# Early detection of inflammation-associated amyloid in murine spleen using thioflavin T fluorescence of spleen homogenates: Implications for amyloidogenesis

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ABBREVIATIONS: AA: INFLAMMATION ASSOCIATED AMYLOIDOSIS, AEF: AMYLOID ENHANCING FACTOR, CR: CONGO RED. SAA: SERUMAMYLOID-A, THT: THIOFLAVIN T

## Abstract

*Amyloid is a generic term which refers to extracellular protein deposits with specific staining, morphological and structural properties, that are seen in a variety of clinical disorders, the most common of which is Alzheimer's disease. In rapid induction models of murine inflammation-associated (AA) amyloid, amyloid deposits cannot he detected in the spleen by conventional histochemical techniques earlier than 36-48 h after administration of the amyloidogenic stimulus. The fluorescent dye Thioflavin T exhibits specific excitation emission wavelength shifts when bound by amlyloid. In the present studies, an existing Thioflavin T fluorescence assay was modified and evaluated for its ability to detect anlyloid formation during accelerated murine splenic AA amyloidogenesis. Concentrations of fibrils as low as 50 ug/ml were measurable. Furthermore, the assay reliably identified amyloid in splenic homogenates 18 h following the anlyloidogenic stimulus. a time point 18-24 h prior to the initial histological detection of amyloid in tissue sections. These data demonstrate the marked sensitivity of the Thiojlavin T assay and show the rapidity with which the onset of amyloid formation occurs in vivo following an induction stimulus. The rapidity of thc model provides a unique system to test compounds that may protect against amyloid formation.*

## Introduction

Amyloid is a fibrillar, usually cxtraccllular, proteinaceous deposit which possesses very specific morphologic, staining and structural characteristics. Histochemical and immunohistochcmical procedures have been used to identify amyloid deposits in tissue sections. When stained with traditional dyes. such as hematoxylin and eosin, amyloid appears histologically amorphous. However, after being stained with Congo red (CR) and viewed with polarized light, amyloid exhibits a characteristic green birefringcnce, indicating some type of organized, underlying structure[1,2]. In rapid induction models of murine inflammation-associated amyloid A (AA) amyloidosis, histochemical techniques have demonstrated

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that the initial deposits of amyloid are detectable in the spleen 36-48 h after induction3. More sensitive techniques would presumably detect AA amyloid at shorter intervals after induction.

Thioflavin T (ThT) is a fluorescent compound that displays a specific shift in excitation and emission maxima when bound to amyloid[4,5]. In tissue sections, ThT binds to elastic fibrils and to nuclei as well as to amyloid, consequently ThT fluorescence is not specific for amyloid under these circumstances. This is due to the variety of wave lengths of excitation light in light microscopy and the inability of the eye to discriminate a single wave length of emitted light. However, when ThT-stained sections are differentiated with acetic acid or pre-stained with hematoxylin, elastic fibril and nuclear ThT fluorescence is prevented and the stain is considered specific for amyloid4. In contrast to light microscopic techniques, the measurement of ThT bound to amyloid in tissue homogenates using a fluorometer has had more success5-7, since single wavelengths of excited and emitted light can be used.

The purpose of the present study was to establish a ThT fluorescence assay which is more sensitive than CR histochemistry for the detection and quantification of splenic AA amyloid in tissue homogenates. Application of the ThT technique to the measurement of amyloid accmmulation during accelerated murine AA amyloidosis demonstrated that splenic amyloid fonnation occurs significantly earlier in this model than had been shown previously with conventional histochemical methods.

## Methods

### Animals

Swiss white CD, 6-8 week old female mice (Charles River, Montreal, QC) were used for all amyloid experiments.

### Chemicals

Chemicals were purchased from BDH (Poole, UK), Fisher Scientific (Ottawa, ON), or Sigma Chemicals (St. Louis, MO). ThT was obtained from Aldrich Chemical Co. (Milwaukee, WI). All chemicals were of analytical grade or better. The BioRad DC Protein assay kit, a modification of the Lowry assay, was purchased from BioRad (Hercules, CA).

### Amyloid induction

Amyioid was induced according to the method of Axelrad *et al.*". Briefly, 0.1 mg AEF (by protein) was administered by intravenous injection using the tail vein of the mouse. Amyloid fibrils isolated by the method of Pras *et* al' were used as AEF as described by Baltz et al. Inflammation was induced by a subcutaneous injection of 0.5 ml of 2% (w/v) silver nitrate. Mice were killed by cervical dislocation at different intervals after amyloid induction (12-96h). Controls consisted of untreated animals, or those treated with AEF alone, or silver nitrate alone, for the equivalent time periods. The spleens were removed and frozen at -20$^{o}$C until use. Purified AA amyloid fibrils were prepared according to the method of Pras et al [9].

### Detection and quantitation of splenic AA amyloid

Amyloid fibrils were isolated using a modification of the method of Pras et al[9]. All procedures were carried out at room temperature without the use of protease inhibitors, as the amyloid fibrils have been shown to be resistant to proteolysis. For each data point, three or four sets of three murine spleens were hand homogenized in a glass D ounce type homogenizer (17x2.5cm, clearance 0.12-o. 15 cm) using l0-15 strokes and a smooth tipped Teflon pestle in 30 ml of 0.15 M NaCl. The homogenates were centrifuged at 10,000g for 30 m. The splenic capsule (which remained on the pestle) was discarded. This step was repeated once and the salt supematants pooled for subsequent analysis for amyloid fibrils.

The pellets were sonicated using a probe sonicator (Vibracell, Sonic and Materials Inc., Danbury, CT), with brief bursts at 60 amplitude on the scaler provided, for 15 s in 1 ml of distilled water and either frozen at -20°C or analyzed directly. Immediately before analysis, the suspension was dissolved by adding an equal volume of 0.5 M NaOH to give a fmal volume of 2 ml at 0.25 M NaOH. Such treatment, as shown previously, does not destroy amyloid fibrils". A 20uL aliquot of the solution was added to 1.5 ml of fluorescence buffer (10 uM ThT/50 mM glycine. pH9.0) as described by Naiki et al[5,6]. ThT fluorescence was measured within 15 m on a Perkin Elmer LS-50 fluorometer. The excitation wavelength was 450 nM with a slit width of 5 nM. To ensure that the emitted light at 482 nM was maximal, the emission wave lengths were scanned from 470 to 490 nM with a slit width of 10 nM. The protein concentration of the sample was determined by the Biorad DC Protein Assay according to the manufacturer's specifications. The amount of amyloid in the samples was expressed in terms of intensity per unit protein concentration ($I_{482}$/mg protein) and when multiplied by the splcnic weight gave a measure of the quantity of amyloid per spleen. A standard curve derived from the addition of known amounts of purified AA fibrils to normal spleen homogenates was used to detennine the concentration of the AA fibril protein in the experimental spleen homogenates.

The presence of amyloid fibrils in the pooled salt washes was assessed after these washes were dialyzed to

remove salt, concentrated by lyophilization and reconstituted to a volume of 1 ml. This material was then assayed in the same manner as the tissue pellet which contained the amyloid as described above.

## Results

The standard curve in which AA fibril-associated ThT fluorescence was plotted vs. AA protein concentration, demonstrated that as little as 50 50mg/mLAA-fibril protein could be detected in this assay (Figure 1). The threshold concentration of fibrils in the ThT fluorescence buffer was significantly lower. The two fold dilution of the standards by the NaOH solution and the 20/l 520 dilution in the ThT solution gave a threshold concentration of standards which ranged from 2.30 to 0.33 ug/ml.The ThT fluorescence buffer contained normal spleen homogenate to show that the signal was detectable above background tissue ThT fluorescence. Furthermore, the signal increased linearly with increasing amounts of AA-fibril protein. Fluorescence was at background levels when ThT was added to non-fibrillar fortns of serum amyloid A (SAA), the AA amyloid precursor, or SAA containing HDL [(the natural lipoprotein carrier of SAA) (data not shown)] preparations. Moreover, the addition of an equal volume of 4 M guanidine-HCl abolished the fluorescence signal at 482 nM (Figure 1, solid circles), demonstrating that this signal was specific for amyloid fibrils.

Initial work with ThT and whole spleen homogenates showed decreasing fluorescence intensity with increasing amyloid induction time, a result which contradicted the clear presence of, and increasing quantity of, amyloid as detected with CR. In these early ThT experiments, the fluorescence intensity was not standardized against total tissue protein concentration. During amyloid induction, as a result of the inflammatory stimulus, spleen cells begin proliferating after 2 days, so that the protein concentration of the homogenate would be expected to increase at a faster rate than AA amyloid formation. This would account for the paradoxical decrease in ThT amyloid signal. In addition to nonnalizing the signal against the protein concentration, several modifications in the preparation of the homogenate were performed to correct this problem. The splenic capsule was removed, since homogenized fragments caused light scatter that interfered with the amyloid dependent ThT signal. The tissue was washed twice with large volumes of 0.15 M NaCl to remove soluble splenic proteins, but not to remove amyloid tibrils which are insoluble at this salt concentration. These modifications enriched the residual pellet for fibrils and increased the sensitivity of the assay. To ensure that fibrils were not lost in these washes, the ThT fluorescence of the dialyzed and lyophilized material from the salt washes was examined (Figure 2, Panel C). No signal was detected, indicating that if fibrils were lost, the amount of loss was less than the assay could detect. Finally, the homogenates were dissolved in 0.25 M NaOH immediately before the samples were assayed, which provided more reproducible measurements. This treatment reduced the turbidity of the samples without destroying the fibril or structure11.

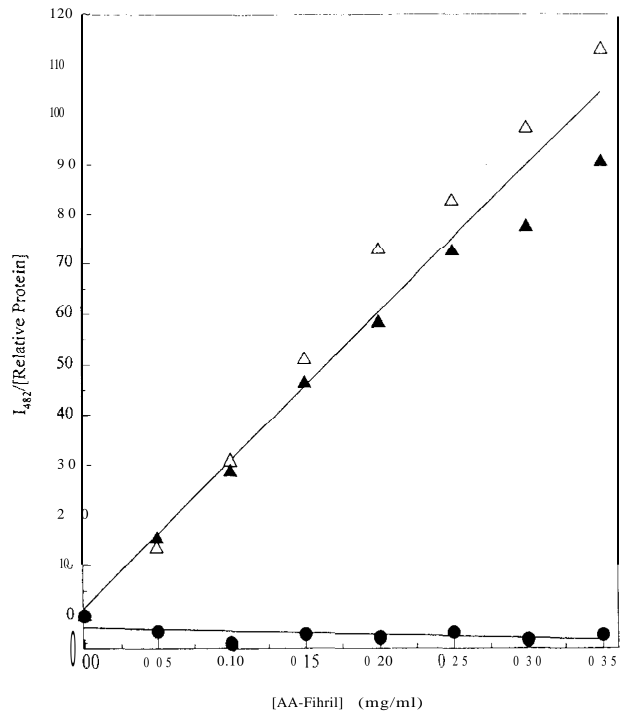


**FIGURE 1.** Thioflavin T fluorescence intensity as a function of AA-fibril protein concentration. Isolated AA amyloid fibrils were added to ThT fluorescence buffer containing control tissue homogenate without (triangles), and with (closed circles), an equal volume of 4 M guanidine-HCl. The excitation wavelength was 450 nM, and emitted light was measured at 482 nM. The signal was normalized by dividing I482 by the protein concentration of the sample, then subtracting the control tissue I482 The open and closed triangles represent replicate determinations. The lines were determined by the method of least squares.

The time course of splenic AA amyloid accumulation is shown in Figure 2. In Panel A the fluorescence emission of splenic homogenates of mice treated with silver nitrate and AEF (amyloid induction Protocol), silver nitrate alone (inflammation control), or AEF alone are compared. The

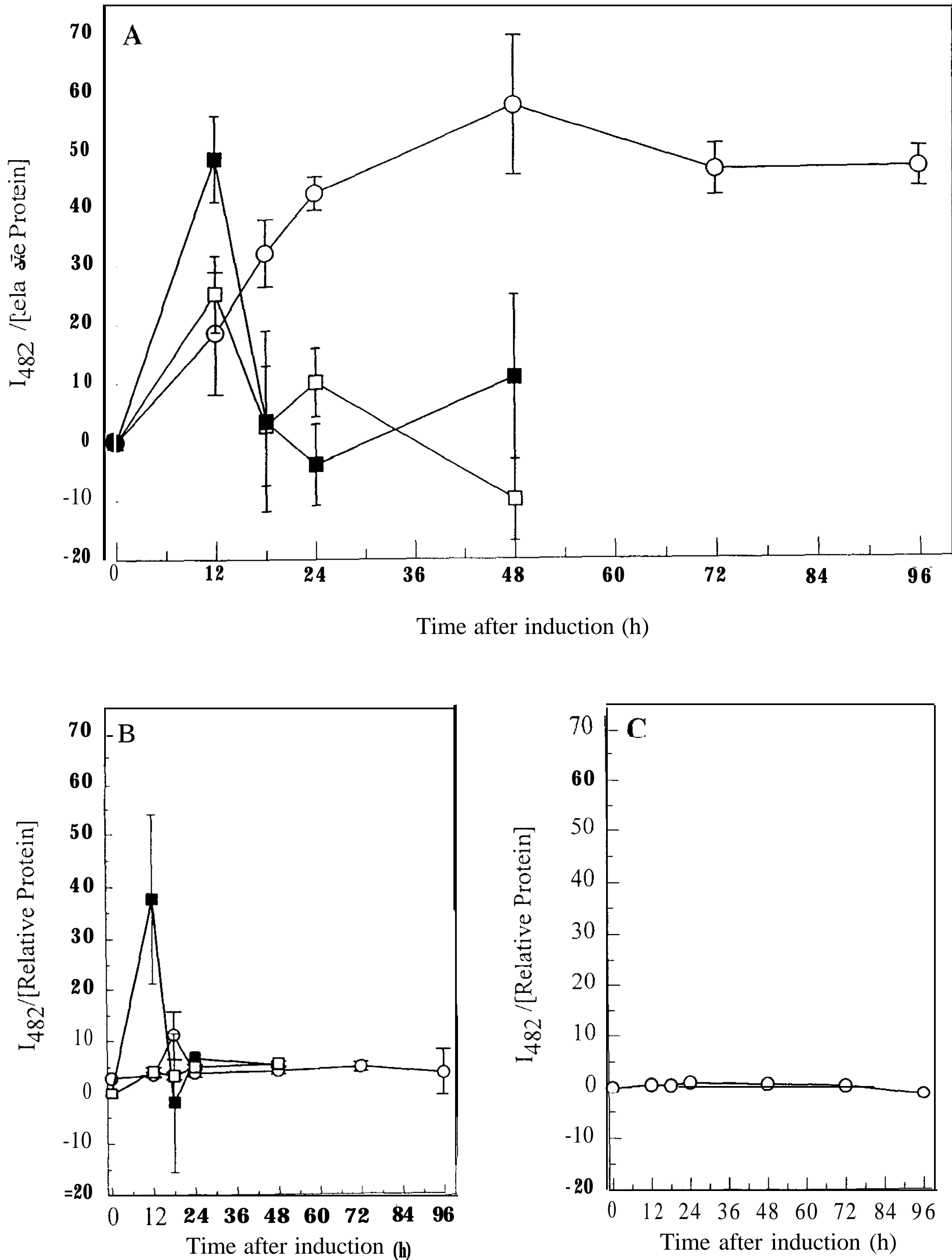


**FIGURE** 2. A time course of splenic amyloid deposition as measured by ThT fluorescence. Panel A: Spleen homogenates (20 ug/ml) from animals forming AA amyloid +/-(silver nitrate and AEF, open circles), or controls (silver nitrate, closed squares; AEF, open squares) were added to ThT fluorescence buffer. The excitation wavelength was 450 nM and emitted light was measured at 482 nM. The signal measured was normalized by dividing I482 by the protein concentration of the sample and then subtracting control tissue I482/protein concentration. Values are means of n=4 ± S.E.M. (silver nitrate and AEF), and means of n=3± S.E.M. (silver nitrate or AEF). Panel B: ThT fluorescence of spleen homogenates in the presence of an equivolume of 4 M guanidine-HCl. Values are means of n=3 ± S.E.M. Panel C: ThT fluorescence of salt washes from spleen homogenates of silver nitrate and AEF injected mice.

fluorescence signal in mice depositing amyloid steadily increased until 48 h, at which point it reached a plateau. However, when the I482/[protein] measurement was multiplied by the splenic mass, it became apparent that amyloid was accumulating in the spleen throughout the time course of the experiment (Figure 3).

Mice which received either silver nitrate, or AEF alone, showed no significant increase in splenic ThT fluorescence, except at 12 h after injection. The addition of an equal volume of 4 M guanidine-HCl destroyed the ThT fluorescence of homogenates from AEF treated mice, but not of homogenates from mice receiving silver nitrate alone. This demonstrated that the 12 h signal in the latter group was due to some factor other than the fonnation of AA amyloid (Figure 2, Panel B). The ThT signal in the former is probably the result of the fibril AEF preparation used in the present studies. When AEF was injected 24 h prior to the inflammatory stimulus, a time sufficient to maintain AEF activity[8,10,12], but long enough to allow for the 12 h AEF ThT "signal" to disappear, and then followed by a silver nitrate injection (i.e. completion of the induction protocol), spleen homogenates showed a ThT fluorescence signal above untreated mice 12 h later. This observation suggested that AA amyloid deposition occurred 12 h after completion of the induction protocol. Treatment of this sample with an equal volume of 4 M guanidinc-HCl again decreased the fluorescence to background levels, demonstrating that the "signal" was probably caused by amyloid fibrils. However, the tentative conclusion that amyloid induction occurs in 12 h requires confirmation by additional studies.

## Discussion

The ability rapidly to induce inflammation associated amyloid (AA amyloid) in mice using amyloid enhancing factor (AEF) and a non specific inflammatory stimulus (e.g. subcutaneous silver nitrate) has provided a model of amyloidogencsis which can be dissected for factors directly associated with amyloid formation. Conclusions drawn about apparent temporal relationships are, however, dependent on the sensitivity of techniques used to measure the parameters of interest.

The detection of amyloid in tissue sections is usually done with CR staining and using this procedure, AA amyloid is clearly detectable in murine spleen within 36-48 h following induction with AEF and silver nitrate[3,12,13]. More recently, ThT has been shown to be useful in quantifying the apoA-II form of amyloid in mouse tissues[5,6], and in monitoring the fonnation of other fonns of amyloid *in vitro*[7,14] AA amyloid in tissue homogenates has been examined with ThT fluorescence procedures, but with apparent low sensitivity[6]. The results of the apoA-II and *in vitro* studies suggested that the ThT fluorescence procedure could be modified to a greater sensitivity in order to monitor AA amyloid fonnation than had previously been reported[6], and could thus be used to monitor splenic AA amyloid accumulation in a rapid amyloid induction protocol.

The specificity and sensitivity of the ThT fluorescence procedure was determined with a standard curve of increasing amounts of AA fibrils (Figure 1). These results showed that over the range examined there was a linear relationship between ThT fluorescence and the concentration of AA fibrils. The sensitivity of the assay was at least 50 ug/ml, even in the presence of normal spleen homogenate. Guanidine-HCI reduced the ThT fluorescence to that of background levels, thus demonstrating that the fluorescence was due to a denaturable component, and not to an increased amount of light scattering as the quantity of fibril protein increased.

With the fibril enrichment procedure used in the present studies, spleen homogenates from all mice showed a ThT fluorescence intensity above background at 12h after treatment with AEF, silver nitrate, or AEF + silver nitrate (i.e. amyloid induction regimen). In the case of the AEF group this can be explained by the use of a fibril containing AEF preparation[10]. In the case of the silver nitrate treated group the ThT signal did not decrease after treating the tissue homogenate with guanidine-HCl, indicating this was not a signal from amyloid fibrils. Beyond 12 h, only the spleens from AEF + silver nitrate treated animals showed increasing ThT fluorescence. When the increasing spleen weights were factored into the results, total splcnic amyloid could be shown to be increasing over a four day period.

The finding that AA amyloid is detectable as early as 18 h in the rapid model of AA amyloid deposition, raises questions about the early temporal events thought to be involved in amyloidogenesis. The first concerns AEF, and the second concerns the relationship of basement membrane proteins to amyloid formation.

When using standard AA amyloid induction protocols such as the daily subcutaneous injection of azocasein in CBA/J mice, AEF activity is detectable 24-48 h before amyloid can be histologically detected'. This early work suggested that AEF and amyloid are separate entities. However, it has been shown that amyloid fibrils, whether obtained from tissue[10], or made *in vitro*[15], have AEF activity. The present work with ThT shows that histologically undetectable amyloid fibrils or protofibrils are actually present at least 18-24 h earlier than previously suspected. Thus, when using standard amyloid induction regimens, AEF extracted from apparently preamyloidotic spleens may contain small quantities of amyloid or amyloid protofibrils which may then function in much the same way as amyloid

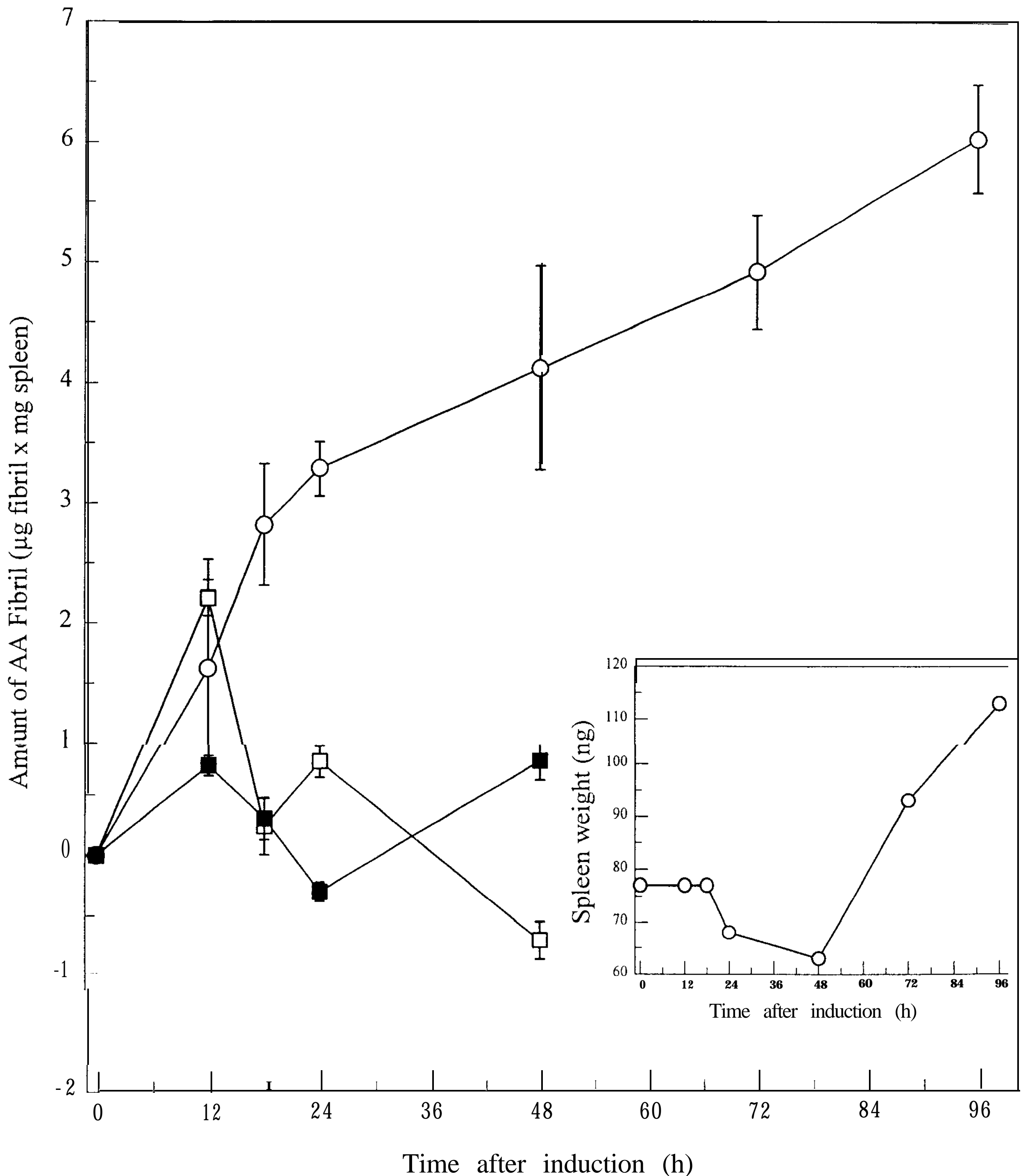


**FIGURE** 3. Time course of ThT fluorescence of spleen homogenates from amyloidotic mice after multiplication by the increase in spleen mass. Combining the increase in spleen weight with ThT fluorescence provided a measure of the total amount of amyloid accumulating overtime. The fluorescence signal measured after treatment with an equivolume of 4 M guanidine-HCl was subtracted from these values. The 12 h time point was from mice where the two injections, AEF and silver nitrate were separated by 24 h. The standard curve in Figure 1 was used to convert I482/protein] to ug AA fibrils per spleen. Silver nitrate and AEF, (open circles); silver nitrate alone, *(closed squares);* AEF alone, (open *squares).* Values are means of n=4 ± S.E.M. (silver nitrate and AEF), and means of n=3 ± S.E.M. (silver nitrate, or AEF). Inset, Change in splenic weight during amyloid induction. Values are means of n=5.

fibrils assembled *in vitro*[15]. Such a view would also be consistent with the idea that AEF functions as a nidus, or scaffold, on which further amyloid may grow in an accelerated fashion[16]. It may, however, be premature to reach this conclusion. Work with the CE/J mouse strain, one incapable of forming AA amyloid, still possesses splenic AEF activity after repetitive inflammatory stimuli[17]. This suggests either that there may be histologically undetectable amyloid deposits or protofibrils in CE/J mice, or that AEF is a separate entity.

The present data, which show the presence of AA amyloid 18-24 h earlier than previously suspected, raise an additional issue related to the potential role of altered basement membrane metabolism in amyloidogenesis. The concept that altered basement membrane protein metabolism is an early step in the process of amyloidogenesis, and one that might precede actual amyloid deposition, is based on two lines of evidence. The first is the co-accumulation of basement membrane proteins with demonstrable amyloid whether examined by immunohistochetnistry or electron microscopy[18,19]. The second is the accumulation of the relevant mRNAs in the tissues concerned before amyloid is demonstrable with CR[20,21]. Thus, the hypothesis that the heparan sulfate proteoglycan (HSPG) perlecan may be involved at an early stage in amyloidogenesis comes from observations that HSPG is deposited concomitantly with AA amyloid'", that it is part of the AA fibril as assessed by electron microscopic techniques[19], that HS influences amyloidogenic fonns of SAA to take on more B-sheet conformation[22,23] and that perlecan mRNA levels, as assessed by a reverse transcriptase polymerase chain reaction, increase before AA amyloid is detected in murine spleens[20]. This temporal relationship was deduced using CR to detect amyloid. In the light of our present results, CR is not sensitive enough to detect small, early amyloid deposits. The increases in perlecan splenic mRNA, and collagen IV mRNA[21], are clearly closely related to amyloid deposition, but which precedes the other is not discernible with existing techniques. It is possible that fibrillogenesis begins as soon as aberrant synthesis of basement membrane proteins occurs. If so, trying to resolve which event comes first may be irrelevant, as they may be inseparable from a practical point of view. Determining the precise role of these constituents of amyloid deposits awaits alternative technologies and experimental approaches.

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