahama and Cohen 1973). Recent biochemical research in amyloidosis has shown that the amyloid fibrils in primary and secondary systemic amyloidosis are derived from proteolytic cleavage of precursor proteins (Glenner et al. 1973). This proteolytic cleavage is thought to occur preferentially in extracellular regions of involved tissues (Lavie et al. 1980; Uchino et al. 1985). However, several researchers have reported that the polymerization of amyloid fibrils takes place intracellularly in various locations, such as in myeloma cells (Kjeldsberg et al. 1977; Raman and van Slyck 1983), pituitary adenoma cells (Mori et al. 1985), islet cell tumours of the pancreas (Yamashita et al. 1985), and RE cells (Zucker-Franklin and Franklin 1970; Shirahama and Cohen 1975).

Two theories have been proposed to explain the intracellular formation of amyloid fibrils. One hypothesis, as suggested by Gueft and Ghidoni (1963), Shirahama and Cohen (1975), and the present authors (Uchino et al. 1985), is that the formation of amyloid fibrils occurs in membrane-bound cytoplasmic organelles, such as lysosomes, rough endoplasmic reticulum, or Golgi apparatuses. Another possibility, although less likely, is that it develops in situ in the cytoplasm of degenerating or disintegrating cells (Kazimierczak 1972).

With the recent application of immunoelectron microscopy, evidence has been accumulated showing that serum amyloid A protein (SAA), the putative precursor of amyloid fibril protein AA, is synthesized mainly by hepatocytes (Shirahama and Cohen 1985; Takahashi et al. 1985a; Miura et al. 1985). However, little attention has been paid to the site of amyloid fibril formation or to the role

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of RE cells in amyloidogenesis. Thus, there is still controversy whether intracellular amyloid fibrils in RE cells are actually polymerized within the involved cells or indicate the phagocytosis of extracellular amyloid by such cells.

Our purpose is to define the precise site of early amyloid deposition in the spleen using protein A-gold immunoelectron microscopy. The main aim of the present study is to clarify the role of the RE cells in the formation and resorption of amyloid fibrils by comparing the features of both stages in an experimental model of rapid amyloid induction devised by Axelrad and Kisilevsky (1980).

Materials and methods

Amyloid-enhancing factor (AEF) was prepared from the spleens of amyloid-laden mice according to the method of Axelrad and Kisilevsky (1980). AKR mice, 8–10 weeks old, were given a single intraperitoneal injection of 0.5 mL AEF and daily subcutaneous injections of a 10% casein solution in 0.3 M $NaHCO_3$. Groups of 3 mice were sacrificed at 0, 1, 2, 3, and 4 days after the amyloidogenic stimuli. The liver, spleen, and kidneys were removed under ether anaesthesia, and the tissue fragments were fixed in a 5% buffered formalin solution for light microscopy. Small pieces of the spleen were fixed in a 2.1% glutaraldehyde solution for electron microscopy.

For the induction process of amyloidosis, AKR mice received daily subcutaneous injections of 0.5 mL of a 10% casein solution in 0.3 M $NaHCO_3$ for 8 weeks. After amyloid deposition in the spleen was confirmed by partial splenectomy, injections of casein solution were stopped. The mice were sacrificed at 6, 8, 12, or 16 weeks after the last injection of casein. The spleens were processed for light and electron microscopy as described above.

Antiserum to murine protein AA was prepared in white rabbits as previously described (Imada 1981). Normal goat serum, goat anti-rabbit immunoglobulin, and rabbit peroxidase-antiperoxidase complexes were obtained from Dakopatts (Santa Barbara, USA). Protein A-colloidal gold particles (15 nm in diameter) were purchased from EY Laboratories (San Mateo, USA).

Paraffin-embedded sections of the spleens were stained for amyloid deposits using the peroxidase-antiperoxidase (PAP) technique of Sternberger et al. (1970). For the protein A-gold immunocytochemical study, the blocks were prepared using standard procedures for electron microscopy. Briefly, murine splenic tissues were fixed in a 2.1% glutaraldehyde solution and postfixed in a 1% osmium tetroxide solution. The tissues were dehydrated in graded ethanol and embedded in Epoxy resin. Before immunocytochemical labelling, thin sections were pretreated for 60 min in a saturated aqueous solution of sodium metaperiodate, according to the method of Bendayan and Zollinger (1983). After washing in 0.01 M phosphate-buffered saline (PBS), pH 7.4, the pretreated thin sections were incubated for 30 min in 1 % bovine serum albumin in PBS in order to block nonspecific binding sites. They were then transferred onto a drop of anti-mouse AA antiserum and incubated for 4 h. After washing rapidly in PBS, they were incubated with protein A-colloidal gold complex for 1 h. All incubations were performed at room temperature. After washing in PBS, rinsing in distilled water, and drying, sections were stained with uranyl acetate and lead citrate. They were then examined under a Hitachi H-800 electron microscope. The specificity of immunostaining was confirmed by replacing primary rabbit antiserum with non-immune rabbit serum or antiserum absorbed by purified murine AA protein.

Results

Two days after the administration of AEF and casein solution, the splenic white pulp became enlarged. Histiocytic cells characterized by an abundance of clear cytoplasm and vesicular nuclei with conspicuous nucleoli increased in number at the border between the white pulp and the marginal zone (perifollicular area). At this stage, small, isolated aggregates of immunoreaction products, corresponding to the early amyloid deposits, were scattered in the perifollicular area and in the white pulp, separate from the border (Fig. 1). These deposits were dot-like or globular in configuration and measured approximately 10 to 20 μm in diameter. They were located adjacent to histiocytic cells, but the relationship between the amyloid deposits and these cells was not clearly defined at the light microscopic level.

Three days after the administration of the amyloidogenic stimuli, the spleens showed a massive, band-like amyloid deposition in the perifollicular area and, in lesser amounts, in the white pulp.

Electromicroscopically, two days after the administration of the amyloidogenic stimuli a small amount of amyloid fibrils were seen extracellularly along the cytoplasmic processes of reticular cells and collagen fibers in the perifollicular area and the white pulp. Amyloid fibrils, measuring 10 to 14 nm in width, tended to be aggregated and arranged in a well-oriented fashion in the cytoplasmic invaginations of histiocytic cells, whereas they were at random and loosely packed in the stroma. Trace amounts of amyloid fibrils in the stroma were not easily distinguished from other microfibrils. Occasionally, amyloid fibrils were observed in the perifollicular area and the white pulp, specifically at the deep cytoplasmic invaginations of the histiocytic cells (Fig. 2). These cells had abundant cytoplasm which often extended from the cell, in the form of large cytoplasmic processes, into the surrounding tissues. The cytoplasm of such cells was relatively electron-lucent. It contained a considerable number of polysomes, scattered mitochondria, a few intermediate filaments, well-developed Golgi apparatuses and rough endoplasmic reticulum. Another conspicuous features of these cells were the presence of a large number of lysosomal dense bodies within the cytoplasm but heterophagosomes were rare among them. The nucleus was often round or oval in shape, smooth in out-

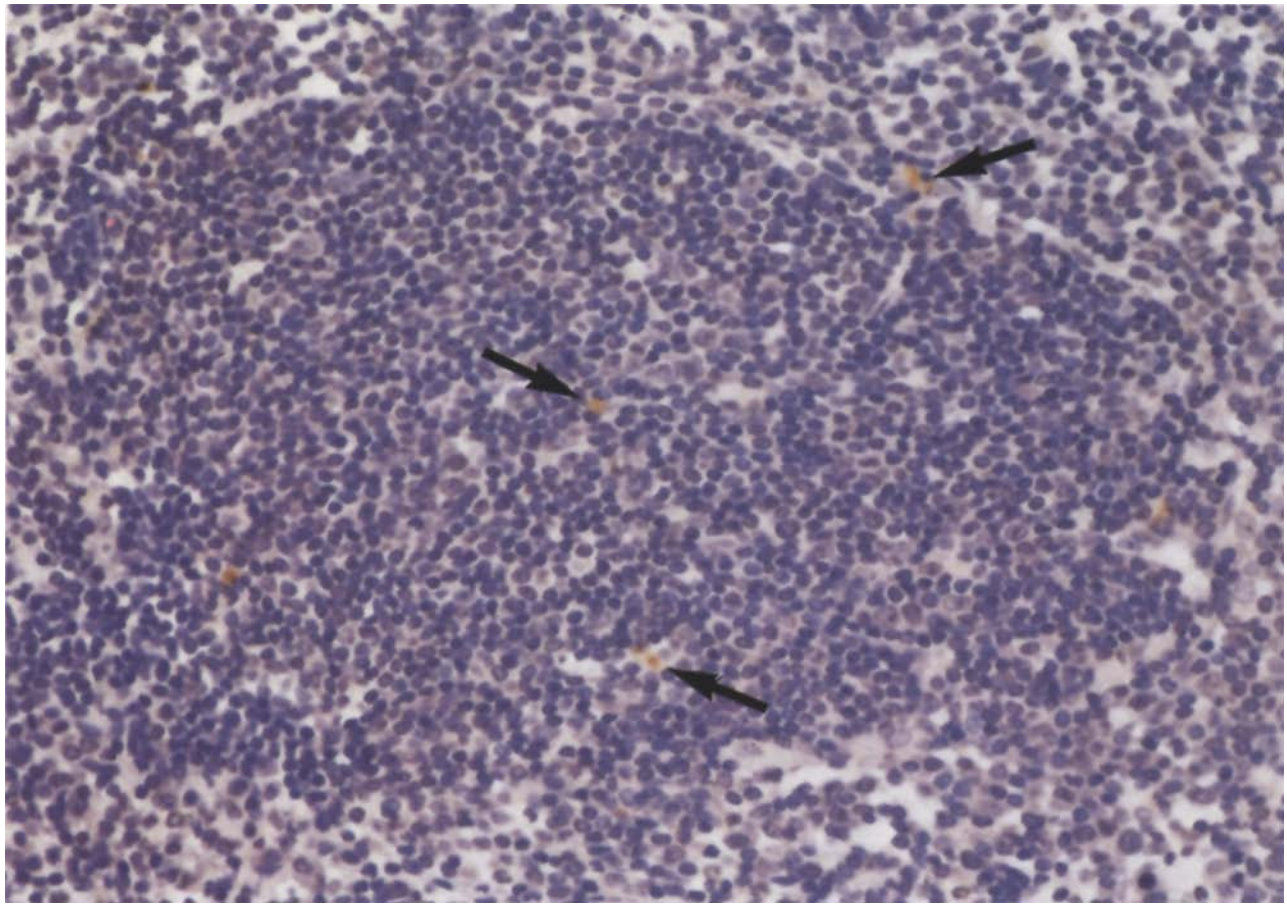


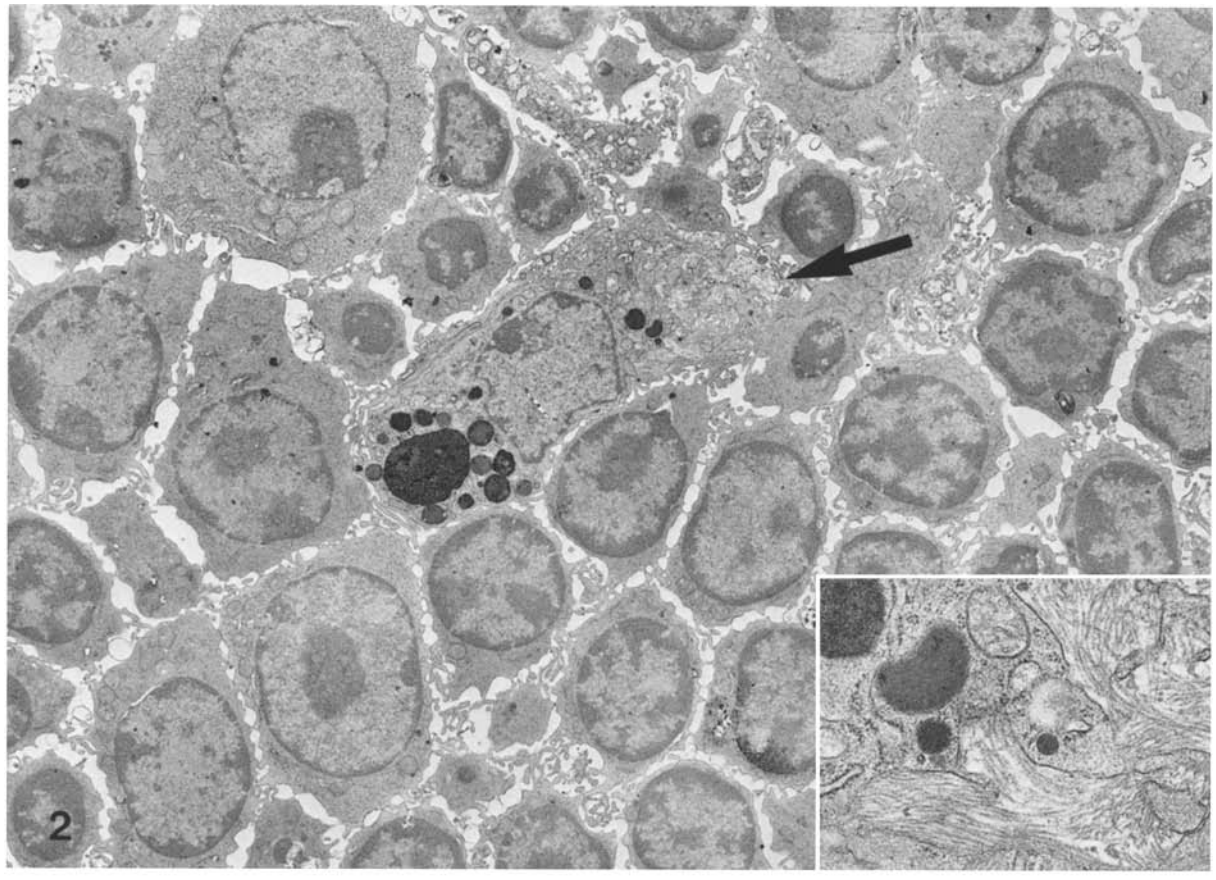
Fig. 1. Two days after the administration of AEF and casein, initial amyloid deposits (*arrows*) are present in the perifollicular area and in the white pulp. (PAP method, anti-mouse AA, haematoxylin counterstain, ×150)

line, and contained relatively few heterochromatin. Prominent nucleoli were frequently noted. Morphologically these cells were thus identified as macrophages (Burke and Simon 1970), and they were clearly distinct from the surrounding lymphocytes and fibroblast-like or dendritic reticular cells.

In the perifollicular area, some macrophages contained fibrillar structures measuring 10 to 15 nm in width within membrane-bound, oval to fusiform dense bodies (Fig. 3). These dense bodies were ultrastructurally comparable to the lysosome-derived structures. Phagosomes or phagolysosomes were rarely seen among them. The fibrillar structures in the dense bodies were arranged in a relatively well-oriented fashion and parallel to the long axis of the bodies (Fig. 3, inset). Morphologically they were similar to amyloid fibrils. Extracellular amyloid deposits were commonly, but not always, observed around the macrophages containing these dense bodies. Morphologically the macrophages appeared to be activated and showed no signs of degeneration or disintegration.

In the immunoelectron microscopy preparations, gold particles labelled the extracellular amyloid deposits specifically. In the white pulp, a small amount of amyloid fibrils labelled by gold particles were detected along the cytoplasmic processes of reticular cells (Fig. 4). These cells contained aggregated bundles of intermediate filaments (10–12 nm in width) arranged parallelly to the long axis of the cells. The matrix of the reticular cells showed an increased electron-density compared to the surrounding lymphoid cells. On the basis of their location and morphological features, the reticular cells were thought to be follicular dendritic cells (Chen et al. 1978).

In the perifollicular area, gold particles labelled several inclusions within a few macrophages. The inclusions were uniformly membrane-bounded and measured 0.5–1.0 μm in width and 1–3 μm in length (Fig. 5). Some of the inclusions showed a long fusiform shape and contained fibrillar structures, which were comparable to amyloid fibrils (Fig. 6a). The others were oval in shape (probably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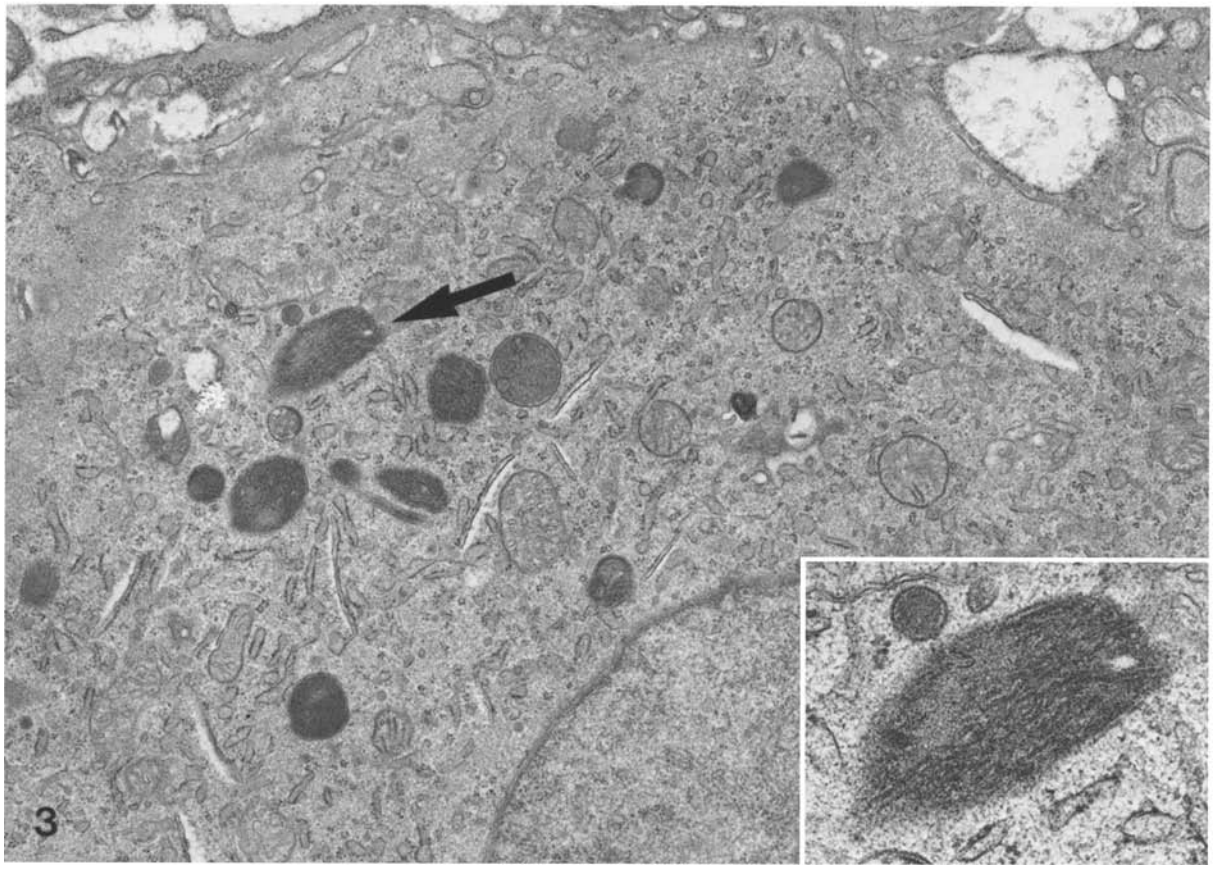
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a cross or oblique section of the inclusions) and consisted of a fine, granular matrix not having any recognizable fibrillar structures (Fig. 6b). At the early amyloid stage, no gold particles were detected in the cytoplasm of lymphoid or reticular cells, nor in the control specimens.

In the stage of amyloid resorption, on light microscopy the splenic tissue obtained by partial splenectomy 8 weeks after casein injections showed massive deposition of amyloid around the white pulp. In contrast, the spleens taken 8 weeks or more after the last casein injection showed a significant decrease in the amount of the deposits. In this later stage, gold particles labelled irregularly-shaped, electron-dense phagosomes in the cytoplasm of macrophages. Most of the phagosomes contained granular or flocculent material in various stages of digestion, with only a few amyloid fibrils still discernible (Fig. 6). These immunolabeled organelles were more varied in shape and size than those seen in the stage of amyloid induction. In the amyloid resorption stage, engulfed, intact-appearing amyloid fibrils, which were invariably labelled by gold particles, were commonly observed within the cytoplasm of these macrophages. The engulfed amyloid fibrils tended to be loosely packed, with a felt-like appearance.

Discussion

In the present study, the first amyloid deposits were detected in the spleen 2 days after the administration of AEF and casein. This experimental model of rapid amyloid induction has allowed us to examine the early amyloid deposition or formation in the spleen prior to amyloid fibril resorption.

In light microscopic immunohistochemistry, early amyloid deposits were observed in the perifollicular area and also in the white pulp. This latter location in the spleen has been overlooked in previous reports in which the amyloid deposits were reported to appear first at the border between the white pulp and the marginal zone (Ranløv and Wanstrup 1967; Kazimierczak 1972; Takahashi et al. 1979). This discrepancy of the location of early amyloid deposits may have been due to the differences in stimuli used for amyloid induction, or as seems more likely, due to the methods for the detection of amyloid. Immunocytochemical staining techniques in the current study are more sensitive for the detection of small amyloid deposits than histochemical staining with Congo red.

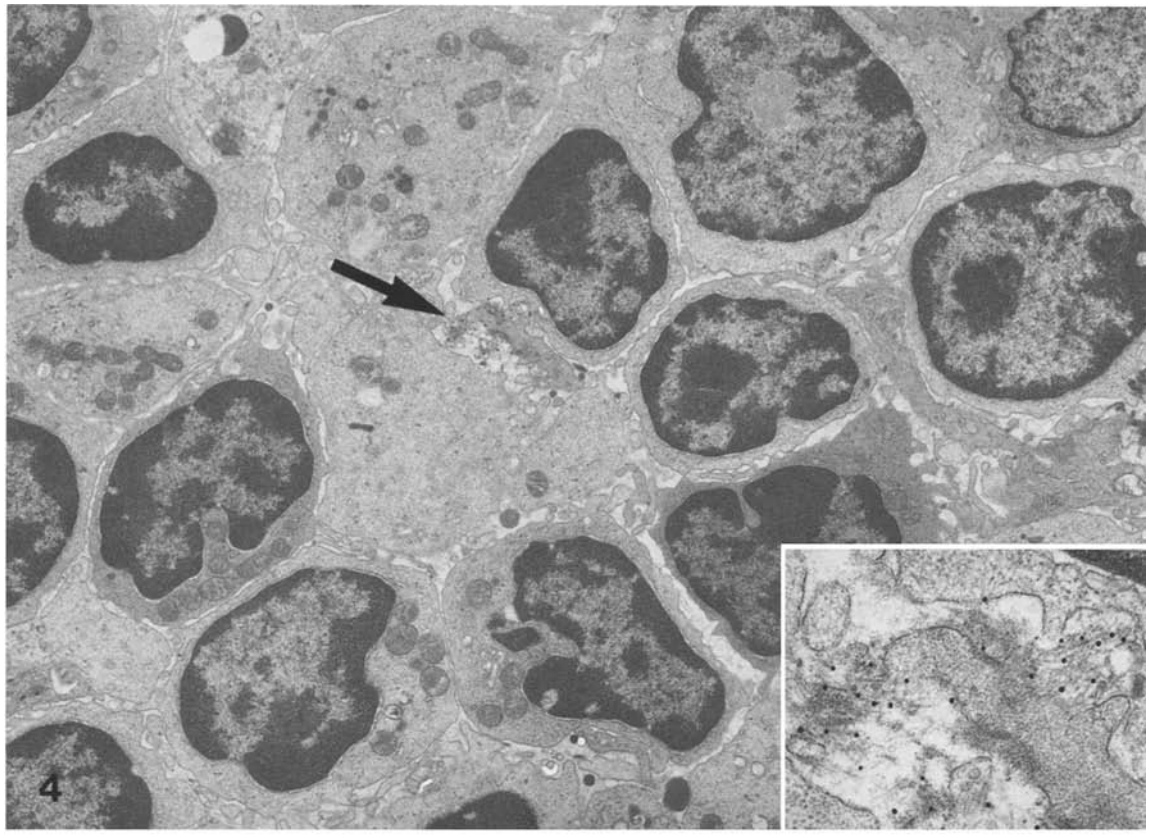
By immunoelectron microscopy, it was also apparent that initial amyloid deposits were detected in the white pulp, separate from the border. It is well documented that intravenously injected materials reach the splenic marginal zone shortly after administration and appear later in the white pulp (Nossal et al. 1966; Groeneveld et al. 1983). Chen et al. (1978) reported that injected horseradish peroxidase was seen in most of the cells in the white pulp within an hour after administration; they suggested that the route probably involved entry into the marginal zone vascular spaces and flowed through the white pulp nodule. They stressed that the follicular dendritic cells played a crucial role in the retention of foreign material. Taking these data into consideration, it is speculated that SAA reaches the marginal zone and the white pulp via the blood and is retained there by the cytoplasmic processes of reticular cells; polymerization of the amyloid fibrils, according to this model, takes place *in loco*. However, the mechanism of extracellular polymerization of SAA is unclear and requires further study.

It is of great interest that amyloid fibrils were also noted in the cytoplasmic invaginations of some macrophages in the perifollicular area and in the white pulp 2 days after the amyloidogenic stimuli. A plausible explanation is that polymerization of amyloid fibrils results from degradation of SAA by membrane-associated enzymes of macrophages, as suggested by Lavie et al. (1980) and Zucker-Franklin and Fuks (1986).

Another feature to be elucidated is the presence of intracytoplasmic amyloid fibrils observed in some macrophages at the stage of amyloid induction. One possible explanation is that they merely represent phagocytosis of extracellular amyloid fibrils by the macrophages. Similar intracytoplasmic amyloid fibrils, possibly phagocytized, have been reported in RE cells in human amyloidosis (Taka-

Fig. 2. Two days after the amyloidogenic stimuli, amyloid fibrils are seen in the cytoplasmic invaginations (*arrow*) of a macrophage in the white pulp. (×3200) *Inset;* higher magnification of the portion indicated by the arrow (×15000)

Fig. 3. At 2 days, several lysosome-derived dense bodies are seen in a macrophage. Note that phagosomes are not seen among them. (×16000) *Inset;* higher magnification of the portion indicated by the arrow shows fibrillar structures in the dense body (×43000)



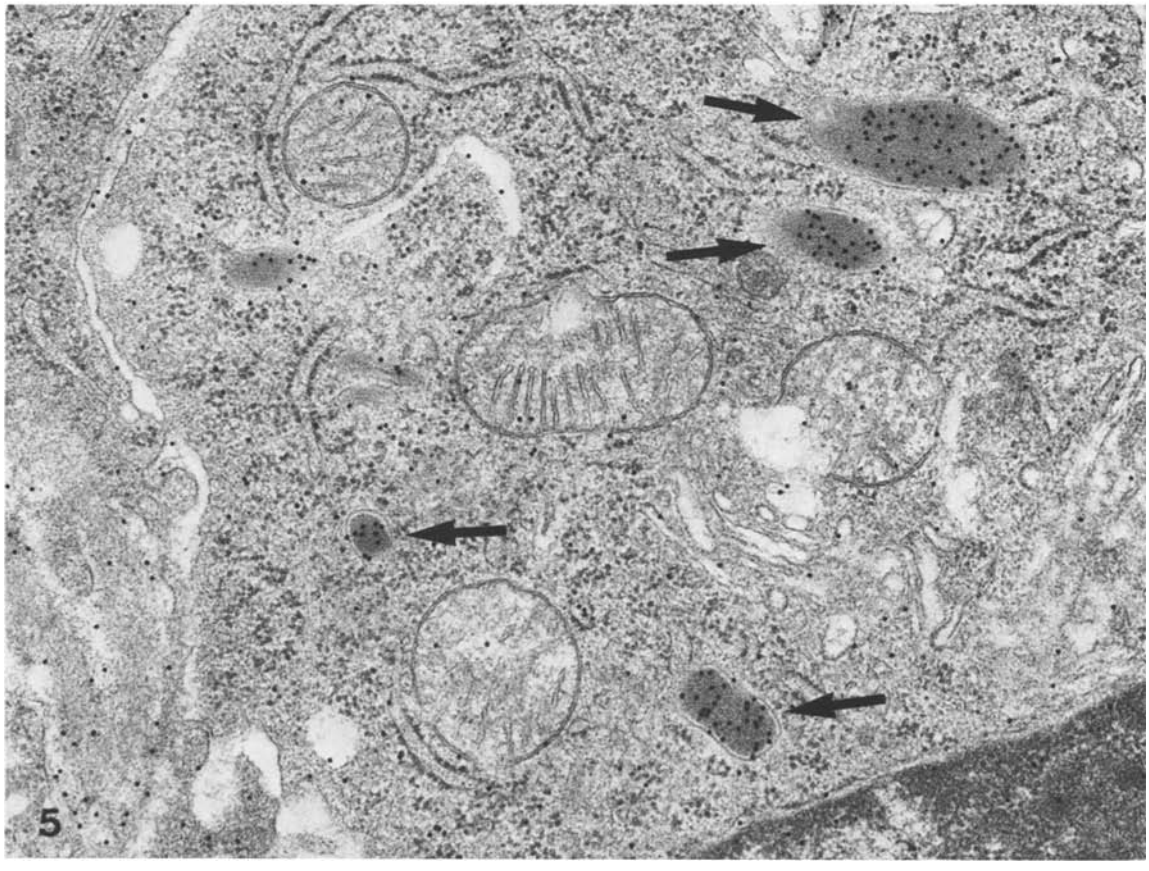
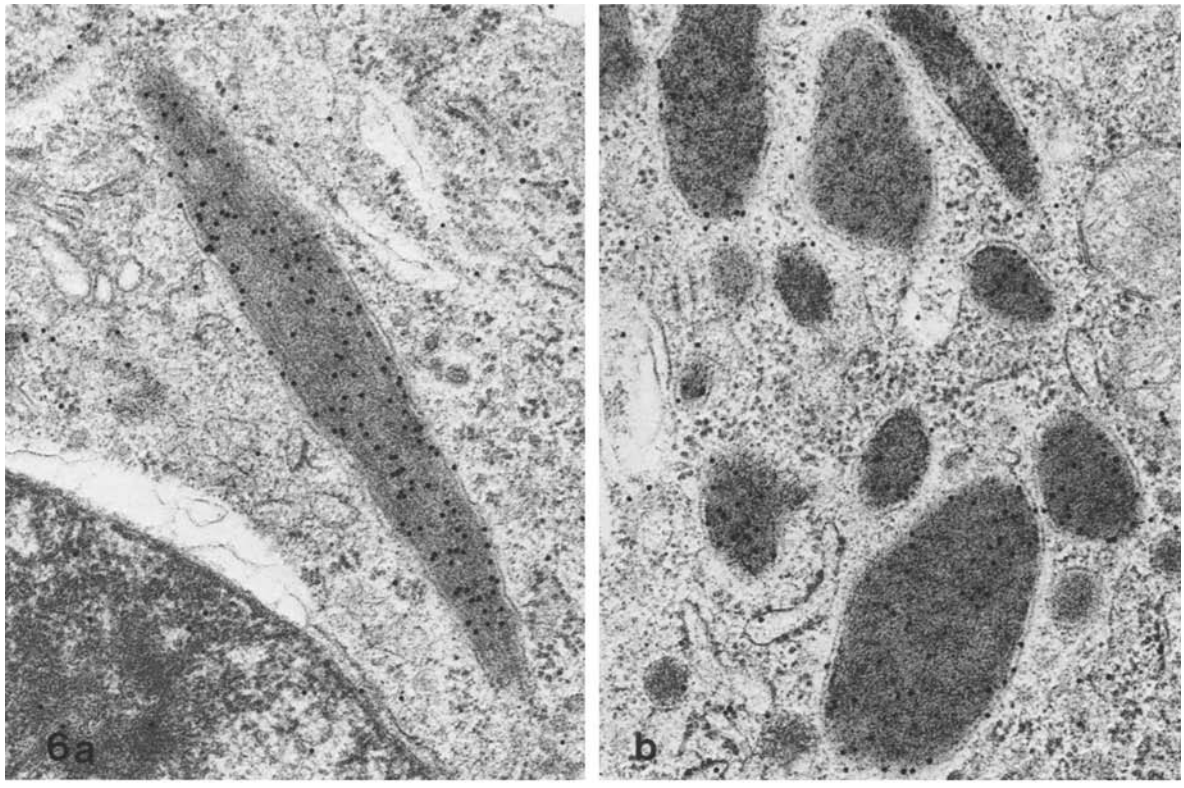


Fig. 4. Two days after the amyloidogenic stimuli. In the white pulp, a small amount of amyloid fibrils are observed in the stroma (*arrow*). (Immunoelectron microscopy, anti-mouse AA, ×5100) *Inset;* higher magnification of the portion indicated by the arrow. Amyloid fibrils labelled by gold particles are seen along with the cytoplasmic process of a reticular cell (×29000)

Fig. 5. At 2 days, gold particles label several lysosome-derived dense bodies in a macrophage (*arrows*). (Immunoelectron microscopy, anti-mouse AA, ×40000)



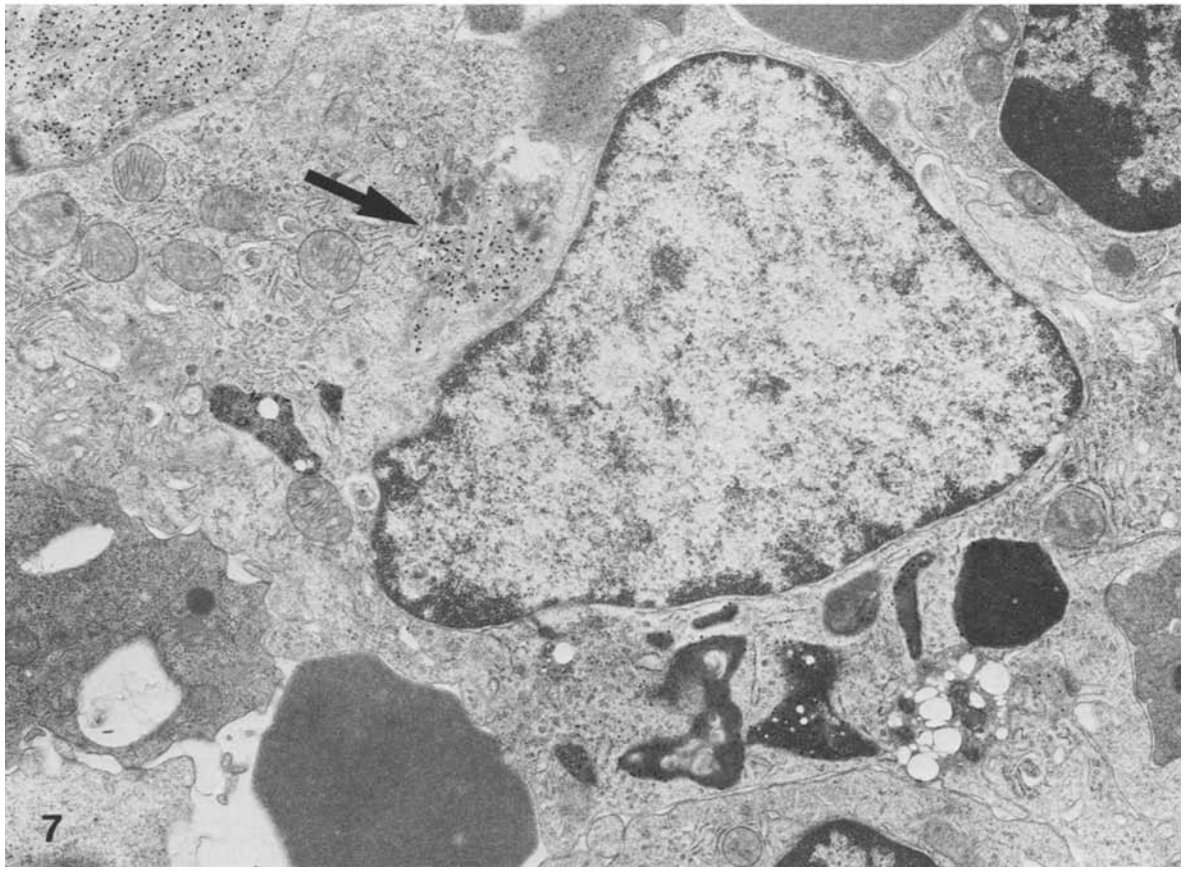


Fig. 6. a Fibrillar structures are discernible in a fusiform-shaped dense body labelled by gold particles. **b** Gold particles label several dense bodies consisted of a fine, granular matrix not having recognizable fibrillar structures. (Immunoelectron microscopy, anti-mouse AA, ×44000)

Fig. 7. At the resorption stage of amyloid deposits (8 weeks after the last casein injection), most immunolabelled organelles in a macrophage are irregular in shape and contain various phagocytized materials within them. *Arrow* shows newly-phagocytized amyloid. (Immunoelectron microscopy, anti-mouse AA, ×9600)

hashi et al. 1985b). Another possible explanation is that it represents synthesis of new amyloid by the macrophages *in situ*. Shirahama and Cohen (1975) and Ishihara and Uchino (1975) have suggested that amyloid fibrils are formed in lysosome-derived organelles of Kupffer cells. Amyloid fibrils have also been found in the cytoplasm of myeloma cells (Kjeldsberg et al. 1977; Raman and van Slyck 1983) which have weak phagocytic capability. At present, the intracytoplasmic formation of amyloid fibrils in RE cells is still controversial and no direct evidence has been shown in support of the latter hypothesis.

In the kidneys, at the stage of amyloid induction, nonfibrillar, immunolabelled vesicles, which seemed to be reabsorbed SAA, have been observed in the cytoplasm of proximal convoluted tubule epithelium (unpublished observations). These observations suggest that immunoelectron microscopy performed in this study can detect pinocytized SAA in the involved cells. We have recently reported that, using a pre-embedding immunoelectron microscopic technique, immunoreaction products for SAA were found in phagosomes of Kupffer cells at the nonamyloidotic stage in experimental amyloidosis (Takahashi et al. 1985a). No reaction products have been observed in the organelles of Kupffer cells involved in protein synthesis at this stage. These data provide additional support that some macrophages pinocytize SAA under some conditions.

In the stage of amyloid induction, gold particles labelled not only fibrillar structures in lysosome-derived organelles in some macrophages but also dense bodies consited of a homogeneous, granular matrix not having any recognizable fibrillar structures. These immunolabelled organelles were also observed during the stage of amyloid resorption, but were irregular in shape and frequently contained other phagocytized materials. Furthermore, these macrophages usually contained newly-phagocytized, intact-appearing amyloid fibrils in the cytoplasm. From these findings, it may be considered that the immunolabelled organelles in the stage of amyloid resorption represent heterophagosomes and result from the digestion of phagocytized amyloid fibrils. In contrast, in the stage of amyloid induction, most of the immunolabelled organelles were regular in shape and consisted of a homogeneous, granular matrix. If these features represent the process of the digestion of phagocytized amyloid by lysosomes, one must inevitably see the spectrum of various stages of the digestion, as seen in the stage of amyloid resorption. Zucker-Franklin (1970) and Shirahama et al. (1971) reported that in vitro system the sequential steps of the digestion of amyloid fibrils were observed within the cytoplasm of the leukocytes or macrophages. In addition, they stressed that altered amyloid fibrils, such as those following treatment with antiserum specific for amyloid or Congo red staining, were susceptible to phagocytosis, whereas intact amyloid fibrils were resistant to phagocytosis. From these data, it is unlikely that newly-formed amyloid fibrils in the stage of amyloid induction are phagocytized by macrophages and digested into homogeneous materials within a day. Thus, the present results, taking previous studies (Shirahama and Cohen 1975; Uchino et al. 1985) into consideration, support the idea that; at least some amyloid fibrils are formed within lysosome-derived organelles of macrophages following proteolytic cleavage of pinocytized SAA. The procedure of *in situ* hybridization has recently shown messenger RNA of SAA in Kupffer cells (Shirahama et al. 1988). Thus, the possibility of the intracellular formation of amyloid fibrils from SAA synthesized by splenic macrophages cannot be excluded.

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