DEGRADATION OF SERUM AMYLOID A PROTEIN BY SURFACE-ASSOCIATED ENZYMES OF HUMAN BLOOD MONOCYTES*

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Amyloidosis encompasses a group of diseases characterized by the deposition of large amounts of certain types of extracellular fibrillar proteins in a variety of organs. Sections of the involved tissues reveal, in addition to the proteinaceous deposits, an infiltration of large pyroninophilic cells. Two types of amyloid occur most frequently. One of these, seen in patients with multiple myeloma, macroglobulinemia, and primary amyloidosis, is immunoglobulin-related amyloid which has as its major component fragments of light chains of varying size (1–3). The second is a nonimmunoglobulin form (4–8) which develops in association with various chronic inflammatory diseases and in almost all experimental models of amyloidosis (9–11). The major component of this type of amyloid fibril is the 8,500 dalton amyloid A (AA)[1] protein (4–8). In addition to these two types, there exist a number of rare forms including a variety of familial types, senile amyloid deposits, (12), and the amyloid that is localized in the skin and in certain endocrine glands (13), all of which seem to be derived from a precursor synthesized either locally or at a distant site. Whereas some information is available concerning the mechanism of degradation of L chains to L chain-related amyloid by proteolytic enzymes or tissue lysosomes (14–16), little is known about the origin of protein AA or the removal of amyloid deposits in the rare instances where recovery occurs.

Normal serum contains small amounts of a 12,500-dalton component known as serum amyloid A related protein (SAA) whose concentration increases several fold during many inflammatory and neoplastic states and returns to normal levels rapidly with recovery (17). SAA has antigenic properties similar to protein AA and has an identical amino acid sequence for at least the first 55 residues (18–21). Because SAA differs from protein AA in having a carboxy terminal extension, it seems likely that this part is cleaved when the AA protein is formed. The absence of protein AA from blood suggests that cleavage occurs in the tissues where amyloid is deposited. We therefore initiated a series of studies to investigate the mechanisms of degradation of SAA and AA in the hope of providing insights into the pathogenesis of the disease and into the causes for its development in only a fraction of subjects exposed to

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[1] *Abbreviations used in this paper:* AA, amyloid A; DFP, diisopropyl fluorophosphate; EACA, ϵ-amino caproic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SAA, serum amyloid A related; SDS, sodium dodecyl sulfate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

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prolonged periods of overproduction of SAA. The study is focused on the blood monocytes which, together with the closely related tissue macrophages, play a major role in the elimination of foreign materials through a process of engulfment and degradation within lysosomes (22) and through the secretion of many proteases such as collagenase (23), elastase (24), and plasminogen activators (25, 26) into the extracellular medium. Accordingly, we developed an in vitro system which shows that blood monocytes can degrade SAA to products very similar to protein AA and which enabled us to study the fate of the AA protein. This communication also presents the mechanism by which such degradation occurs and the location of the enzymes that may be involved in the process. The results raise some interesting questions concerning mechanisms which may be operative in the pathogenesis of the various forms of the disease.

Materials and Methods

Preparation of SAA. SAA was purified from the serum of a patient with macroglobulinemia and another one with an IgA myeloma. Serum was dialyzed against water, the supernate was made 10% in formic acid, and passed sequentially through Sephadex G-100, G-75, and G-50 columns (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) as previously described (21).

Preparation of Protein AA. Protein AA was prepared from the liver of a patient with secondary amyloid by distilled water extraction followed by chromatography on Sephadex G-100 in 5 M guanidine, and 1 M acetic acid (1, 27).

Cells. Peripheral blood monocytes from the blood of 20 normal donors, 6 patients with primary amyloidosis, and 4 patients with secondary amyloidosis were isolated on Ficoll-Hypaque (Pharmacia Fine Chemicals Inc.) and cultured as described elsewhere (28–30). Briefly, the cells were plated in 100-mm tissue culture dishes (BioQuest, BBL, & Falcon Products, Cockeysville, Md.) at a concentration of 10^7 cells per plate in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) medium, supplemented with 15% fetal calf serum, and incubated at 37°C in an atmosphere of 10% CO_2, 90% air for a 24-h period. The culture dishes were washed with RPMI medium, refrigerated for 1 h, and the cells removed from the plates with a rubber policeman. The harvested cells consisted of 99% monocytes on morphologic and functional grounds (29, 30).

Degradation of SAA and Protein AA by Cells. The monocytes purified as described above were suspended at a concentration of 10^6 cells/tube in 10-ml plastic test tubes containing 0.75 ml serum-free Dulbecco-s medium (Grand Island Biological Co.) with penicillin-streptomycin to which SAA or protein AA was added at a final concentration of 500 μg/ml. Aliquots of the medium were collected after different periods of incubation, in most cases, 2, 24, and 48 h, frozen, lyophilized, and examined on 10–20% sodium dodecyl sulfate (SDS)-polyacrylamide gradient slab gels (31). Where quantitative data were required the stained gels were scanned at 550 nm on a Gilford 250 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) equipped with a scanner and recorder.

Release of Enzymes by Cells. Degradative activity for SAA in the supernates of cultured monocytes, and the effects of SAA and aggregated gamma globulin on the release of such enzymes was assessed as follows: monocytes were incubated in serum-free Dulbecco's medium, or in medium containing either 50 μg/ml of SAA or 100 μg/ml of aggregated gamma globulin for 0.5 and 3 h. After incubation, the cells were spun down at 300 *g* for 5 min, and the supernates were collected and incubated with SAA at a concentration of 500 μg/ml at 37°C for an additional 3- and 22-h period. The supernates were lyophilized, assayed on SDS-polyacrylamide gel electrophoresis (PAGE), and scanned at 550 nm. The aggregated gamma globulin was prepared by heating a 10-mg/ml solution of human gamma globulin to 65°C for 30 min.

Inhibitors. The inhibitory effects of a series of enzyme inhibitors including diisopropyl fluorophosphate (DFP) (Sigma Chemical Co., St. Louis, Mo.) at a 5×10^{-4}-M concentration, 1-mg/ml ε-amino caproic acid (EACA) (Sigma Chemical Co.), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) at a 5×10^{-4}-M concentration and 5×10^{-4}-M L-1-tosylamide-2-

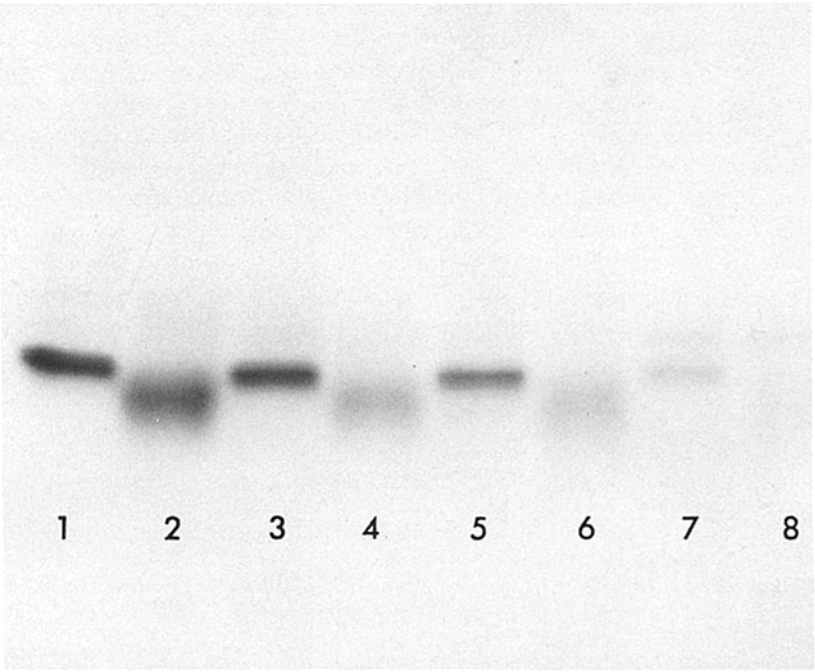

FIG. 1. Degradation of SAA and protein AA by peripheral blood monocytes. Slots 1 and 2 contain the initial medium solution with SAA and protein AA, respectively, before digestion with the cells. Slots 3, 5, and 7 are samples of SAA containing supernates incubated with cells for 2, 7, and 22 h, respectively, and slots 4, 6, and 8 are the AA protein containing supernates digested by the cells for 2, 7, and 22 h, respectively (staining was done with Coomassie Blue).

phenylethyl chloromethyl ketone (TPCK) were also tested.

Degradation of SAA by Glutaraldehyde Fixed Cells. To inhibit pinocytosis and phagocytosis completely, monocytes were fixed with 0.1%–0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min at room temperature. The fixed cells were then washed three times with PBS and incubated (1×10^6 cells/tube) with a 500-μg/ml solution of SAA in Dulbecco's medium. Cells incubated under identical conditions but not treated with glutaraldehyde were used as controls. Samples of the medium were collected and assayed on gels for SAA content after 5, 24, and 48 h of incubation. Samples of the glutaraldehyde-treated cells were tested for their ability to phagocytose by incubating them with latex particles for 30 min and examining them by electron microscopy (29, 30).

Results

Degradation of SAA and AA by Monocytes. To determine whether monocytes can degrade SAA and AA, cells obtained from the peripheral blood of 20 normal donors, 3 of whom were studied twice, 6 patients with primary amyloidosis, and 4 with secondary amyloidosis were isolated and tested for their ability to degrade these proteins under the conditions described in Methods. Fig. 1 shows the result of one experiment performed with cells from a normal donor. Both SAA and protein AA were almost completely eliminated from the medium after incubation with the cells. Among the 30 individuals tested, three patterns of degradation of SAA were noted: (*a*) a pattern in which SAA was completely degraded without the appearance of any intermediary products on the acrylamide gels (Figs. 1 and 2A). This degradation profile was found in eight normal individuals and none of the patients with amyloi-

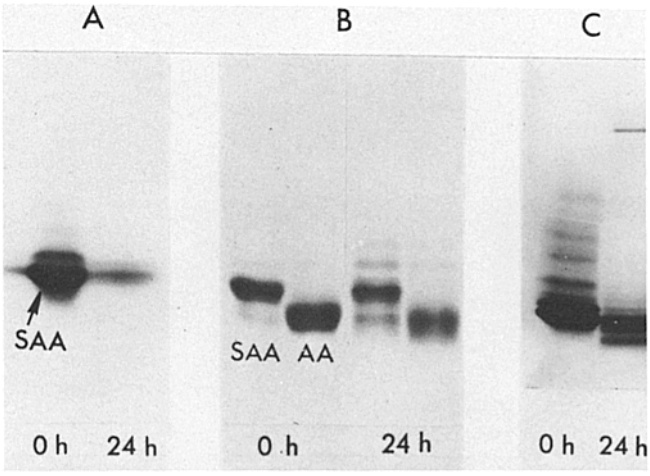

FIG. 2. Patterns of degradation of SAA by macrophages: (A) Elimination of SAA after incubation with macrophages without the appearance of detectable degradation products on the gel. (B) Degradation of SAA by macrophages to a product comigrating with protein AA. Samples were collected after 24 h of incubation with the cells. (C) The cleavage of SAA by macrophages to an 8,000 mol wt product which remains intact and is not further degraded.

dosis; (*b*) a pattern in which the 12,500-dalton SAA molecule was degraded to a product with a mol wt of 7,000–9,000 daltons which comigrated on SDS-PAGE with protein AA isolated from amyloidotic tissues, and reacted with antibodies to protein AA in Ouchterlony double diffusion plates. This pattern of degradation (Fig. 2 B) was observed in cells of 8 normal individuals and all 10 patients with amyloidosis. Because this intermediate was degraded further with time, only a small portion of the protein could be found in this form when supernatant samples were assayed between 2 and 20 h of incubation. In most cases, the intermediate disappeared when the incubation period exceeded 24 h (Fig. 2 B); (*c*) In four instances, a pattern of degradation was observed in which SAA yielded one or more degradation products having molecular weights ranging between 7,000 and 9,000 daltons which remained even after incubations longer than 48 h (Fig. 2 C). The rate of elimination of SAA by cells from a subject that degraded SAA without intermediates (pattern I) was determined in a kinetic experiment in which SAA in concentrations ranging from 0.25 to 2.5 mg/ml was incubated with monocytes. Samples of the medium were collected after 1, 2, 3, 4, 5, and 16 h. The results (Fig. 3) show that 30% of the protein is degraded by the cells during the first incubation hour when the initial concentration of SAA was 0.25 mg/ml. During the initial incubation period, degradation ranged between 100 and 200 μg/h per 10^6 cells depending on the SAA concentration.

The frequencies of these three degradation patterns among normal cell donors and patients with amyloidosis are summarized in Table I. An indication that these differences are not technical artifacts is found in the fact that the patterns of SAA degradation for three individuals who were retested remained constant.

Because these observations suggested that the SAA degradation pathway by macrophages involves a multistep reaction, with cleavage of the 12,500-dalton SAA molecule to an intermediate product with a mol wt of 8,000 daltons, which is then followed by a series of subsequent steps which eliminate the intermediate AA-like

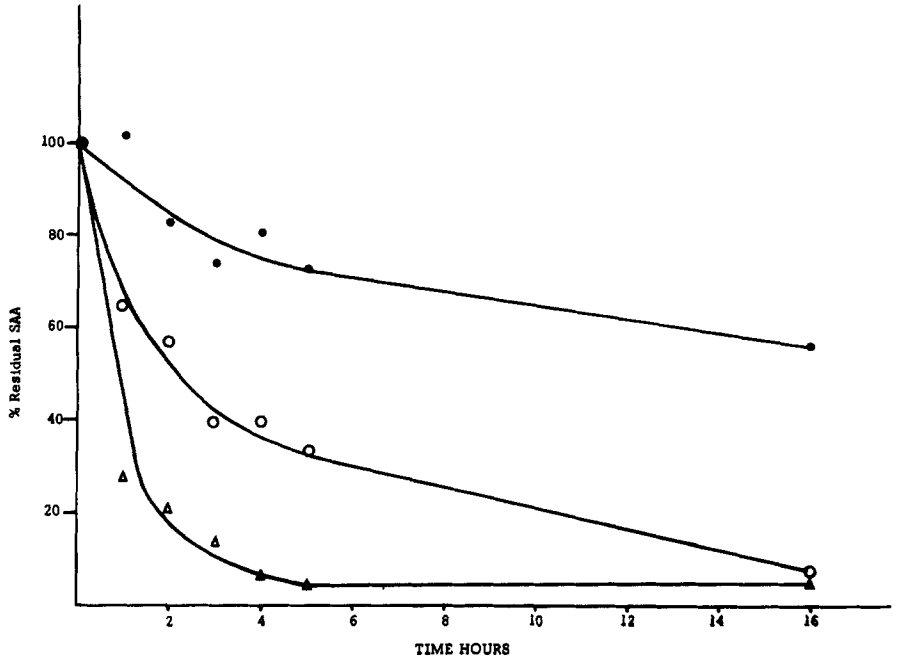

FIG. 3. The kinetics of SAA degradation by monocytes. 10^6 cells were incubted with 0.4 ml of Dulbecco's medium containing 2.5 (●), 1.5 (○), and 0.25 mg/ml (△) of SAA. 0.05-ml Samples of the medium were collected after 1–5, and 16 h of incubation, assayed on SDS-PAGE stained with Coomassie Blue and scanned. The results are expressed as percent SAA left in the medium after the incubation.

TABLE I

Patterns of Degradation of SAA and AA by Monocytes from Normal Donors and Patients with Amyloidosis

Diagnosis	Degradation of SAA				Degradation of AA	
	No	Complete	8,000 Dalton intermediate		No	Degradation
			Further degradation	No further degradation		
Normal donors	20	8	8	4	18	16
Primary amyloid	6	0	6	0	6	6
Secondary amyloid	4	0	4	0	3	3

product, we studied the handling by these cells of AA protein obtained from the liver of a patient with secondary amyloidosis after treatment of the fibrils with 5 M guanidine.

Fig. 1 shows that monocytes are also capable of degrading protein AA. The rate of elimination of protein AA was similar to that of SAA. Though in general the handling of AA appeared similar for cells from normal subjects and patients with amyloidosis, cells from two of the normal donors which degraded SAA to a stable 8,000 dalton component also failed to degrade protein AA, even when the incubation period was extended to 48 h.

The Mechanisms of SAA Degradation by Macrophages. Two mechanisms are primarily

TABLE II

Comparison of SAA Degradation by Monocytes and Monocyte Supernates Obtained Under Equivalent Conditions

	Amount of SAA left after 3-h incubation	Amount of SAA left after 20-h incubation
	μg	μg
Incubation of 100 μg SAA with:		
Intact cells	10.3	2.9
Supernates after 0.5-h incubation of cells with:		
Dulbecco's medium	92.5	76.4
Dulbecco's medium + aggregated γ-globulin	89.4	84.0
Dulbecco's medium + latex particles	93.1	90.6
Dulbecco's medium + SAA*	87.7	78.3
Supernates after 3-h incubation of cells with:		
Dulbecco's medium	90.7	87.3
Dulbecco's medium + aggregated γ-globulin	89.1	82.6
Dulbecco's medium + latex particles	99.5	87.2
Dulbecco's medium + SAA*	88.9	80.0

* Corrected for the amount of SAA added after the incubation with the cells.

involved in the degradation of proteins and particulates by macrophages (22). The first is a process of endocytosis in which engulfment of extracellular material into phagosomes or pinosomes is followed by fusion with lysosomes and degradation by lysosomal enzymes. The second is through the release of active proteases by the cells into the extracellular environment where degradation of proteins such as collagen (23), elastin (24), and plasminogen (25), is believed to take place. Pinocytosis or phagocytosis appeared unlikely because intracellular degradation is usually complete and not known to be associated with the discharge of partially degraded products. Therefore, we first examined the possibility that the cells secrete a proteolytic enzyme which cleaves the SAA molecule outside the cell.

The degradation of SAA by 1×10^6 intact monocytes was compared with that by supernates obtained from the same cells under a variety of conditions. Cells were incubated for 0.5 or 3 h in 0.50 ml of serum-free Dulbecco's medium, the same medium with 100 μg/ml aggregated gamma globulin, latex particles, or with low concentrations of SAA (50 μg/ml). The cells were then spun at 300 *g*, the medium was collected, and incubated with 0.2 ml of SAA (500 μg/ml) for 3 and 20 h after which the supernates were lyophilized, assayed on SDS-PAGE, and scanned to determine the amount of residual SAA. The results presented in Table II show that after 3 h of incubation, the intact cells were nine times more active than the supernates obtained from cells that had been previously incubated for the same time period, even when the cells had been exposed to aggregated gamma globulin or SAA. Differences in enzyme activity between tubes containing supernate or intact cells were even greater after a 20-h incubation period with SAA. Almost no SAA remained in the sample incubated with intact cells, whereas ≅ 80% remained in the samples exposed to the culture supernates. Because these results suggested that degradation takes place predominantly by cell-associated enzymes and only to a small extent by enzymes released into the medium, experiments were designed to distinguish between cell surface as opposed to intracellular degradation.

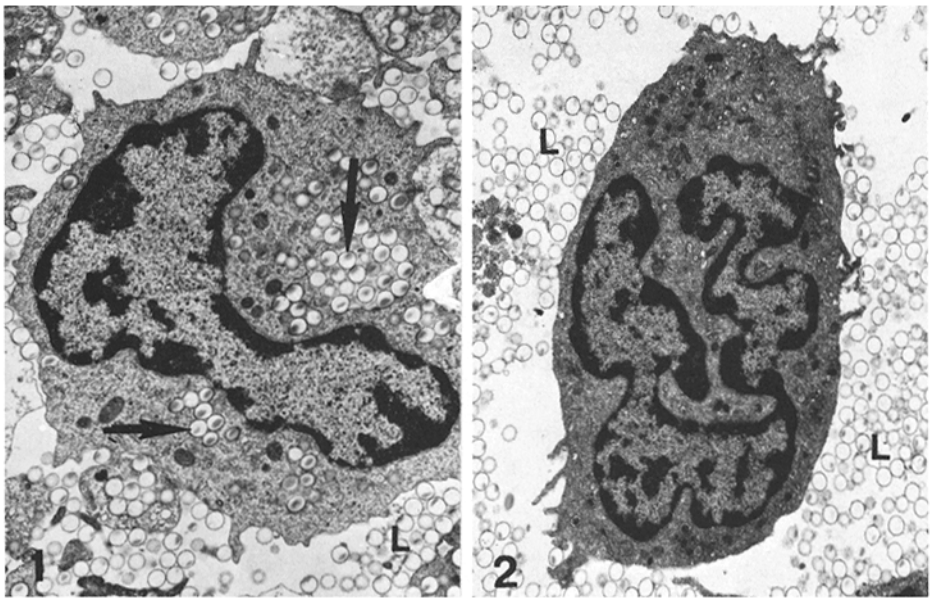

FIG. 4. (1) Monocyte incubated with latex particles (L) before fixation. As was to be expected, numerous particles are seen within phagocytic vacuoles (arrows) (magnification ×7,000). (2) Monocyte fixed with 0.1% glutaraldehyde for 5 min before incubation with latex particles (L). Fixation completely inhibited interiorization of the particles (magnification ×7,000).

TABLE III

The Effect on Degradation of SAA and AA of Treatment of Monocytes with 0.1% Glutaraldehyde

Time of incubation	SAA left after incubation with		Protein AA left after incubation with	
	Intact cells	Glut Ald* fixed cells	Intact cells	Glut Ald* fixed cells
h	%		%	
5	37.4	69.9	59.1	42.9
20	13.2	22.0	30.3	16.4
48	—	—	12.4	8.9

* Glut Ald, glutaraldehyde.

Evidence of Enzymatic Activity Bound to the Cell Membrane and its Possible Nature. To test the possibility that the enzymes may be associated with the outer part of the cell membrane, we treated the cells with 0.1%–0.5% glutaraldehyde in PBS for 5 min and assayed for the ability of these cells to degrade SAA and to interiorize latex particles. Whereas electron microscopy showed that latex particle interiorization was entirely inhibited by 0.1% glutaraldehyde (Fig. 4), there was no significant decrease in the amount of AA degraded (Table III) and only moderate inhibition of SAA degradation after 5 h of incubation and no inhibition after 20 h. The mild treatment with glutaraldehyde did not appear to significantly affect the activity of the enzyme which could still degrade SAA and AA. Release of enzymes subsequent to glutaraldehyde fixation was excluded by showing that supernates obtained after 3 h of incubation

TABLE IV

The Effect of Enzyme Inhibitors on Degradation of SAA by Monocytes

Inhibitor	SAA degraded after 24-h incubation of	
	Cells	Cells + inhibitor
	%	
DFP, 5×10^{-4} M	88.0	1.2
EACA, 100 μg/ml	18.3	21.9
TLCK, 5×10^{-4} M	86.8	91.1
TPCK, 5×10^{-4} M	88.0	79.3

with SAA in a manner similar to the studies in Table II did not degrade SAA (data not shown). This experiment provides additional support for the assumption that the enzymes which degrade amyloid-related proteins are located on the surface of the cells. Treatment with 0.3 and 0.5% glutaraldehyde resulted in inactivation of the enzyme.

Table IV shows that SAA degradation can be inhibited by 5×10^{-4} M DFP, a concentration which was found not to impair cell viability or to inhibit interiorization of horse radish peroxidase by the cells (data not presented). TPCK, which inhibits chymotrypsin-like activity, and TLCK, which inhibits trypsin-like activities, had no effect on this enzyme when tested at the same concentration. This suggests that the enzyme may be a serine esterase not related to trypsin-like or chymotrypsin-like enzymes. Soy bean trypsin inhibitor, pepstatin, leupeptin, antipain, and chymostatin had no effect. The possibility that the enzyme that degrades AA is a plasminogen activator or a complement component known to be elaborated by these cells seems unlikely because EACA, which inhibits plasminogen- and complement-activator activities, had no effect either on SAA or on protein AA degradation (Table IV).

Discussion

The structural relationship of tissue protein AA to SAA has led to the belief that AA protein results from the cleavage of SAA, a protein whose serum concentration rises and remains elevated for long periods of time during a variety of inflammatory and other pathological states. Because proteolysis appears to play an important role in the genesis of all types of amyloid, it seems possible that phagocytic cells could play a role in the mechanisms of amyloid formation as well as removal. Such studies could perhaps also shed light on factors predisposing some individuals to develop the disease whereas others subjected to the same stimulus resist it.

Our experimental results suggest that monocytes are capable of degrading SAA and AA through the action of one or more cell surface-associated enzymes rather than by interiorization or release of enzymes into the extracellular medium. In the case of SAA, the protein appears to be cleaved to an 8,000 dalton AA-like molecule and then further to smaller peptides. The detection of intermediate degradation products in some individuals and failure to find them in others may imply that there are multiple complex mechanisms involved in the degradation process of these two components. It seems possible that the two steps in the degradation of SAA involve separate enzymes or enzyme systems because unlike the majority of normal subjects and patients with amyloidosis who degrade both SAA and AA, the monocytes from four normal individuals, two of whom were tested twice, were found to be deficient in the

enzymatic activity responsible for the second stage. The cells of these donors degraded SAA to a persistent 8,000 mol wt product reactive with antisera to AA and also failed to degrade AA protein obtained from amyloidotic tissue. However, no other properties such as profiles of the effects of inhibitors or distribution of the enzymes on the cells could so far distinguish the enzymatic activities. If two separate enzymes are indeed involved, relatively larger amounts or higher rate of turnover of the second enzymatic system, which degrades the 8,000 dalton intermediate and protein AA, may lead to the rapid elimination of these proteins and result in the first pattern of degradation. In contrast, a slower rate of degradation of AA than SAA may result in the accumulation of the intermediates yielding the second pattern of degradation. Thus it is possible that the first two patterns of degradation which we classified as A and B simply reflect differences in the relative amounts of the two enzyme systems. Though it is not clear that the second enzymatic system is absent in cells of donors in whom the intermediate degradation products persist (type C), this possibility receives support from the finding that the cells of two such donors failed to degrade protein AA derived from amyloidotic tissue. Because the in vitro assay system is not sufficient to permit any conclusions relating a defect in either step of processing to the pathogenesis of the disease, other types of experiments will be required to study that problem and to document the nature of the enzyme(s) involved. In addition, the function of this degradative system in the processing of other proteins remains to be determined. Some specificity for degrading AA-related proteins is suggested by the failure to observe a similar rapid degradation by monocytes of other proteins such as Bence Jones proteins, ovalbumin, and serum albumin (G. Lavie, D. Zucker-Franklin, and E. C. Franklin, unpublished observations), and the lack of inhibitory effect of EACA which points away from an association of these proteolytic enzymes with complement activators or clotting factors. Similarly, though it is not known whether the activity is specific for monocytes or whether it is associated also with other cell types, preliminary data from studies currently in progress indicate that human lymphocytes isolated from peripheral blood as described elsewhere (29) are also capable of degrading SAA, although perhaps less efficiently (unpublished observations). Mouse peritoneal macrophages also appear to possess similar enzymes on their surface.

The mechanism by which the cells degrade the SAA and AA proteins is not a very common one. The almost complete preservation of the enzymatic activity after a brief treatment of the cells with glutaraldehyde together with the finding of extracellular intermediate breakdown products which disappear upon prolonged incubation, speak against a process of interiorization and intracellular digestion and point to a surface-associated enzyme. A major role for proteolytic enzymes released by the cells after exposure to SAA or associated with phagocytosis of latex particles or aggregated gamma globulin appears unlikely because cell supernates were not nearly as effective as intact cells. Based on these positive results, and the inhibition of the process with DFP, the most likely mechanism appears to involve a serine protease which is predominantly associated with the cell membrane and is released by the cells only in minute amounts. Similar types of membrane-associated proteolytic activities, have been observed in the brush border of kidneys (32) on cytotoxic lymphocytes (33), on the membrane of mouse peritoneal macrophages which appear to contain an amino peptidase (34) and on the surface of polymorphonuclear leukocytes (35).

The possible relevance of the observed findings to the pathogenesis of amyloidosis

remains unanswered. However, some of the differences in the behavior of cells from normal subjects and those with amyloidosis suggest a possible role in the pathogenesis of amyloidosis. Whereas 40% of normal cells degraded SAA without the appearance of any intermediates, all 10 patients with amyloidosis showed the transient appearance of a molecule with size and antigenic properties of AA, perhaps indicating a quantitative or even a qualitative deficiency in the second degradative step. During prolonged inflammation, when blood SAA levels are markedly increased, this type of degradation may result in the formation of large amounts of protein AA intermediates, the further degradation of which may be incomplete. The undegraded protein AA may find favorable conditions in the tissues to polymerize and form amyloid fibrils. The finding of a similar pattern in 12 of the normal subjects, 4 of whom failed to degrade the intermediate further, may perhaps identify individuals predisposed to the development of amyloidosis once subjected to the inciting stimulus. Studies of larger numbers of subjects, family members, and strains of mice differing in their susceptibility to amyloidosis will be required to answer some of these questions.

Summary

Peripheral blood monocytes incubated in a serum-free medium degraded serum amyloid A (SAA) protein along three pathways. Of 20 normal subjects, 8 degraded SAA completely with no detectable intermediates. Eight subjects transiently produced an amyloid A (AA)-like intermediate which comigrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) with tissue AA protein and reacted with antisera to AA, whereas four subjects yielded a persistent AA-like intermediate on PAGE. This group also failed to degrade tissue AA protein. Cells from 10 patients with amyloidosis fell into the second group. The responsible enzymes appear to be serine proteases because they are inhibited by diisopropyl fluorophosphate. They were not affected by ε-amino caproic acid, L-1-tosylamide-2-phenylethyl chloromethyl ketone, or *N*-α-*p*-tosyl-L-lysine chlormethyl ketone. It appears possible that the enzymes are associated with the outer membrane of the cell because only a small fraction of the activity is secreted into the medium and because enzyme activity remains after fixation of the cells with glutaraldehyde which completely stops phagocytosis. Perhaps differences in patterns of proteolysis may play a role in the predisposition to amyloidosis.

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