Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 306-312

# FIBRILS FROM SYNTHETIC AMYLOID-RELATED PEPTIDES ENHANCE DEVELOPMENT OF EXPERIMENTAL AA-AMYLOIDOSIS IN MICE

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Received January 10, 1994

Amyloid enhancing factor is an incompletely characterized activity of extracts from many amyloid-containing tissues and which greatly shortens the preamyloidotic phase during experimental induction of AA-amyloidosis. In this communication we show that amyloid-like fibrils made *in vitro* from synthetic peptides, corresponding to segments of amyloid fibril proteins, have amyloid enhancing factor-like activity. Thus, there is a possibility that amyloid enhancing factor activity depends on small fibrils serving as nucleation centers for fibril elongation. © 1994 Academic Press, Inc.

Secondary, systemic amyloidosis (AA-amyloidosis) is not an unusual complication in many chronic inflammatory diseases, most commonly rheumatoid arthritis. The main constituent in the AA-fibril, protein AA, is derived from the apolipoprotein acute phase reactant, serum AA (SAA), by proteolytic cleavage at which a C-terminal part of the molecule is split off resulting in a 5-10 kDa fibril-forming protein.

AA-amyloidosis can also be induced experimentally in animals by induction of long-lasting inflammation, e.g. by subcutaneous injections of casein or silver nitrate (1). Experimental amyloidosis has been described to run through two phases, one lag phase and one phase of amyloid formation (2,3). The lag phase typically lasts for several weeks during which no amyloid can be detected in any organ and can be drastically shortened by intravenous injection of cells or extracts from amyloid of other animals (3-5). Many studies have been devoted to the elucidation of the nature of this/these amyloid enhancing factor(s) (AEF) but no definite explanation for the enhancement effects has been achieved.

In this communication we show that amyloid-like fibrils made from synthetic peptides corresponding to part of known amyloid fibril proteins and given intravenously, shorten the lag phase at experimental induction of AA-amyloidosis in mice.

## MATERIALS AND METHODS

**Animals.** Outbred female NMRI mice aged 8-11 weeks and weighing 20-25 g at the beginning of the experiments were obtained from ALAB AB, Södertälje, Sweden. The animals had free access to water and pellets (Type R36; Lactamin, Vadstena, Sweden).

0006-291X/94 $5.00
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**Preparation of amyloid enhancing factor.** Animals were given a single intravenous (iv.) injection of 0.1 ml (100 μg) AEF (kindly donated by Dr. R. Kisilevsky, Department of Pathology, Queens University, Kingston, Ontario, Canada), immediately followed by a subcutaneous (sc.) injection of 0.5 ml 2% $AgNO_3$ in the back. The animals were then given 0.1 ml of $AgNO_3$ twice weekly for three to four weeks, and were then sacrificed. The livers were dissected out, homogenised in 0.15 M NaCl and centrifuged at 15 000 x g for 30 minutes at 4°C. The pellet was resuspended, homogenised and centrifuged once more; this step was repeated ten times. The amyloid fibrils were then extracted with distilled water (6) and the supernatants from the second and third distilled water extractions were pooled, stored at 4°C with 0.02% sodium azide as a preservative, and used as AEF in the experiments. The fibril concentration was measured by protein determination using the Bio Rad protein assay kit (Bio-Rad laboratories, München, Germany).

***In vivo* titration of AEF.** Prior to injection, the AEF preparations were sonicated for 40 seconds. The animals were divided into seven groups with two mice in each group. The animals in the experimental groups received 0.1 ml AEF serially diluted neat - 1:64 iv. and 0.5 ml of 2% $AgNO_3$ sc. at onset of the experiment. The controls were given a sc. injection of 2% $AgNO_3$ only. All animals were then given a sc. injection of 0.1 ml $AgNO_3$ on day 3, 6, 9, and were sacrificed on day 10. Pieces from the spleen, liver and kidney were fixed in 10% buffered formalin and embedded in paraffin.

**Preparation of synthetic peptide fibrils.** Synthetic, C-terminally amidated peptides corresponding to positions 20-29 of human islet amyloid polypeptide [IAPP(20-29), amino acid sequence S-N-N-F-G-A-I-L-S-S] and position 24-35 of human transthyretin [TTR(24-35), amino acid sequence P-A-I-N-V-H-V-F-R-K] were prepared as described previously (7,8). Both peptides were dissolved in 10% acetic acid at a concentration of 10 mg/ml, left at room temperature for 24 hours and then neutralised with concentrated $NH_3$ solution (7).

**Induction of amyloidosis.** The animals were divided into seven groups with 6 to 12 mice in each group. The fibril suspensions were diluted in distilled water to a concentration of 1 mg/ml or 0.2 mg/ml. The animals in the experimental groups received an iv. injection of either 0.1 ml AEF (1.32 mg/ml), 0.1ml IAPP(20-29) fibrils (1mg/ml or 0.2 mg/ml), or 0.1 ml TTR(24-35) fibrils (1 mg/ml or 0.2 mg/ml) at the beginning of the experiment. Prior to injection, the AEF and the fibril suspensions were sonicated for 40 seconds. Controls were injected iv. with 0.1 ml of distilled water, or 10% acetic acid neutralised with concentrated $NH_3$ and diluted ten times with distilled water (vehicle). Immediately after the intravenous injection, all mice received a subcutaneous injection of 0.5 ml 1% $AgNO_3$ . The animals were then injected with 0.1 ml of 1% $AgNO_3$ on day 7 and 14. One to 3 animals from each of the experimental and control groups were sacrificed on day 2, 4, 10 and 14. Pieces of the spleen, liver, kidney and heart were fixed in 10 % buffered formalin and embedded in paraffin.

**Light microscopy.** Sections were deparaffinized and were stained with Congo red and hematoxylin (9). The sections were examined in polarised light and a green birefringence was taken as evidence of amyloid deposition. The number of deposits and the amount of amyloid was graded in the spleen as follows: - : no amyloid found; 1+ : minute amount of amyloid; 2+ : small amount of amyloid; 3+ : moderate amount of amyloid; 4+ : extensive amyloid deposits. The coded sections were examined without knowledge of treatment given.

**Immunohistochemistry.** Rabbit antiserum to mouse protein AA was obtained by subcutaneous injection of 0.3 mg purified protein AA, dissolved in 0.001 M NaOH and mixed with Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). The protein AA had been purified as previously described (10) from the liver of two amyloidotic mice. The purity of protein AA was assessed from sodium dodecyl sulphate polyacrylamide gel electrophoresis. Immunohistochemically, the antiserum reacted only with amyloid deposits in mouse tissues and the reaction was abolished after preabsorbtion with purified protein AA. Immunohistochemistry was performed on deparaffinized sections with the peroxidase-antiperoxidase method (11) using the anti protein AA antiserum diluted 1:400-1:800. The reaction was visualised with diaminobenzidine. For controls, the anti protein AA antiserum was replaced by normal rabbit serum in appropriate dilution.

**Electron microscopy.** Samples of IAPP(20-29) fibrils, TTR(24-35) fibrils and AEF were diluted with distilled water and small drops were placed on formvar-coated copper grids (200 mesh/inch). Negative staining was performed with 1% sodium phosphotungstate at pH 7.0. The samples were air dried and examined in a JEOL 1200 electron microscope at 80 kV.

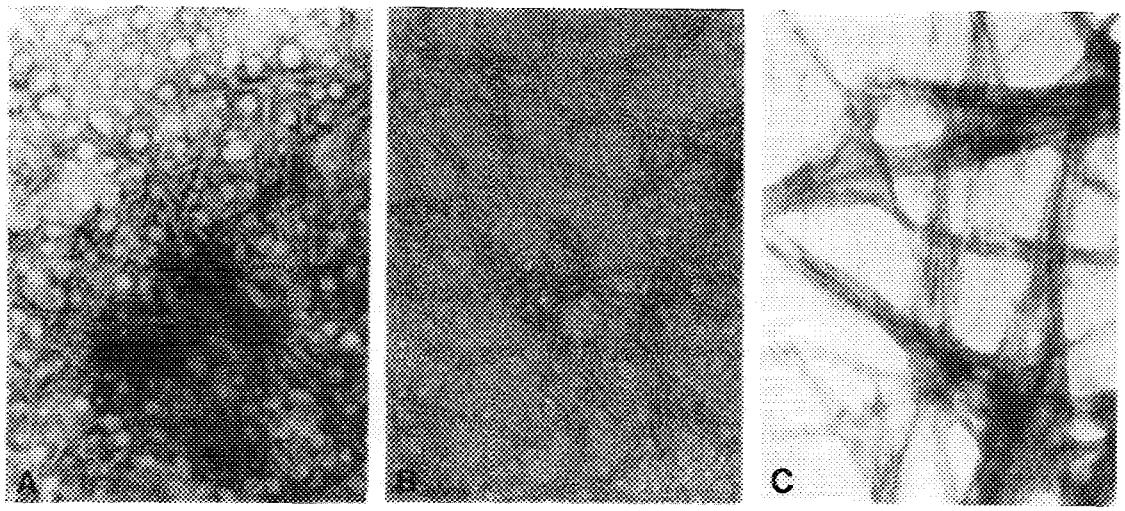


Fig.1. Electron microscopic appearance of AA-fibrils extracted from murine material (A), TTR(24-35) fibrils (B) and IAPP (20-29) fibrils (C), negatively contrasted with 1% sodium phosphotungstate at pH 7.0. x 95,000.

## RESULTS

**Fibrils.** The synthetic fibrils and the extracted murine material, used as AEF showed strong green birefringence after Congo red staining. Electron microscopically, the AA-fibrils were straight and short. The synthetic fibrils tended to be longer (Fig.1), and had the appearance described elsewhere (7,12).

**Induction of amyloidosis by AgN03.** In a first series of experiments, the time for induction of amyloidosis by subcutaneous injections of AgN03 (without AEF) was estimated. Amyloid deposits did not appear within 24 days of treatment (data not shown). From these results and the results with AgN03 combined with AEF, the experimental time span 2-16 days was chosen (Table 1).

**Effects of AEF.** In all mice treated with AEF and AgN03 amyloid deposits were detected (by positive Congo red staining) on day 2. The spleen showed perifollicular amyloid deposits. In the liver, amyloid was confined to the central vein areas. Small amyloid deposits were seen in the heart, localized in the interstitium and the vessel walls. The kidneys were without amyloid. After 10 days, amyloid deposits were seen surrounding the splenic follicles, and the liver showed amyloid deposits confined to the central vein areas, sinusoids and parenchyma. In the heart, the deposits had increased in number and size. The kidneys exhibited amyloid deposits mainly in the papillas, but also along the tubules. No deposits were seen in the glomeruli. On day 16, amyloid deposits had further increased in all examined tissues but remained in the same localisations, except for slight depositions along the glomerular capillaries. The controls injected with distilled water and AgN03 showed no amyloid deposits after 2, 4 and 10 days of treatment. At day 16, amyloid was found in the liver and spleen in one control mouse. The deposits in this mouse were similar in amount and pattern to those observed in the AEF-treated mice, sacrificed 2 days after beginning of the experiment (Table 1).

Table 1. Splenic amyloid deposits induced by treatment with $AgN0_3$ sc. and AEF iv. as compared to controls treated with $AgN0_3$ sc. and $H_20$ iv.

| No. of $AgN0_3$ inj. | Duration of experiment | AEF | | | $H_20$ | | |
|---|---|---|---|---|---|---|---|
| | | No. of mice | No. of positive mice | Grading of amyloid | No. of mice | No. of positive mice | Grading of amyloid |
| 1 | 2d | 3 | 3 | 1+ to 2+ | 3 | 0 | - |
| 1 | 4d | 3 | 3 | 2+ to 3+ | 3 | 0 | - |
| 2 | 10d | 3 | 3 | 3+ to 4+ | 3 | 0 | - |
| 3 | 16d | 3 | 3 | 3+ to 4+ | 3 | 1 | 3+ |

***In vivo* titration of AEF.** In all mice treated with serially diluted AEF and $AgN0_3$ and sacrificed on day 10, small to extensive amyloid deposits were detected perifollicularly in the spleen (Table 2). Amyloid was also seen in all livers and in the kidneys from 9/11 of these mice. There was no evident correlation between the amount of AEF injected and the amount of amyloid deposited. Furthermore, the mouse given the lowest dose of AEF, which died 3 days after beginning of the experiment, demonstrated minute amount of amyloid in both spleen and liver.

**Effects of TTR(24-35) and IAPP(20-29) fibrils.** Treatment with TTR(24-35) fibrils + $AgN0_3$ or IAPP(20-29) fibrils + $AgN0_3$ accelerated the induction of amyloidosis (Table 3). TTR(24-35) fibrils caused heavier deposits of amyloid than IAPP(20-29) fibrils. Congo red stained sections showed minute to moderate amyloid deposits, localized in the perifollicular areas of the spleen from 6/13 mice treated with TTR(24-35) fibrils, and from 3/14 mice treated with IAPP(20-29) fibrils on day 10 - 16. Furthermore, amyloid deposits confined to the central vein areas were seen in the livers of 3/13 mice given TTR(24-35) fibrils, and in 1/14 mice given IAPP(20-29) fibrils. The amount of amyloid deposited was not dependent on the dose of fibrils given (Table 4). Amyloid deposits were neither found in the kidney nor in the heart of mice given TTR(24-35) or IAPP(20-29) fibrils. The controls treated with vehicle and 1-3 injections of $AgN0_3$ showed no amyloid deposits.

**Immunohistochemistry.** The Congo red amyloid deposits in tissues of mice treated with TTR(24-35) fibrils + $AgN0_3$ or IAPP(20-29) fibrils + $AgN0_3$, were shown by immunostaining with anti-AA antiserum to contain AA amyloid (Fig. 2). No staining was detected in the preparations when the anti-AA antiserum was replaced by normal rabbit serum.

Table 2. Splenic amyloid deposits on day 10 as induced by treatment with $AgN0_3$ sc. and serially diluted AEF iv.

| AEF dilution | Duration of experiment | No. of mice | No. of positive mice | Grading of amyloid |
|---|---|---|---|---|
| 1:1 | 0.13 | 1[a] | 1 | 3+ |
| 1:2 | 0.065 | 2 | 2 | 3+ to 4+ |
| 1:4 | 0.033 | 1[b] | 1 | 3+ |
| 1:8 | 0.016 | 2 | 2 | 2+ to 4+ |
| 1:16 | 0.008 | 2 | 2 | 3+ to 4+ |
| 1:32 | 0.004 | 2 | 2 | 3+ |
| 1:64 | 0.002 | 1[c] | 1 | 3+ |

a one mouse died on day 6, AA deposits grad 2+.
b one mouse died on day 6, AA deposits grad 3+.
c one mouse died on day 3, AA deposits grad 1+.

Table 3. Splenic amyloid deposits induced by treatment with $AgN0_3$ sc. and TTR(24-35) fibrils or IAPP(20-29) fibrils iv. as compared to controls treated with $AgN0_3$ sc. and vehicle iv.

| No. of $AgN0_3$ inj. | Duration of experiment | TTR | | | IAPP | | | Vehicle | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of mice | No. of positive mice | Grading of amyloid | No. of mice | No. of positive mice | Grading of amyloid | No. of mice | No. of positive mice | Grading of amyloid |
| 1 | 2d | 1[a] | 0 | - | 3[b] | 0 | - | 2[b] | 0 | - |
| 1 | 4d | 4 | 0 | - | 4 | 0 | - | 2[b] | 0 | - |
| 2 | 10d | 4 | 3 | 1+ to 3+ | 4 | 2 | 1+ to 2+ | 2[b] | 0 | - |
| 3 | 16d | 4 | 3 | 2+ to 3+ | 4 | 1 | 1+ | 3 | 0 | - |

a 3 mice died at the beginning of the experiment.
b 1 mouse died at the beginning of the experiment.

## DISCUSSION

The research on AEF goes back to the 1960's when it was shown that splenic cells from pre-amyloidotic mice rapidly induced amyloidosis (13) and it was suggested that an "Amyloid Enhancing Factor" responsible for the initiation of amyloid formation exists. It was further shown that AEF is not species-specific since homogenates of spleens from humans with primary, myeloma associated and secondary amyloidosis have the same activity (14). Extensive research on AEF has demonstrated that its activity can be recovered in various fractions at attempts to purify a distinctive component (15,16), and that AEF is closely associated with the AA-amyloid fibril (16). The mechanism through which AEF produces its dramatic effect has remained unclear, however.

Our findings can lend support to one of many proposed theories about the nature of AEF, namely that something in AEF serves as a nucleus around which fibril growth takes place (3,17,18). The nature of this nucleus has been discussed (3,18) but our study indicates that fibrils themselves can have this property without further constituents. A similar mechanism has been demonstrated *in vitro* with amyloidogenic peptides of the scrapie prion protein (A-Pr) (19) and the amyloid ß-protein A-ß (20). It has been shown in these *in vitro* studies that fibrillogenesis is strongly speeded up by seeding the peptide solutions with a small amount of preformed fibrils. It should be noted that TTR(24-35) and IAPP(20-29) fibrils used in our study were not as efficient as native murine AA-fibrils. The reason is not known but it is possible that a chemical identity between seed and amyloidogenic protein is a prerequisite for optimal fibrillogenic conditions. Although the studies do not give a definite solution of the AEF problem, defined synthetic fibrils offer a means for further studies of amyloidogenesis.

Table 4. Splenic amyloid depositis in mice treated with $AgN0_3$ sc. and different doses of synthetic fibrils iv., sacrificed on day 10 and 16

| Synthetic fibrils | Dose of synthetic fibrils (mg/mouse) | No. of mice | No. of positive mice | Grading of amyloid |
|---|---|---|---|---|
| TTR 24-35 | 0.1 | 4 | 2 | 1+ to 3+ |
| TTR 24-35 | 0.02 | 4 | 4 | 1+ to 4+ |
| IAPP 20-29 | 0.1 | 4 | 2 | 1+ to 2+ |
| IAPP 20-29 | 0.02 | 4 | 1 | 1+ |

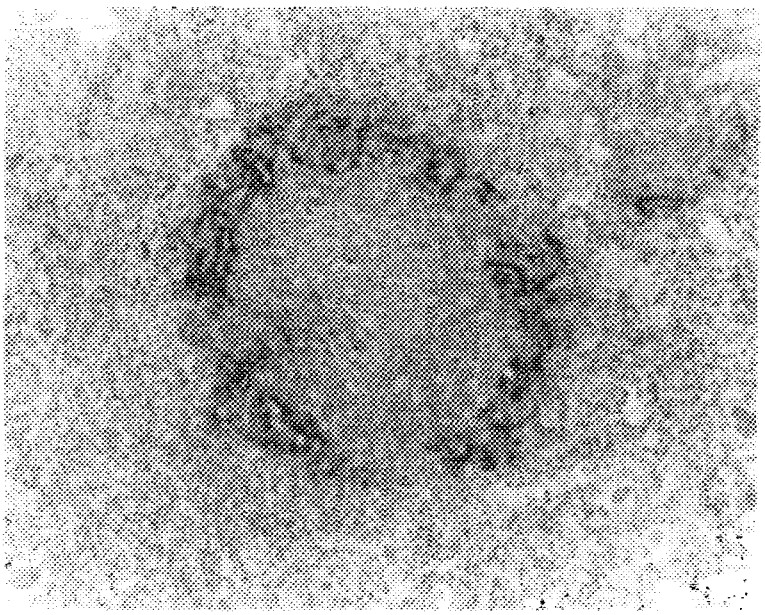

Fig.2. Section of spleen of a mouse with amyloidosis, induced with silver nitrate and TTR(24-35) fibrils as enhancing factor and immunolabelled with antiserum to murine protein AA. Amyloid is seen perifollicularly. X 120.

**Acknowledgments.** Supported by the Swedish Medical Research Council. Thanks are due to Christer Bergman for skilled assistance.

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