

IMPAIRED KUPFFER CELL FUNCTION PRECEDES DEVELOPMENT OF SECONDARY AMYLOIDOSIS

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Secondary amyloidosis is a condition characterized by the deposition of an extracellular fibrillar protein, amyloid protein A (AA),¹ which may lead to the failure of vital organ functions. As a rule, this type of amyloidosis develops in response to chronic stimuli associated with infectious, inflammatory, or neoplastic diseases. In some instances, e.g., familial Mediterranean fever, the etiology of the inflammatory condition is obscure, but amyloidosis develops in a very high percentage, if not all, of the affected patients (1).

The AA protein (8,000 mol wt) is believed to be derived from the acute phase reactant, serum amyloid A (SAA) (12,500 mol wt), by proteolytic cleavage of its carboxy terminus (2-4). A series of studies designed to identify the cells involved in SAA degradation have shown that proteinases associated with the plasma membrane of peripheral blood monocytes can mediate this function (5-7). Mononuclear leukocytes of most healthy individuals degraded SAA completely, whereas the cells of patients with amyloidosis yielded an intermediate product that had electrophoretic mobility and immunological crossreactivity with AA (5). This observation led to the hypothesis that the deposition of AA might be attributable to faulty processing of SAA, especially since SAA attains very high serum levels in patients with conditions prone to the development of amyloidosis. In line with this postulate, it seemed appropriate to investigate whether fixed macrophages are involved in the catabolism of SAA. In fact, cells of the reticuloendothelial system have long been implicated in amyloidosis (8-13). Kupffer cells (KC), in particular, have been held responsible for amyloid synthesis and deposition in the liver (10, 11), and the literature is replete with illustrations showing the relationship of AA fibrils to the plasma membrane of these cells (12). The existence of a thoroughly studied animal model for secondary amyloidosis (14, 15) has provided us with the opportunity to investigate whether KC, isolated from animals with experimentally induced amyloidosis, have the same defect in the proteolysis of SAA as peripheral blood mononuclear leukocytes obtained from patients afflicted with this condition. Accordingly, KC were isolated from the livers of normal mice and of mice sacrificed at various time intervals during the induction of amyloidosis with casein. The cells were cultured

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¹ *Abbreviations used in this paper:* AA, amyloid protein A; DME, Dulbecco's modified Eagle's medium; KC, Kupffer cells; SAA, serum amyloid A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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with SAA and their ability to degrade this protein was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the culture media. KC of healthy animals degraded SAA completely, whereas supernatants of KC obtained from animals with amyloidosis revealed not only residual SAA, but also an intermediate product indistinguishable from AA. Even more significantly, the AA product was found in KC cultures prepared from animals that had received few injections of casein, i.e., before amyloid deposits could be demonstrated by Congo Red staining or electron microscopy of their intact organs. The addition to these cultures of KC from healthy animals led to complete degradation of the AA intermediate product. Thus, altered KC function appears to precede deposition of fibrillar amyloid. The observations leading to this conclusion are the subject of this communication.

Materials and Methods

Preparation of Animals and Tissues. Animals were male, 4–5-mo-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) weighing ≥ 30 g at the beginning of the experiments. They were housed in groups of five or six per cage, fed Purina mouse chow, and given water ad libitum. Each animal received daily subcutaneous injections of 0.5 ml of 10% casein (Fisher Scientific Co., Pittsburgh, PA) in 0.5 M sodium bicarbonate. Control animals received no treatment. Tissues and cells were prepared from groups of mice sacrificed after having received 0, 8, 13, 18, 20, and >30 injections. The spleens, livers, and kidneys from representative animals of each group were processed for light and electron microscopy. Frozen and embedded tissues were stained by the alkaline Congo Red method (16) for the detection of amyloid. Samples for electron microscopy were fixed in 3% glutaraldehyde, postfixed in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated, and embedded in Poly/Bed 812. Thin sections, obtained with an LKB ultratome (LKB Instruments, Inc., Gaithersburg, MD), were stained with uranyl acetate and lead citrate and viewed with a Siemens Elmiskop I electron microscope.

Isolation of KC. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal; Abbott Laboratories, Irving, TX) in saline 5 mg/ml (0.06 mg/g of body weight). The perfusion-isolation procedure was modified from a procedure devised by us for the isolation of KC from rats (17). Briefly, the portal vein was cannulated with a 21 gauge needle connected with plastic tubing to the perfusate container. The perfusate was kept at 37°C. A syringe equipped with a Millipore filter (Millipore Corp., Bedford, MA) was inserted between the container holding the perfusate and the tubing leading to the portal vein. The flow rate was maintained at 2 ml/min with a peristaltic pump (Scientific Industries, Inc., Bohemia, NY). After perfusion was established, the thorax was opened and the inferior vena cava was cut to permit the perfusate to drain. Perfusion was initiated for 8–10 min with Dulbecco's modified Eagle's medium (DME), containing 4.5 mg glucose per milliliter, 2% heat-inactivated fetal calf serum, and 0.2% heparin (1,000 U/ml), penicillin, and streptomycin. This was followed by 10 min of enzyme perfusion consisting of the same medium to which had been added 0.1% collagenase type IV (260 U/mg; Sigma Chemical Co., St. Louis, MO), 0.2% pronase (70,000 PUK/g; Calbiochem-Behring Corp., La Jolla, CA), 5 mM CaCl₂, and 0.8 mg% deoxyribonuclease. The entire procedure was carried out under sterile conditions. At the end of the perfusion, the liver became soft. It was excised quickly and transferred to a sterile petri dish containing cold culture medium. The gall bladder was removed and the remaining liver pieces were transferred to another petri dish containing the same enzyme solution. With the help of two pointed forceps, the organ was then completely disrupted. The cell suspension and remaining pieces of tissue were transferred to a 75 cm² tissue culture flask containing 50 ml of the same enzyme solution. This suspension was agitated for 60 min on a rocker kept in a 37°C incubator with 5% CO₂ and 100% humidity. At the end of the incubation period, the cell suspension was passed through a two-layer gauze into a 30 ml plastic test

tube, which was then centrifuged at 1,200 rpm for 5 min. The pellet was resuspended in 35 ml DME, placed on a 17 ml Ficoll-Hypaque gradient prepared as described (18), and centrifuged at 400 *g* for 30 min at room temperature. The cells at the interface were washed twice, resuspended, and counted. Cell viability was checked by trypan blue exclusion and phagocytosis of latex particles. Without exception, the cells used in these experiments were >95% viable and phagocytic.

KC Cultures. The isolated washed cells were plated at a concentration of 7×10^6 /ml on 35-mm petri dishes (Falcon Labware, Oxnard, CA) and incubated at 37°C in 10% CO₂ and 100% humidity. After 24 h, the supernatant containing unattached cells and debris were removed and the medium was replaced with fresh DME containing 10% fetal calf serum. In some instances, sterile glass coverslips were placed into the plastic culture dishes to permit KC attachment to glass which is necessary for Congo Red staining and other histochemical procedures.

Incubation of KC with SAA. After 48 h in culture, the serum-containing medium was washed from adherent KC and replaced with 1 ml of serum-free DME, to which 200 µg SAA had been added. On some occasions, the KC were scraped off the culture dish and incubated with the SAA-containing medium in suspension. The preparation of SAA has been described in detail (19). Supernatants of KC incubated with or without SAA were collected at 0, 4, 8, and 18 h. Each specimen was dialyzed against distilled H₂O for 24 h. After lyophilization, the samples were placed on 10–20% SDS-polyacrylamide slab gels (20). Cytochrome *c* (13,000 mol wt) was used as a marker. The gels were stained with Coomassie Blue and the products of enzymatic digestion were quantitated at 516 nm with a Guilford 250 spectrophotometer equipped with a scanner and recorder. Values were expressed as the percentage of residual SAA or AA after incubation with KC.

The culture medium of control KC was also incubated with SAA to determine whether the relevant proteolytic activity is secreted or whether, as in the case of monocytes, it remains intimately associated with cells. In addition, the KC as well as the medium were incubated separately with orcein-impregnated elastin (Sigma Chemical Co.) to assay for elastase-like enzymes as described previously (21, 22).

Electron Microscopy and Histochemistry of Cultured KC. Samples of control KC and KC incubated with SAA obtained from each group of animals were fixed in situ overnight with 3% phosphate-buffered glutaraldehyde. All specimens were dehydrated and flat-embedded in Poly/Bed 812 as described in detail (23). The cells were sectioned mostly at right angles to the surface that was attached to the substrate. Electron microscopy was carried out as described for tissues.

Light microscopy and histochemistry was usually performed on cells attached to the glass coverslips that had been placed in the culture dishes. These were stained with Wright's and Giemsa, alkaline Congo Red (16), and examined for the presence of acid phosphatase, peroxidase (24), α-naphthyl acetate esterase (25), and glucose-6-phosphate dehydrogenase (26).

Results

Under the conditions of our protocol, amyloid deposition could not be detected in the livers, spleens, or kidneys of animals that had received <18 injections of casein. Only two of the mice that had been injected 18 times revealed traces of Congo Red-positive material in their kidneys. No amyloid deposits were ever found, even upon electron microscopy of tissues prepared from animals that received 13 or fewer injections of the antigen.

The yield of purified KC obtained from healthy animals was $1.5\text{--}2.0 \times 10^7$ per liver. The cells were >95% viable and phagocytic. Their KC identity was established unequivocally by ultrastructural and histochemical analyses. The cells were strongly positive for glucose-6-phosphate dehydrogenase, an enzyme that is only weakly expressed by endothelial cells (26). The likelihood that they were

contaminated by fibroblasts was ruled out by our observation that fibroblasts are killed by pronase (unpublished results). Since the KC of unstimulated mice were only loosely attached after 18 h of incubation, experiments on the degradation of SAA were begun after 48 h. As illustrated in Fig. 1, the morphology of normal KC was fairly homogeneous. Although there was some spreading, the majority of control cells remained spindle-shaped, exhibited oval nuclei, and had few inclusions. During the course of amyloid induction, there was a progressive increase in the number of cells harvested per liver as well as a remarkable change in their morphology. KC obtained from mice that had received as few as eight subcutaneous injections of casein already showed physiologic and morphologic changes. Attachment and spreading took place within a few hours. There was a decrease in the nucleocytoplasmic ratio, an increase in the number of inclusions and vacuoles, and, in general, the cytoplasm had developed the "busy" appearance of stimulated macrophages (27). As expected, there was a marked increase in acid phosphatase. However, the content of some lysosomal enzymes had decreased. As seen in Fig. 2, the number of KC harvested from mice with 18 injections had doubled; in mice receiving 30 injections, there was a fivefold increase in the yield. When the livers had become grossly amyloidotic, the morphology of the cells isolated as KC was very heterogeneous. There was contamination with monocytes, lymphocytes, and, occasionally, neutrophils. Since this was not the case in animals that had received <18 injections of casein, nor before amyloid was grossly evident, the heterogeneity of the cell population harvested from amyloidotic livers was not considered relevant to the aim of these studies.

Degradation of SAA by KC. KC isolated from healthy, unstimulated mice degraded SAA completely without the appearance of the intermediate cleavage product, protein AA. After 4 h of incubation, the concentration of SAA was reduced to $33.0 \pm 5.4\%$ of the original amount of SAA added (Figs. 3 and 4). KC of mice injected eight times with casein showed $50.0 \pm 3.28\%$ remaining SAA after 4 h of culture. When supernatants of these cells were analyzed after 8 and 18 h of culture, the residual SAA proved to be $18.0 \pm 2.44\%$ and $16.5 \pm 3.36\%$, respectively. More importantly, a band corresponding to the AA protein had appeared that was digested on overnight incubation (Fig. 3). KC from animals that had received 13 injections were able to degrade only $31.5 \pm 4.20\%$ of the protein during the first 4 h, $78.0 \pm 3.33\%$ in 8 h, and $78.0 \pm 2.08\%$ after 18 h in culture. Moreover, the AA band that represented $19.62 \pm 1.88\%$ of the residual protein at 8 h and $24.30 \pm 3.09\%$ after 18 h of culture had become conspicuous (Fig. 3). It should be recalled that animals that had received 13 or fewer injections of casein did not have amyloidosis, nor could amyloid fibrils be demonstrated in their KC cultures to which SAA had been added (see below). It is thus likely that the AA cleavage product found in the culture medium had not yet polymerized into insoluble amyloid fibrils. KC obtained from mice with 18 or more injections of casein revealed an even greater impairment in their ability to degrade SAA. As seen in Figs. 3 and 4, KC of mice with 18 injections had processed only $\sim 1/3$ of the substrate SAA on overnight incubation, and a distinct AA band became visible on SDS-PAGE of the 8-h culture medium. This was not degraded by the cells even after incubation for 18 h. Quantification of the

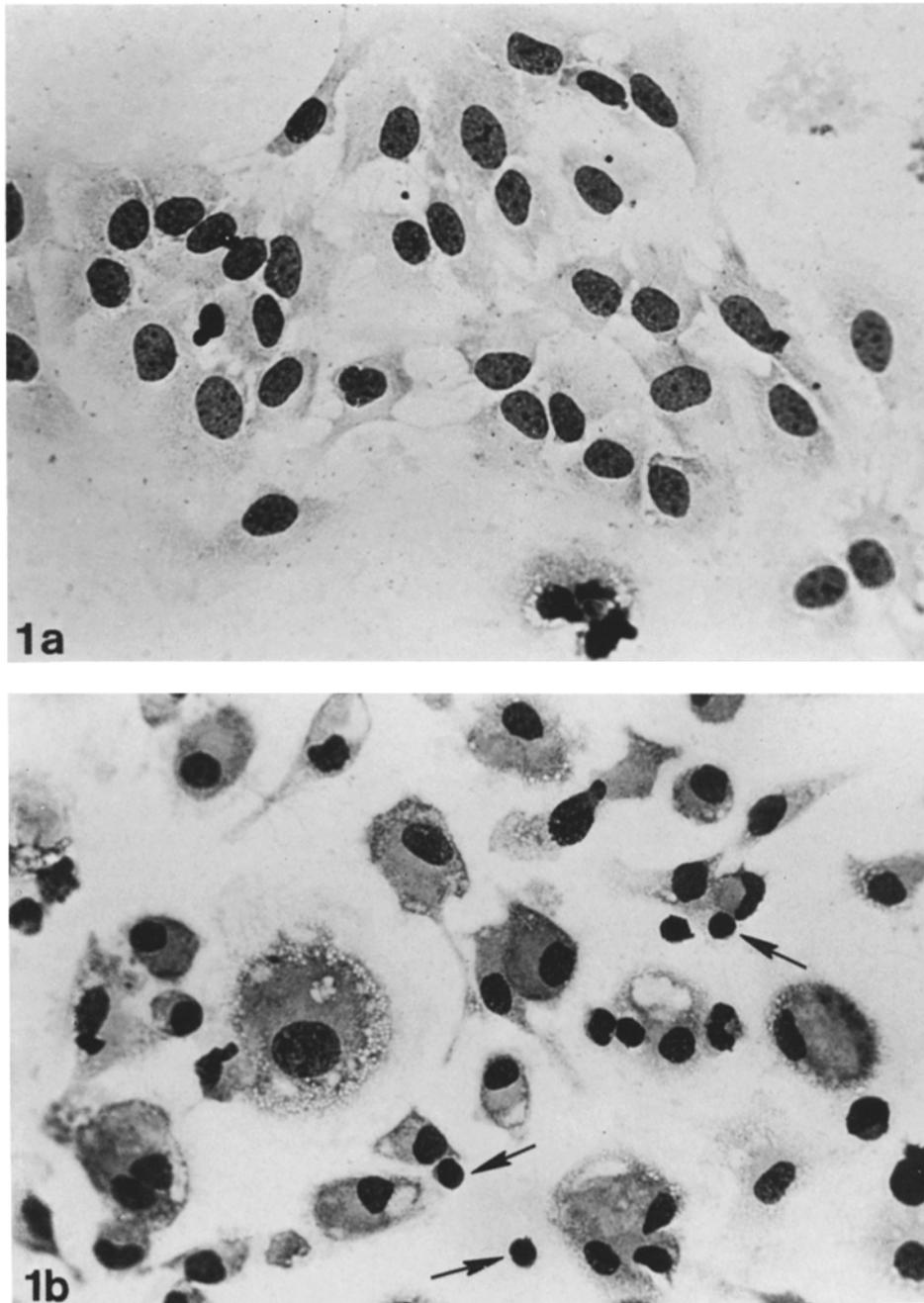


FIGURE 1. (a) KC isolated from a normal, unstimulated mouse, cultured overnight as described in text. Stained with Wright's and Giemsa. (b) KC isolated from the liver of a mouse that had been stimulated with casein. Note that the cells have become heterogeneous in size and shape. They display numerous vacuoles and inclusions. There is some admixture with smaller cells (arrows) that have the appearance of lymphocytes or undifferentiated stem cells.

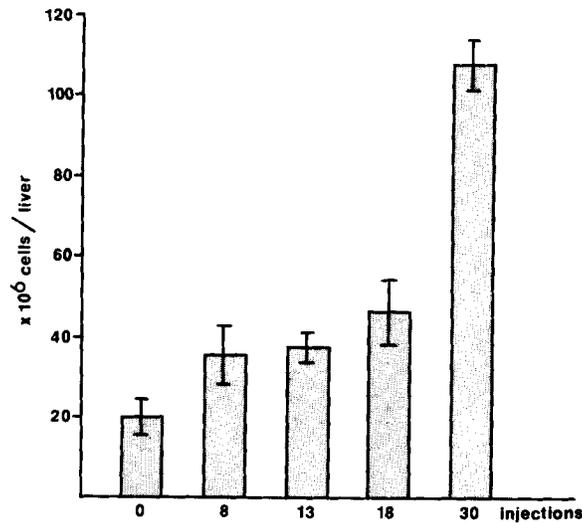


FIGURE 2. Effect of casein treatment on the yield of KC. There appears to be a progressive increase in the number of cells harvested per liver. Bars, \pm SD.

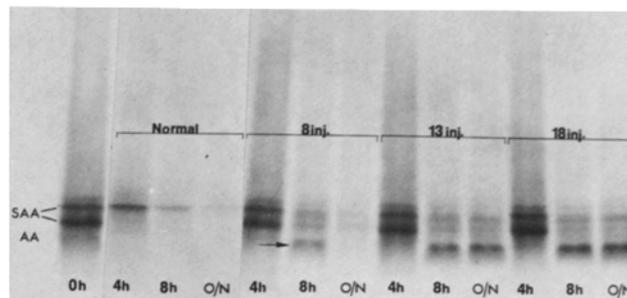


FIGURE 3. SDS-PAGE patterns with supernatants of KC cultures. The KC were isolated from the livers of mice that had received 0, 8, 13, or 18 injections of casein. The cells were incubated with SAA for 4 h, 8 h, and overnight (O/N). Control shows the two SAA isotypes characteristic for mice. Arrow indicates AA band. Stained with Coomassie Blue.

amount of residual SAA and AA in supernatants of KC cultures is shown graphically in Fig. 4. It is obvious that there was an ever-increasing amount of residual SAA as well as larger quantities of AA during the course of amyloid induction.

To determine whether the presence of intact KC was a requirement or whether enzymes secreted into the medium could also mediate SAA degradation, supernatants of control and stimulated KC were used as a source of enzymatic activity. As seen in Fig. 5, neither the supernatant of normal KC cultures nor the supernatants of stimulated KC could degrade SAA, indicating that the enzymes responsible for SAA hydrolysis are cell-associated as had been shown previously to be the case for blood monocytes (6, 7). On the other hand, supernatants of normal and stimulated KC degraded elastin-orcein, indicating that some elastase(s) were secreted by the cells into the medium (data not shown).

Reversibility of Processing Defect. In a few experiments, normal KC harvested

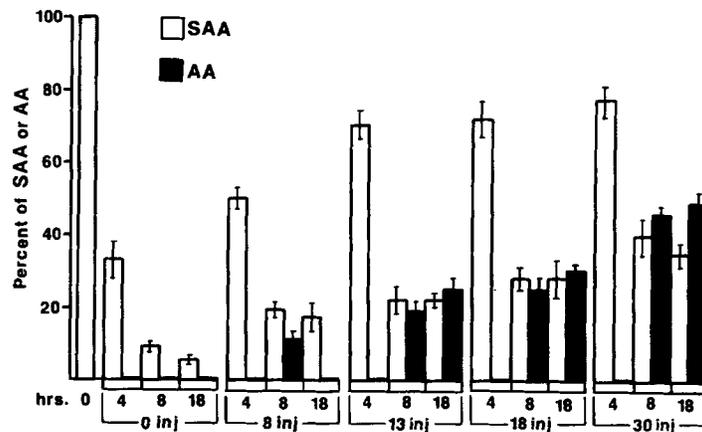


FIGURE 4. Spectrophotometric quantitation of residual SAA and AA in supernatants of KC. The cells were derived from mice that had received 0, 8, 13, 18, or 30 injections of casein and were incubated for 4, 8, or 18 h with SAA. Larger amounts of residual SAA and increasing quantities of AA appeared during the amyloid induction period. When the animals had been given more than eight injections of the stimulant, their KC were no longer able to eliminate the AA intermediate product.

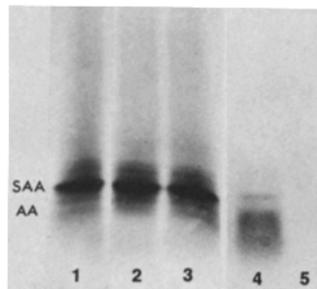


FIGURE 5. SDS-PAGE showing the following: (1) SAA; (2) SAA incubated with supernatant of normal KC cultures, showing that the medium does not contain enzymes responsible for SAA degradation; (3) SAA incubated with supernatant of KC culture prepared from an amyloidotic liver; (4) pattern obtained when SAA was incubated with KC from a mouse that had received 18 injections of casein, showing the AA intermediate product; (5) result obtained when normal KC were added to the supernatant shown in lane 4. The intermediate AA fragment was eliminated.

from control animals were added to medium derived from KC cultures that had produced the AA intermediate product. To this end, 2×10^6 KC isolated from healthy animals were added to 1 ml of supernatant derived from cultures that had produced AA, as identified by SDS-PAGE. The normal KC were able to complete the degradation process within 4 h (Fig. 5), suggesting that faulty degradation is not attributable to an aberrant or "indigestible" structure of the protein. It should also be noted, when KC obtained from the 25-injection group were cultured with SAA 6 wk after cessation of casein treatments, the AA intermediate product was seen in 8-h cultures, but it could still be processed on overnight incubation. When animals from the 25-injection group were rested 10 wk, the ability of their KC to degrade SAA to completion within 4 h was restored.

On the other hand, KC from animals that had received 35 casein injections did not recover their ability to degrade SAA over a 10 wk period. Whether recovery took place after that time of observation was not established, nor has it been determined whether this recovery was due to restored enzyme function of "old" cells or if the KC population had been replenished.

Fibrillogenesis. Lastly, it seemed of interest to examine whether impaired KC would also be responsible for the polymerization of soluble AA, detected on SDS-PAGE, into insoluble fibrils. In this regard, it cannot be stressed enough that KC isolated from amyloid-laden livers could never be freed entirely of adhering or phagocytosed fibrils (Figs. 6 and 7). Therefore, such specimens can probably not be used to distinguish whether the fibrils had formed in vivo or whether they were derived from the SAA which had been added in vitro. Such fibrils have the appearance characteristic of amyloid described in detail elsewhere (28). They are either located in vacuoles, probably when they have been phagocytosed (Fig. 6), or are found in spaces delimited by membranes that are in continuity with the surface (Fig. 7). On only two occasions did a fibrillar substance seem to have formed upon addition of SAA to cultures of KC derived from casein-stimulated mice whose intact organs and control KC were still devoid of amyloid.

An example of such filaments is illustrated in Fig. 8. Their close proximity to

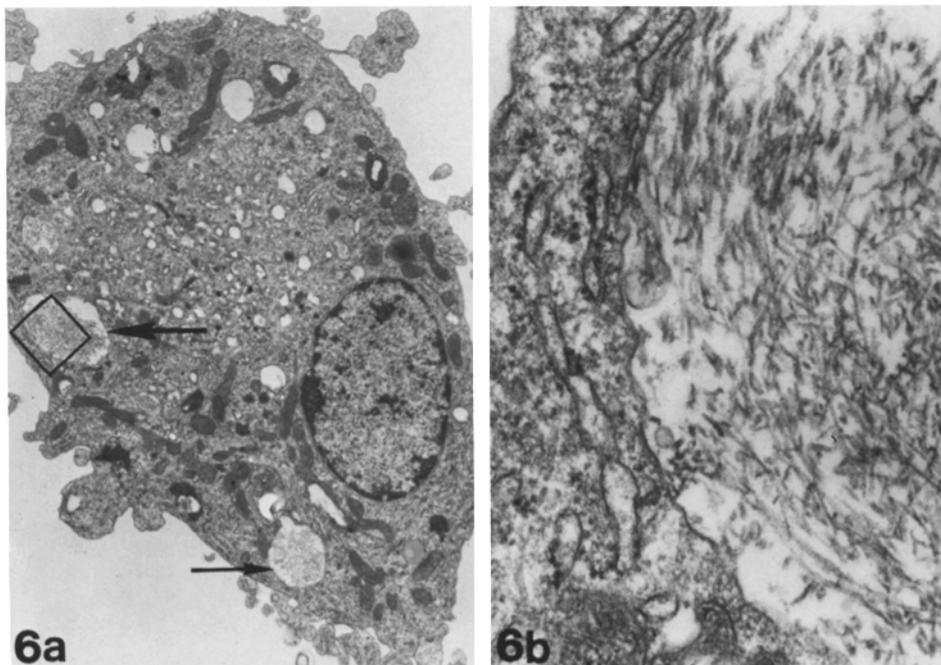


FIGURE 6. (a) KC isolated from an animal that had received 30 injections of casein. The cells were washed three times before being plated. No SAA was added to the culture. Despite this, the cell exhibits two vacuoles (arrows) containing amyloid fibrils. It is possible that these fibrils were taken up by the cell during the isolation procedure. $\times 4,000$. (b) Detail of the vacuole indicated by the rectangle in a showing amyloid fibrils at higher resolution. $\times 42,000$.

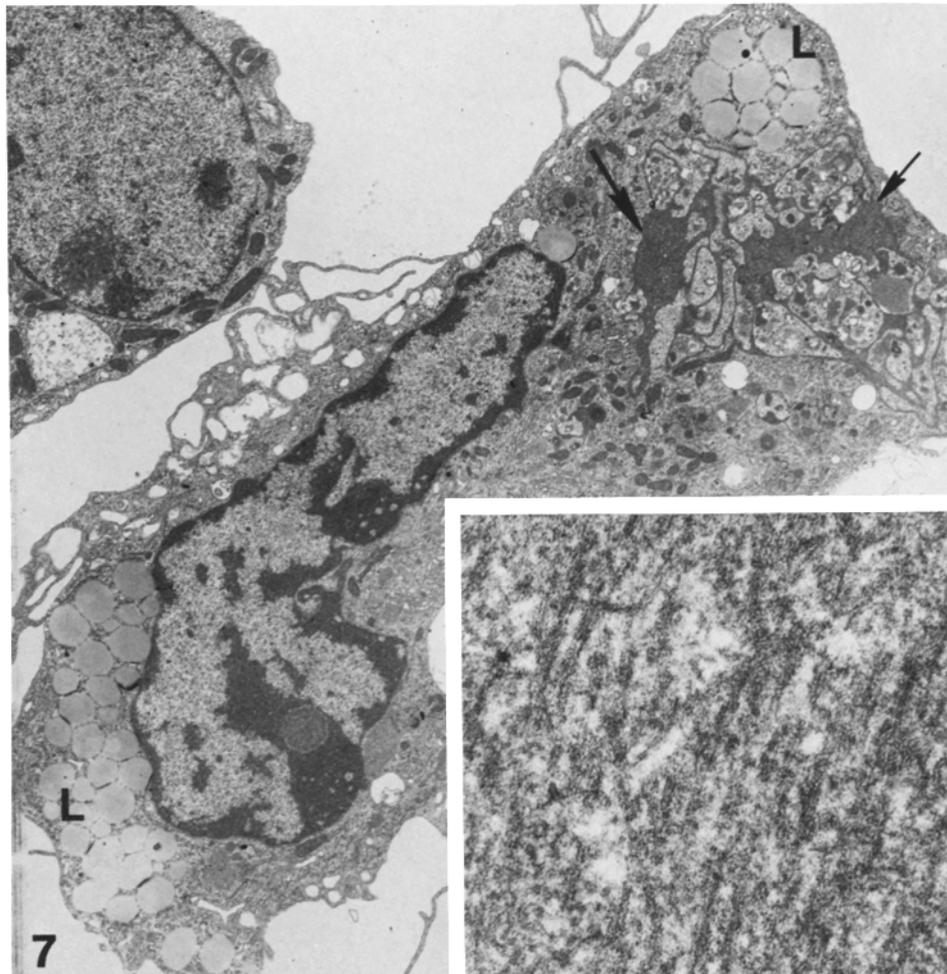


FIGURE 7. A KC from the same specimen as that in Fig. 6. This cell has been selected to illustrate another type of fibril-containing inclusion seen in KC isolated from mice with amyloidosis (arrows). These inclusions have an irregular circumference and appear to be in continuity with narrow channels and the cell surface. These membrane-bound spaces do not resemble phagocytic vacuoles (see text). A large number of lipid bodies (*L*), vesicles, and granules are characteristic for stimulated KC. The inset shows the fibrils of the inclusion indicated by the larger arrow at higher resolution. The fibrils are double-stranded, as is typical for mature, fully polymerized amyloid. $\times 5,000$. Inset, $\times 120,000$.

the invaginated plasma membrane of the cells is noteworthy. In cross section, they measured $\sim 100 \text{ \AA}$, while their length could not be determined in thin sections.

Discussion

During the past decade, the cellular origin and biochemical composition of several amyloids have been elucidated (2-5, 28-31), but it is not clear why, under some conditions, these proteins are deposited in fibrillar form. In second-

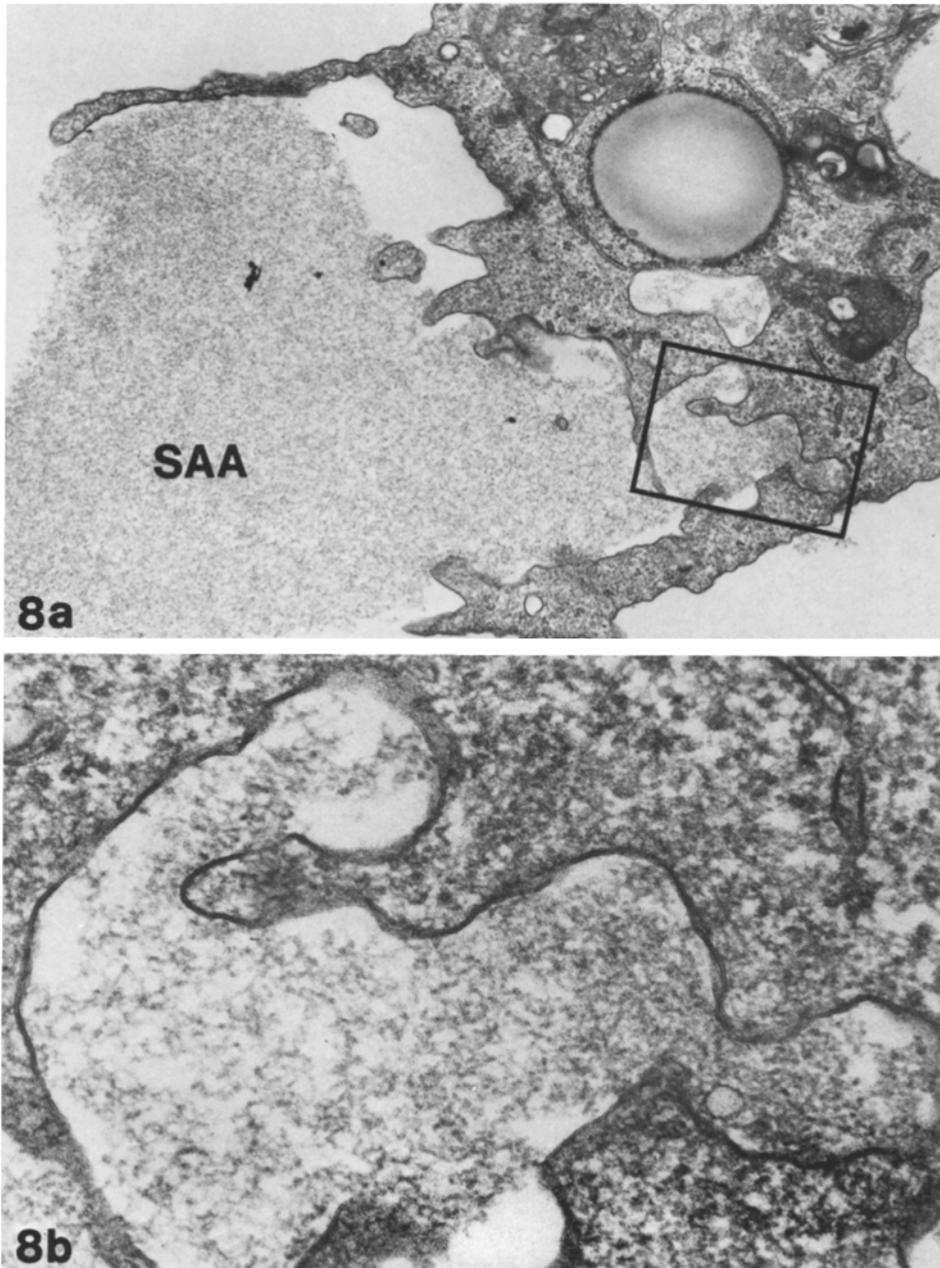


FIGURE 8. (a) Detail of a KC obtained from a mouse that had received 18 injections of casein. No amyloid could be demonstrated in the intact organs or isolated KC. The cell illustrated was taken from a specimen of KC cultured with SAA for 18 h. $\times 19,000$. (b) Higher magnification of the area delineated by the rectangle in a. Filaments have formed within the area of cellular invagination in close proximity to the plasma membrane. Dots represent filaments in cross section. $\times 86,000$.

ary amyloidosis, the congophilic AA fibrils are presumably derived by proteolytic cleavage of a normal plasma component, serum amyloid A (SAA) (2-4). SAA is synthesized by hepatocytes (32) and may attain very high levels in patients with infectious diseases or animals subjected to repeated inflammatory stimuli (14, 15). However, there is no correlation between the serum levels of SAA and the development of amyloidosis (33, 34). In fact, colchicine treatment of patients with familial Mediterranean fever (35) or of mice subjected to amyloid-inducing stimuli (36) prevents amyloidogenesis without reducing the plasma levels of SAA. Therefore, overproduction of SAA alone is not responsible for the condition. Alternative explanations have been sought. Amyloid formation could be due to the synthesis of an aberrant, incomplete molecule, AA, which subsequently polymerizes into fibrils, or it could be due to improper catabolism of SAA with similar results. Indeed, protein AA-crossreacting material has been found in homogenates of livers from casein-stimulated mice before amyloid fibrils could be detected (36). However, these experiments were not designed to distinguish between faulty synthesis or processing, and did not determine which cell type might be involved.

The first clue that AA formation may be caused by faulty processing of SAA was the observation that incubation of SAA with peripheral blood monocytes of patients with amyloidosis yielded an intermediate product that had immunologic and amino acid sequence identity with AA, whereas monocytes of most healthy individuals degraded SAA completely (5). To demonstrate that impaired catabolism of SAA by the macrophage phagocytic system (MPS) may actually precede formation of amyloid deposits necessitated the use of experimental animals. Accordingly, we turned to the well-studied model of murine amyloidosis (14, 51) and focused on KC, since these cells have been implicated in amyloidogenesis for many decades (12). The results were clear-cut. When KC isolated from control mice were incubated with SAA, they degraded the peptide completely, whereas KC obtained from amyloidotic mice yielded the intermediate AA product. Of even greater interest was the observation that impaired KC function could be observed before any amyloid was detected in the organs of mice from which the KC had been derived, and whose isolated KC showed no fibrils on electron microscopy. The appearance of AA in the cultures was accompanied by qualitative and quantitative alterations in the KC (Figs. 1 and 2). They assumed the appearance of "activated" macrophages and exhibited an increase in α -naphthyl acetate esterase and a reduction in α -naphthyl butyrate esterase (data not shown, and 37). With increasing numbers of casein injections, progressively larger amounts of residual SAA and AA were found in cultures (Fig. 4). The addition of normal KC to supernatants of cultures that had produced AA eliminated the peptide (Fig. 5). Therefore, it may be concluded that soluble AA per se is not resistant to hydrolysis by normal cells and that the appearance of this peptide is due to the inability of the stimulated cells to degrade SAA to completion. At first glance, it seemed puzzling that macrophages which appear activated would, at the same time, be defective in some degradative functions. There is, however, precedent for the observation that stimulation of some enzymes may be accompanied by down-regulation of others. This may be particularly relevant to the metabolism of ectoenzymes, where down-regulation

could be attributable to internalization of portions of the surface membrane. Thus, it has been shown that 5' nucleotidase activity becomes reduced when peritoneal macrophages are stimulated (38, 39), and a decrease in receptors after binding and internalization of ligands has been observed in several systems (e.g., 40). We have shown for monocytes that degradation of SAA is surface membrane associated (5, 7, 22). It is likely that the same holds true for KC, especially since SAA degradation also required the continuous presence of the cells. Since activated KC are replete with interiorized material, it is possible that activated KC lose some of their degradative ability by this process, i.e., that portions of their plasma membrane become internalized. This hypothesis requires further study.

In any case, the data presented here seem to permit the conclusion that the production of AA is due to faulty processing of SAA. It is still necessary to explain by what mechanism soluble AA, detected by SDS-PAGE, polymerizes into fibrils. It is of interest to mention that Hoffman et al. (41) have suggested that only one isotype of murine SAA is fibrillogenic. To observe this fibrillogenesis, KC isolated at various stages during amyloid induction were incubated with SAA and subjected to electron microscopy. Our studies were hampered by the observation that KC isolated from amyloidotic mice could not be entirely liberated from either phagocytosed or adhering fibrils. This was particularly surprising since the method for the isolation of KC included extensive enzyme treatments, multiple washes, and cell fractionations. As explained in the results and the legend to Fig. 6, it is possible that some of the fibrils were phagocytosed during the isolation procedure, a process that has been demonstrated to take place *in vitro* (42). However, the configuration of some of the fibril-containing inclusions, e.g., the ones illustrated in Fig. 7, did not resemble phagocytic vacuoles. The membranes of such inclusions were usually in continuity with the plasma membrane. As mentioned before, SAA degradation is mediated by cell surface enzyme(s). It is conceivable that not only AA production, but also its polymerization into fibrils, occurs in intimate contact with the plasma membrane. In considering the topology of the KC with its numerous processes and deep cytoplasmic invaginations, referred to as worm-like structures (43; reviewed in 44), it takes little imagination to conceive how fibrils formed on the surface membrane and its invaginations could appear as though they were both intracellular and extracellular. Proof of this phenomenon would settle the controversy over whether the fibrils go into or come out of the cells. The concept that insoluble filaments can be formed as a result of crosslinking of peptides by enzymes associated with plasma membranes is not novel. Examples include cytosolic proteins of keratinocytes crosslinked to form a water-insoluble envelope (45), and various cytoskeletal proteins of erythrocytes which, when crosslinked by the cells' transglutamidase(s), cause irreversible loss of membrane deformability (46).

Since amyloid fibrils have not yet been produced experimentally *in vitro*, nothing is known about the process of polymerization itself, i.e., whether molecules assemble side to side, end to end, or in any other way. Therefore, it is difficult to interpret images like the one shown in Fig. 8. Since the cells were

incubated with SAA in a serum-free medium and such filaments were not seen in control KC cultures, it is possible that the filaments were derived from SAA.

Thus, there is accumulating evidence showing that amyloidogenesis of the AA type is a multistep process. The present studies have clearly demonstrated that the elaboration of a soluble AA peptide is due to faulty processing of precursor SAA by KC defective in this regard. Whether KC are also involved in polymerization of this peptide into insoluble fibrils awaits further proof.

Summary

It has been demonstrated previously that the acute phase reactant, serum amyloid A (SAA), is subject to degradation by surface membrane-associated proteinases of peripheral blood monocytes. However, monocytes obtained from the blood of patients with amyloidosis degraded SAA incompletely, leaving a cleavage product that, biochemically and immunologically, resembled the amyloid protein A (AA) deposited in their tissues. To investigate the role of fixed macrophages in amyloidogenesis and to establish more definitively that amyloid deposition is attributable to faulty processing of the precursor protein rather than aberrant synthesis, secondary amyloidosis was induced in C57BL/6J mice by serial injections of casein. Kupffer cells (KC) were isolated from livers of mice that had received 0, 8, 13, 18, and >30 injections of the stimulant. The cells were cultured with SAA for 4, 8, and 18 h and then subjected to electron microscopy and enzyme analyses. The medium was analyzed by SDS-PAGE to determine the amount of residual SAA and/or the appearance of AA. KC of healthy animals degraded SAA completely whereas KC of stimulated mice showed increasing amounts of residual SAA and the appearance of the AA cleavage product. The AA peptide appeared in KC cultures early during the course of casein injections and before any amyloid could be demonstrated in the organs of the stimulated mice. The addition of KC isolated from healthy mice to cultures that had produced AA eliminated the abnormal peptide. The results indicate that defective KC function precedes amyloidosis. The abnormal AA cleavage product formed by such cells is still susceptible to hydrolysis by normal cells. In addition, ultrastructural evidence is presented that suggests that KC may also play a role in fibrillogenesis of the AA protein.

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