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Amyloid enhancing factor activity is associated with ubiquitin

Kamel Alizadeh-Khiavi, Josée Normand, Soula Chronopoulos, Ambereen Ali, and Zafer Ali-Khan

Department of Microbiology and Immunology, McGill University, 3775 University Street, Lyman Duff Medical Building, Montreal, Quebec H3A 2B4, Canada

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Summary. Crude amyloid enhancing factor (AEF) drastically reduces the pre-amyloid phase on passive transfer and induces amyloid deposition in the recipient mice in 48–120 h. We attempted to purify AEF from murine amyloidotic liver and spleen extracts by using gel filtration, preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis and ion exchange chromatography and isolated a 5.5 kDa peptide. In the mouse bioassay, this peptide induced accelerated splenic AA deposition in a dose-dependent manner. Based on structural, electrophoretic and immunochemical criteria the peptide was identified as ubiquitin. A polyclonal rabbit anti-bovine ubiquitin IgG antibody (RABU) abolished the in vivo AEF activity of crude murine AEF in a dose-dependent manner. Monomeric ubiquitin and its large molecular weight adducts were isolated from crude AEF using cyanogen bromide-activated sepharose conjugated to RABU and size exclusion chromatography methods. These were assayed and were found to possess AEF activity. Furthermore, increased levels of ubiquitin, a phenomenon similar to that of AEF, were detected by immunocytochemistry in mouse peritoneal leucocytes prior to and during amyloid deposition. Since AEF shares a number of biological and functional properties with ubiquitin, we suggest a possible role of ubiquitin as an AEF, and that serum amyloid protein A and ubiquitin, the two reactants generated during inflammatory stress conditions, may converge to induce AA amyloid deposition

Key words: Amyloid enhancing factor – Ubiquitin – Amino acid sequencing – Anti-ubiquitin antibody – Immunocytochemistry

Introduction

The term amyloidosis describes a heterogeneous collection of systemic diseases characterized by the extracellular deposition of β-pleated amyloid fibrils of great chemical diversity in various organs and tissues (Glenner 1980). Structural studies on purified amyloid proteins indicate at least 11 different chemical forms of human amyloids. Proteolytic cleavage and release of amyloidogenic fragments from larger amyloid precursor proteins are believed to occur prior to amyloidogenesis in most forms of amyloid (Stone 1990). The pathophysiology of this process is, however, not well understood. Hall et al. (1960) proposed stress as the cardinal amyloid-inducing factor in non-amyloidogen-induced (reactive) amyloidosis in mice. Intermittent electrical shock, continuous fighting or overcrowding, all stress-related conditions, were shown to affect the incidence of amyloidosis in mice and Peking ducks (Cowan and Johnson 1970; Hall et al. 1960; Page and Glenner 1972). However, in the absence of a definitive pathophysiological entity to confirm amyloidosis as a possible sequel of stress, these earlier findings have generally remained unnoticed.

In the mouse, one of three major apoprotein isotypes of serum A protein (SAA), SAA_2, has been shown to be the precursor protein of AA amyloid (Hoffman et al. 1984). Sustained high SAA levels during the pre-amyloid phase in conjunction with amyloid enhancing factor (AEF), is believed to predispose mice to amyloid deposition during the second stage of the disease (Axelrad et al. 1982; Kisilevsky 1983; Sipe et al. 1978). Recent studies indicate a causative role for AEF in amyloidogenesis (Abankwa and Ali-Khan 1988; Ali-Khan et al. 1988; Alizadeh-Khiavi and Ali-Khan 1988; Hol et al. 1985; Niewold et al. 1987; Shirahama et al. 1990a; Varga et al. 1986). The mechanism(s) by which AEF promotes accelerated amyloidogenesis is unclear, although its formation is believed to be genetically linked (Gervais et al. 1988). Attempts to purify and chemically identify AEF until now have been unsuccessful. Based on the homogeneous preparations of mouse AEF and its amino acid sequence analysis, as well as the results from immunochemical and passive transfer experiments using anti-bovine ubiquitin IgG antibody, we present evidence that AEF activity lies within ubiquitin (UB). Subsequent to

Offprint requests to: Z. Ali-Khan

the present discovery, we extended this work to Alzheimer-brain-derived AEF (Ali-Khan et al. 1988); it was purified, sequenced, biologically characterized and was also found to be UB (Alizadeh-Khiavi and Ali-Khan 1990; Alizadeh-Khiavi et al. 1991).

Materials and methods

Male C57BL/6 mice (Charles River, Montreal, Canada), 6–8 weeks old, were infected intraperitoneally (i.p.) with 250 alveolar hydatid cysts (AHC); these mice develop multi-organ amyloid (AA type) deposits 1 week post-infection (Du and Ali-Khan 1990). Mice were killed at 8 weeks post-infection (p.i.) and their spleens and livers were homogenized in cold 100 mM sodium phosphate buffer containing 150 mM sodium chloride, pH 7.4 (PBS; 1 g tissue/10 ml buffer; 3 min, Tekmar Tissuemizer, Ohio, USA). The tissue suspensions was stirred (60 min; 4° C), centrifuged (30000 *g*, 60 min, 4° C) and the supernatant used as crude AEF.

Protein concentrations were determined using the Bio-Rad protein assay kit and bovine serum albumin as the standard.

The identification of low-molecular-weight AEF was done after adjusting the pH of the crude AEF to 5 with 1 M phosphoric acid. The turbid suspension was stirred (45 min, 4° C), centrifuged (30000 *g*, 60 min, 4° C) the supernatant dialysed against distilled water (Spectrapor; molecular weight cut-off 3.5 kDa; 4° C), concentrated against polyethylene glycol (PEG) and lyophilized. The lyophilized sample (300 mg) was dissolved in 3 ml of 25 mM TRIS-hydrochloride, pH 8.0 and applied to a Sephacryl S-200 HR column (1.5 × 85 cm; flow rate 12 ml/h) pre-equilibrated with the above buffer. As indicated (Fig. 1), the F1 to F4 fractions were collected, concentrated against PEG, dialysed against PBS, then assayed (0.2 mg protein/mouse) for AEF activity. The activity co-fractionated with F1 and the small F2 fractions (Fig. 1). In 15% polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970; see below), the F1 demonstrated numerous protein bands, and F2 had five prominent protein bands. Since previous studies had indicated that AEF might be a small-molecular-weight protein (Abankwa and Ali-Khan 1988; Niewold et al. 1987), we chose F2 for electro-elution and AEF bioassay. F2 (2 mg protein) was mixed with 400 µl of 2 × Laemmli's sample buffer and, instead of boiling, which destroys AEF activity (Axelrad and Kisilevsky 1980; also Ali-Khan et al. unpublished), the sample was incubated for 2 h at 37° C, electrophoresed and the gel stained. The Coomassie-stained bands from F2 were excised and electro-eluted (Model 22, Electro-Eluter, Bio Rad, Toronto, Canada) using 50 mM ammonium acetate containing 0.1% SDS. The fractions EP-AEF-a to EP-AEF-c (Fig. 1) were exhaustively dialysed (Spectrapore, molecular weight cut-off 3.5 kDa) against PBS. The protein concentration of the Coomassie-stain-tinged electro-eluted EP-AEF-a to c fractions was adjusted to approximately 100 µg/ml with PBS at 595 nm before determining their AEF potency.

Since EP-AEF-a was the only AEF-positive band in the F2 fraction, it was submitted to repetitive Edman degradation in an AB1 model 470 peptide microsequencer, and the phenylthiohydantoin (PTH)-derivatives of the released amino acids were analysed, on-line with AB1 model 120A PTH-amino acid analyser (Centre for Human Genetics, Shriner's Hospital, Montreal, Canada).

Large scale purification of UB from the crude AEF was carried out following the methods of Matsumoto et al. (1984) with minor modification as described (Alizadeh-Khiavi et al. 1991).

The mouse bioassay for determination of AEF activity was carried out as described (Ali-Khan et al. 1988; Axelrad et al. 1982). Briefly, crude AEF or its various fractions, with or without 10 mM calcium chloride, were injected i.p. to mice. The inflammatory stimulus consisted of either four daily subcutaneous (s.c.) injections of 0.5 ml of 1% silver nitrate (Ali-Khan et al. 1988) or one s.c. injection with 0.5 ml of 2% silver nitrate (Axelrad et al. 1982). Mice were sacrificed 24 h after the last 1% silver nitrate injection and 72 h after the 2% silver nitrate injection. The spleens were sectioned (8 µm), stained with alkaline Congo red and amyloid deposition was graded between ± to 3+ depending upon the approximate splenic perifollicular circumferential area covered with amyloid: ±less than 10%; 1+, 10–25%; 2+, 25–50%; 3+, 50–100% (Ali-Khan et al. 1988). Several controls were used in these experiments: groups of mice received i.p. injection of either crude AEF or murine-tissue-derived monomeric UB only, PBS with 10 mM calcium chloride i.p. and one (2%) or four (1%) s.c. injections with silver nitrate; bovine serum albumin (10 mg/mouse, i.p.), human haemoglobin (10 mg/mouse, i.p.) or azocasein (100 µg/

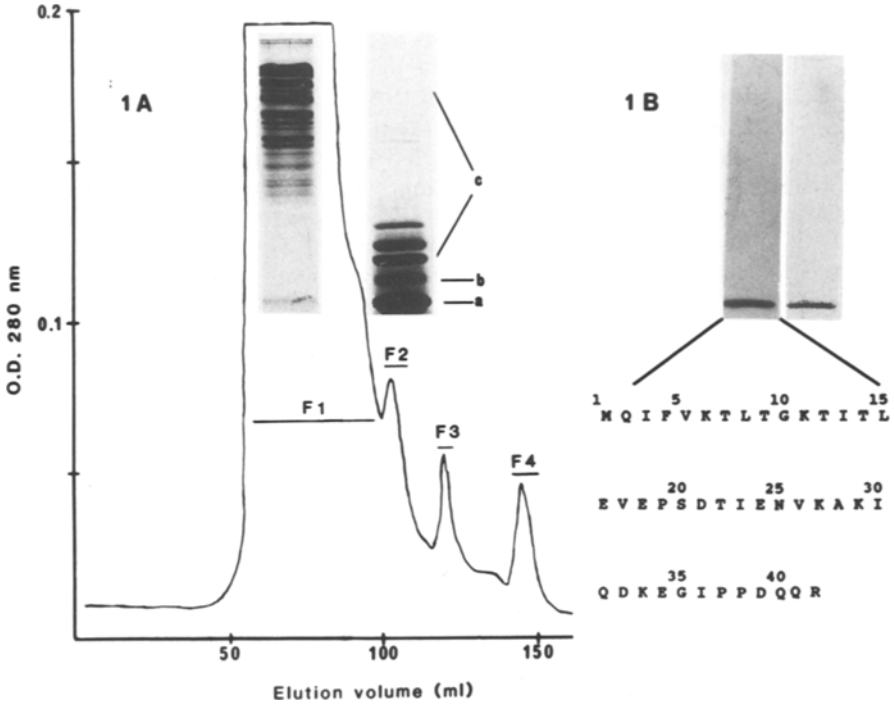

Fig. 1. A Sephacryl S-200 HR chromatography of the pH 5 supernatant obtained from amyloidotic murine liver and spleen extracts; the *insets* represent Coomassie-stained 15% SDS-polyacrylamide gel pattern of peptides in the F1 and F2 fractions (15 µg). **B** Homogeneous 5.5 kDa amyloid enhancing factor (AEF)-positive peptide (*left*) (5 µg) similar to the EP-AEF-a peptide in the F2 fraction, and Western blot analysis of the same peptide using anti-bovine ubiquitin (UB) IgG as the primary antibody; partial amino acid sequence of the 5.5 kDa AEF-positive peptide is shown below

mouse, intravenously) and four daily s.c. injections with 0.5 ml of 1% silver nitrate. Spleen sections from the control mice were processed as described.

The resulting AEF fractions in 5–15 μg aliquots were subjected to SDS-PAGE. Western immunoblotting was performed using rabbit anti-bovine UB IgG antibody (RABU), and goat anti-rabbit IgG conjugated to alkaline phosphatase (Alizadeh-Khiavi et al. 1991). RABU was generated and purified as described by Haas and Bright (1985). In some experiments, parallel immunoblots were prepared using another anti-UB antibody, kindly provided by Dr. A.L. Hass (Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin, USA).

In the immunoaffinity purification of UB and its adducts, crude AEF (2 ml; total protein 8 mg) was incubated (overnight, 4° C) with 7 ml of cyanogen bromide-activated sepharose 4B (Pharmacia, Montreal, Canada) conjugated to 75 mg RABU. After exhaustive washing with 10 mM phosphate buffer, pH 7.4 containing 0.5 M sodium chloride, bound proteins were eluted with 0.1 M glycine-hydrochloride buffer pH 2.8 containing 0.5 M sodium chloride. The pH of the eluate was neutralized immediately with 1.7 M TRIS-hydrochloride. After dialysis against PBS the eluted material was concentrated against PEG and bioassayed for AEF. Since only the eluate from RABU-bound gel contained AEF activity, it was fractionated on a sephacryl S-200 HR column (1.5 × 85 cm; flow rate 10 ml/h) pre-equilibrated with PBS and the two resulting fractions (P1 and P2) were analysed by SDS-PAGE, immunoblotting, and the AEF assay. The specificity of the immunoaffinity purification method was confirmed by incubating crude AEF with 1 M ethanolamine-treated cyanogen bromide-activated sepharose gel in similar proportions as above.

The neutralization of AEF activity in crude AEF by RABU was examined in groups of C57BL/6 mice that received i.p. differing doses of RABU (0.4, 2.0, 5.0 and 10.0 mg/mouse), or 5.0 mg/mouse normal rabbit IgG. After 24 h, mice were challenged i.p. with crude AEF (0.2 mg) and one s.c. injection with 0.5 ml of 2% silver nitrate. In another neutralization experiment crude AEF (0.2 mg) was incubated (overnight, 4° C) with 0.1, 1.0, 2.0 and 4.0 mg RABU or 4 mg normal rabbit IgG; these samples were also assayed for AEF activity.

For indirect immunofluorescent staining (IFA) peritoneal cells were harvested from protease-peptone-treated (2 ml of 10% solution/mouse, i.p.) mice after 72 h or AHC-infected mice at days 1, 3, 6 and 4 weeks p.i. cytocentrifuged onto glass slides and treated with 1:20 dilution of RABU (15 μg protein/ml) and goat anti-rabbit IgG Fab_2-conjugated to fluorescein isothiocyanate (1:40) as described (Chronopoulos et al. 1991 b). Specificity of the staining reaction was determined by exposing the cells to the pre-immune rabbit serum (1:5), or RABU absorbed with bovine UB (5 mg UB/0.2 mg of RABU; overnight; 4° C), and the conjugate. At least 200 leucocytes from duplicate samples were examined under 40 × or oil immersion objective; nuclear morphology was used for leucocyte identification.

In the determination of SAA groups of C57BL/6 mice (3/group) received either silver nitrate (one s.c. injection of 0.5 ml of 1% silver nitrate) only, UB (one i.p. injection, 10 mg/mouse, dissolved in PBS with added 10 mM calcium chloride) or crude AEF (one i.p. injection, 0.2 mg/mouse). Sera were collected after 16 h and assayed for SAA concentrations by enzyme linked immunosorbent assay as described (Zuckerman and Suprenant 1986; Chronopoulos et al. 1991 b).

Results

The crude AEF (0.1 mg/mouse) with or without 10 mM calcium chloride showed AEF activity, although addition of calcium chloride increased significantly the potency of AEF (Table 1). AEF without the silver nitrate stimulus proved to be non-amyloidogenic (Table 1). Following acidification and gel filtration, crude AEF resolved into four fractions (F1 to F4; Fig. 1). The fractions were assayed for AEF activity (0.2 mg protein/mouse). F1 showed potent AEF activity (80–100% of the splenic follicles had 2+ to 3+ AA), marginal AEF activity was present in F2 (1 to 2% of the spleen follicles had ± to 1+ AA) and none in F3 and F4 fractions. Addition of 10 mM calcium chloride and 10 mM dithiothreitol (DTT) to F2 to F4 significantly enhanced the AEF potency of F2 only (50–60% of the spleen follicles had 1+ to 2+ AA); the F3 and F4 fractions remained negative for AEF activity.

In SDS-PAGE, F1 demonstrated at least 18 prominent proteins bands of molecular weights greater than 21.5 kDa (protein standard, soybean trypsin inhibitor) (Fig. 1, inset). F2 (Fig. 1, inset), which was also positive for AEF, contained 5 protein bands of molecular weights under 31.5 kDa (protein standard, bovine carbonic anhydrase). As explained in the Materials and methods section, we chose F2 for further analysis. The Coomassie-stained F2 peptides, as indicated in Fig. 1 (inset, EP-AEF-a to EP-AEF-c), were electro-eluted and

Table 1. Amyloid enhancing factor (AEF) activity of crude AEF and the electro-eluted peptides (EP-AEF-a, EP-AEF-b, EP-AEF-c) obtained from AEF-positive sephacryl S-200 HR chromatography F2 fraction (see Fig. 1)

Group	Crude AEF or electroluted peptide (EP)	Diluent	Number and % of $AgNO_3$ injection	Mice sacrificed	Mice positive for AA/mice examined	% of splenic follicles positive for AA	Grading of AA
1	Crude AEF (0.1 mg)	PBS[c]	1; 2%	72 h	5/6	37.1 ± 12.06	± to 1+
2	Crude AEF (0.1 mg)	PBS, Ca^{2+}	1; 2%	72 h	6/6	93.8 ± 7.055	1+ to 3+
3	Crude AEF (0.1 mg)	PBS, Ca^{2+}	–	72 h	0/4	–	–
4	EP-AEF-a	PBS, Ca^{2+}, DTT[b]	4; 1%	120 h	2/3	88.5 ± 16.2	2+ to 3+
5	EP-AEF-a	PBS, Ca^{2+}, DTT	–	120 h	0/3	–	–
6	EP-AEF-b	PBS, Ca^{2+}, DTT	4; 1%	120 h	0/4	–	–
7	EP-AEF-c	PBS, Ca^{2+}, DTT	4; 1%	120 h	0/2[a]	–	–

[a] One mouse died
[b] Containing 10 mM $CaCl_2$ and 10 mM dithiothreitol (DTT)
[c] Phosphate buffer saline, pH 7.4

F2 fraction was electrophoresed and the electro-eluted peptides (approximately 100 μg protein/mouse) along with crude AEF were assayed for AEF activity. Mice received subcutaneously 0.5 ml of either one injection of 2% or four injections of 1% silver nitrate

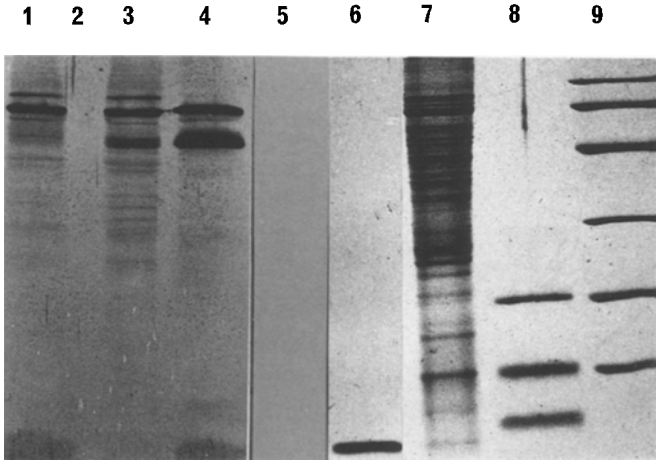

Fig. 2. Coomassie-stained 15% SDS-polyacrylamide gel profile of crude AEF (18 μg, *lane 7*), UB purified from this extract (5 μg, *lane 6*); and low (3.5 μg, *lane 8*) and high (7 μg, *lane 9*) molecular weight markers; *lanes 1–4* represent the immunoblots using anti-bovine UB IgG antibody (RABU) or RABU absorbed with bovine UB (*lane 5*); *lane 1*, crude AEF (18 μg); *lane 2*, eluate from the control 1 M ethanolamine-treated cyanogen bromide-activated sepharose gel incubated overnight with crude AEF; *lane 3* (10 μg), flow through and 0.1 M glycine-hydrochloride, pH 2.8 containing 0.5 M sodium chloride eluate (10 μg, *lane 4*) obtained from cyanogen bromide-activated sepharose gel conjugated to RABU and incubated with crude AEF; *lane 5* (10 μg) same as in lane 4; *lane 8*, low-molecular-weight markers (Combitek; Boehringer, Mannheim, FRG) from top to bottom: soybean trypsin inhibitor 21.5 kDa, cytochrome C 12.5 kDa, aprotinin 6.5 kDa; *lane 9*, high-molecular-weight markers (Bio-Rad) from top to bottom: phosphorylase B 97.4 kDa, bovine serum albumin 66.2 kDa, ovalbumin 42.69 kDa, bovine carbonic anhydrase 31 kDa, soybean trypsin inhibitor 21.5 kDa, egg lysozyme 14.4 kDa

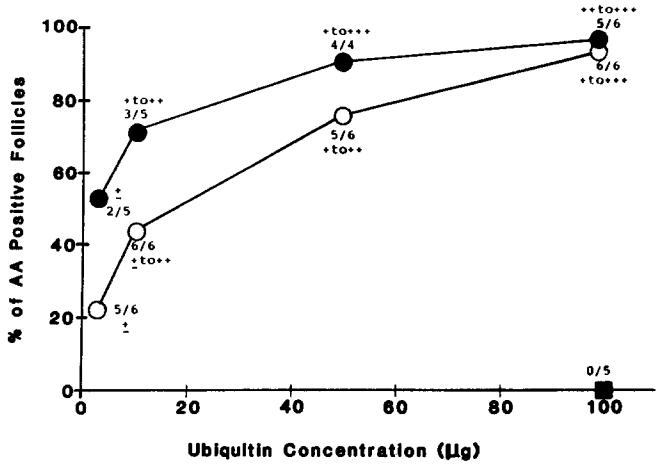

Fig. 3. Dose-dependent AEF activity of murine-tissue-derived UB in 100 mM PBS, pH 7.4 with 10 mM calcium chloride. Groups of four received i.p. 3, 10, 50 and 100 μg UB in conjunction with either one s.c. injection of 0.5 ml of 2% silver nitrate (*open circle*) or from daily s.c. injections of 0.5 ml of 1% silver nitrate (*closed circle*). Mice in control group (*filled square*) received i.p. 100 μg UB only, with no silver nitrate injections, and were sacrificed at 120 h. Mice in the 2% and 1% silver nitrate groups were sacrificed at 48 h or at 24 h after the last silver nitrate respectively and their Congo-red-stained spleen sections graded for amyloid deposits (see Materials and methods)

bioassayed in approximately 100 μg dosage per mouse with added calcium chloride and DTT as stabilizing agents for AEF (Ali-Khan et al., unpublished). Only EP-AEF-a demonstrated AEF activity; EP-AEF-a without the silver nitrate stimuli was negative (Table 1). Thus the AEF activity detected in EP-AEF-a appeared to be specific. Since the F1 fraction (Fig. 1) demonstrated strong AEF activity, the possibility existed that other large-molecular-weight proteins might also possess AEF activity (see below). Subsequent to these initial studies with the electro-eluted fractions, DTT was deleted from the AEF assay. DTT alone does not enhance the AEF activity, whereas addition of calcium chloride does (Alizadeh-Khiavi et al. 1991; compare groups 1 and 2 in Table 1).

EP-AEF-a, which on SDS-PAGE appeared to be a homogeneous peptide, was subjected to 42 cycles of amino-terminal amino acid sequencing and the resulting sequence was found to be identical to that of mammalian UB (Fig. 1B) (Rechsteiner 1989). In Western immunoblotting EP-AEF-a co-migrated with bovine UB, and both the murine and bovine UB samples immunoreacted with RABU (Fig. 1B). RABU absorbed with bovine UB, as shown previously (Alizadeh-Khiavi et al. 1991), did not react with these UB samples.

The ethanol-chloroform method of Matsumoto et al. (1984) as described previously (Alizadeh-Khiavi et al. 1991) was used for the large-scale purification of UB from the crude AEF (Fig. 2, lane 7). The estimated yield of monomeric UB was less than 0.1% of the total protein present in the starting crude AEF. It migrated as a homogeneous 5.5 kDa peptide (Fig. 2; lane 6), immunoreacted with RABU similar to that of EP-AEF-a (Fig. 1B) and showed AEF activity in the mouse bioassay (Fig. 3) in a concentration-dependent fashion (3, 10, 50 and 100 μg/mouse). Both the amount of AA (± to 3+) and the percentage of AA-positive follicles corresponded with the increasing UB dosages. Commercially available bovine UB (Sigma St. Louis, Mo., USA) and calf-thymus-derived UB (Calbiochem, La Jolla, Calif., USA), both purified by the heat-treatment method, as indicated in the catologues, were also assayed for AEF activity in 0.1, 1.0 and 10.0 mg/mouse dosages in conjunction with 4 s.c. injections of 1% silver nitrate. In PBS, none of these bovine UB dosages showed AEF activity, but with added 10 mM calcium chloride, 1 of 3 mice with 0.1 mg, 7 of 11 mice with 1.0 mg and 2 of 3 mice with 10 mg dosages demonstrated ±1 to 1+ grades of splenic AA. Calf-thymus-derived UB was totally negative for AEF activity. Heat treatment is known to abrogate AEF activity (Axelrad and Kisilevsky 1980; Axelrad and Kisilevsky 1980; Axelrad et al. 1982; also unpublished observations); this may explain the residual AEF activity observed in one of the UB samples.

The crude AEF on immunoblotting with RABU demonstrated a weak 5.5 kDa band and at least five major and several minor large-molecular-weight UB immunoreactive bands (>40 kDa) (Fig. 2, lane 1). These finding are in accord with previous reports; both mono-

Table 2. Results from control experiments in which mice were injected with ubiquitin (UB) alone, four daily subcutaneous (s.c.) injections with 1% or 2% silver nitrate (0.5 ml) alone or bovine serum albumin, human haemoglobin or azocasein in conjunction with four daily s.c. injections with 0.5 ml of 1% silver nitrate

Group	Treatment (number)	% and (number) of $AgNO_3$ injections	Mice sacrificed	Mice (+)ve for AA/ mice examined	% AA (+)ve follicles
1	Murine UB (50 µg; i.p.)	None	120 h	0/12	–
2	PBS, Ca^{2+} (10 mM)	1 (4)	120 h	0/8	–
3	PBS, Ca^{2+} (10 mM)	2 (4)	120 h	0/8	–
4	Bovine serum albumin (10 mg; i.p.)	1 (4)	120 h	0/4	–
5	Human haemoglobin (10 mg; i.p.)	1 (4)	120 h	0/4	–
6	Azocasein (100 µg, i.v.)	1 (4)	120 h	0/8	–

Table 3. AEF activity of fractions obtained from cyanogen bromide-activated sepharose gel conjugated with anti-UB IgG antibody and incubated with crude AEF (flow through and 0.1 M glycine-hydrochloride, pH 2.8 eluted fraction)

Group	Immuno-affinity purified AEF	Diluent	Number and % of $AgNO_3$ injection	Mice sacrificed	Number of mice (+)ve for AA/ number examined	% of splenic follicles (+)ve for AA	Grading of AA
1	Immuno-affinity eluted UB and adducts (0.1 mg; i.p.)	PBS, Ca^{2+} [b]	1; 2%	48 h	4/6	54.1±32.3	1+ to 3+
2	Immuno-affinity eluted UB and adducts (0.1 mg; i.p.)	PBS, Ca^{2+}	–	48 h	0/4	–	–
3	Adducts (P1) separated by gel filtration after affinity elution						
	10 µg; i.p.	PBS, Ca^{2+}	1; 2%	48 h	2/6	14.4±10.2	± to 1+
	100 µg; i.p.	PBS, Ca^{2+}	1; 2%	48 h	6/6	77.5±15.6	1+ to 2+
4	UB (P2) separated by gel filtration after affinity elution						
	10 µg; i.p.	PBS, Ca^{2+}	1; 2%	48 h	3/5[a]	50.5±11.5	± to 1+
	100 µg; i.p.	PBS, Ca^{2+}	1; 2%	48 h	5/5	82.8±22.5	1+ to 3+

[a] One mouse died
[b] Phosphate buffer saline containing 10 mM $CaCl_2$

The eluted fraction was fractionated on a sephacryl S-200 HR gel column to obtain P1 and P2 fractions (for details see Materials and methods). These fractions were injected intraperitoneally (i.p.) in conjunction with 0.5 ml of 2% silver nitrate s.c.

meric UB and UB adducts are generally present in cell lysates (Hass and Bright 1985; Rechsteiner 1989). As also demonstrated previously (Alizadeh-Khiavi et al. 1991; Chronopoulos et al. 1991 b) the absorbed RABU failed to react with UB (Fig. 2, lane 5).

To ascertain whether UB adducts possessed AEF activity, crude AEF was passed through immunoaffinity and gel filtration columns (see Materials and methods). On immunoblotting, both the flow through (Fig. 2, lane 3) and the eluted (Fig. 2, lane 4) fractions from the immunoaffinity column demonstrated several UB adducts of similar molecular weights, but monomeric UB was present only in the eluted fraction (Fig. 2; lane 4). Excessive amounts of UB adducts present in the crude AEF, beyond the binding capacity of the immunoaffinity column, may explain the presence of UB adducts in the flow through fraction. In contrast, the 1 M ethanolamine-blocked sepharose gel lacking the ligand failed to bind to proteins present in a crude AEF (Fig. 2, lane 2). This suggests that the binding of monomeric UB and UB adducts to the immunoaffinity gel was selective and specific (Fig. 2, compare lanes 2 and 4). The affinity-purified fraction was further separated using sephacryl S-200 gel into two fractions, P1 (void volume) and P2 (retarded, fraction), and immunoblotted (Fig. 4). P1 contained at least two major and two minor adducts, while P2 apparently contained only the monomeric UB (Fig. 4). Both these fractions demonstrated AEF activity in a dose-dependent manner (Table 3), indicating that UB adducts also contained AEF activity.

Since the F1 fraction (Fig. 1, also see text), had demonstrated potent AEF activity, the question thus remained whether any other protein in crude AEF besides UB (including the adducts might possess AEF activity.

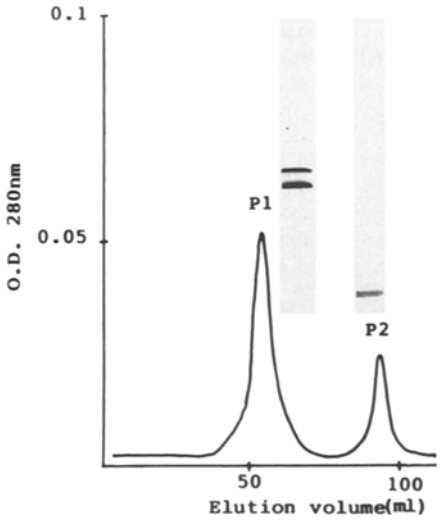

Fig. 4. Sephacryl S-200 HR chromotography profile of 0.1 M glycine, pH 2.8 containing 0.5 M sodium chloride eluate obtained from cyanogen bromide activated sepharose gel conjugated to RABU and incubated overnight with crude AEF. After thorough washing with 10 mM phosphate buffer pH 7.4 containing 0.5 M sodium chloride, glycine buffer was applied to elute the bound protein, which was concentrated, fractionated into P1 and P2 and immunoblotted, using RABU. Two major and two minor large molecular weight UB adducts were seen in P1 and a single peptide, apparently monomeric UB of 5.5 kDa in P2

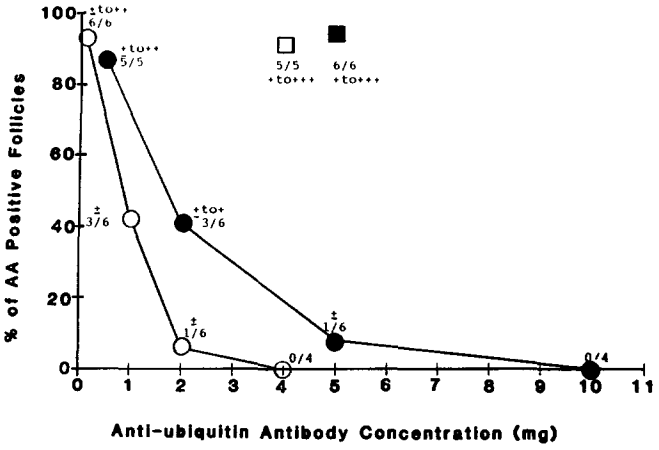

Fig. 5. Effect of RABU on the AEF activity of crude AEF: groups of C57BL/6 mice received i.p. various amounts of RABU (*filled circles*) or 5 mg normal rabbit IgG (*filled square*), 24 h before the i.p. challenge with crude AEF (0.2 mg protein). In the second experiment, groups of mice received i.p. crude AEF (0.2 mg protein) pre-incubated overnight with various amounts of RABU (*open circle*) or with 4 mg normal rabbit IgG (*open square*). Mice in each group received one s.c. injection with 0.5 ml of 2% silver nitrate and 48 h after their Congo-red-stained spleen sections were graded for AA deposits

Results in Fig. 5 show that RABU, whether passively transferred 24 h prior to the challenge with crude AEF or injected after overnight incubation with crude AEF, completely abolished the biological activity of crude AEF in a dose-dependent manner. The optimal concentration of RABU required for complete abolition of

Table 4. Serum amyloid A protein (SAA) response in groups of C57BL/6 mice, 16 h after the administration of crude AEF, UB, bacterial lipopolysaccaride (LPS), aqueous silver nitrate or phosphate buffered saline (PBS)

Groups	Reagent	Amount injected/ mouse	Number/ route of injection	Mean SAA concentration (μg/ml)
1	1% $AgNO_3$	0.5 ml	1/s.c.	536.6 ± 38
2	Bacterial LPS	50 μg	1/i.p.	584 ± 129.7
3	UB	10 mg	1/i.p.	2.3 ± 0.52
4	Crude AEF	0.2 mg	1/i.p.	1.9 ± 0.21
5	PBS	0.5 ml	1/i.p.	<1.0

AEF activity was approximately two-fold higher in the antibody passive transfer experimental group than in that of overnight incubation with RABU. Normal rabbit IgG did not affect AEF activity in the crude AEF. These results further confirm that AEF activity lies within UB and its adducts.

To investigate the pattern of UB response, cytocentrifuged peritoneal cells from control protease-peptone-treated and AHC-infected mice were reacted with RABU and analysed by IFA. Almost all the mature and immature (inset) polymorphonuclear leucocytes (PMN) between day 1 to 4 weeks p.i. contained UB-immunoreactive granules (Fig. 6D); in some PMN the entire cytoplasm appeared as a homogeneous RABU-positive fluorescent mass. The percentage of UB-positive macrophages increased from 38% at day 1 to 54% at 4 weeks p.i. (Fig. 6A). The majority (87%) of protease-peptone-stimulated macrophages contained only nuclear UB-immunoreactive granules; the remaining cells had both nuclear and cytoplasmic granules (Fig. 6B). PMN, although rare in such preparations, showed dull and diffuse cytoplasmic UB immunoreactivity. The immunostaining for UB was specific; neither the pre-immune rabbit serum nor RABU absorbed with bovine UB immunoreacted with the activated leucocytes (Fig. 6C).

Since neither crude AEF nor UB alone, when administered in amyloidogenic dosages, induce amyloidogenesis in mice (Tables 1, 2), we investigated their potential in the induction of SAA. Neither crude AEF, nor UB or PBS induced any significant serum SAA elevations at 16 h (Table 4). In contrast, SAA elevation was higher than 500-fold above the basal level, in the lipopolysaccharide or silver nitrate stimulated mice (Table 4).

Discussion

The data presented provide evidence that UB, purified from murine amyloidotic tissues, possesses AEF activity. This claim is based on the partial amino acid homology between murine-tissue-derived homogeneous AEF and UB (Figs. 1, 2); their identical electrophoretic, immunochemical and in vivo pathophysiological properties (Fig. 1; Tables 1, 3); immunoaffinity purification of AEF-positive monomeric UB and its large-molecular-weight adducts from crude AEF (Figs. 2, 4; Table 3);

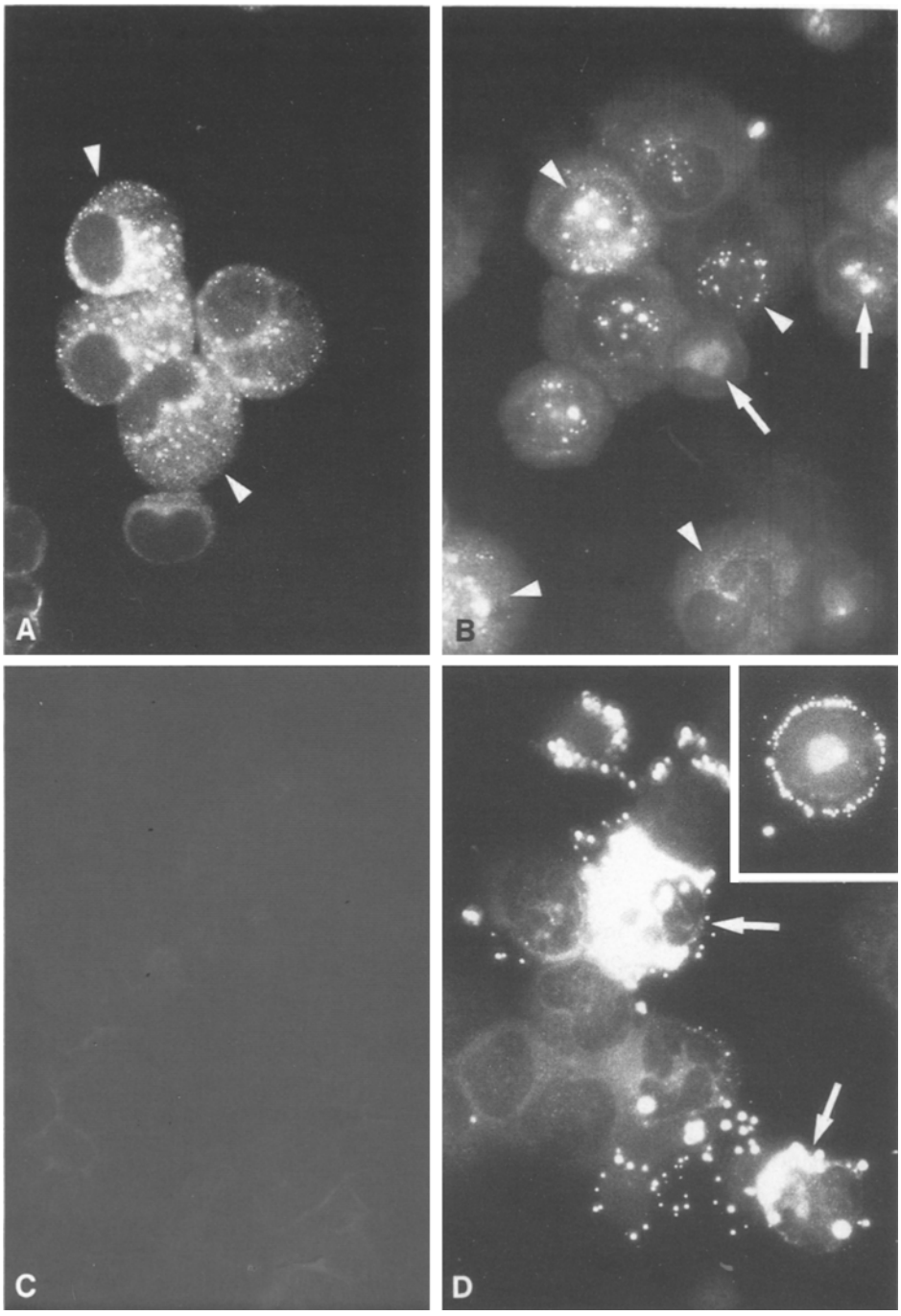

Fig. 6. Ethanol fixed cytocentrifuged peritoneal cells from proteose-peptone-stimulated (1 ml of 10% solution, i.p.) **B** or 250 alveolar hydatid cyst-infected **A**, **C**, **D** mice, stained with RABU **A**, **B**, **D** or RABU absorbed with bovine UB **C** and fluoresceinated goat anti-rabbit Fab_2 antibody. Note the nuclear and cytoplasmic fluorescent granules (FG) in macrophages (*arrowheads*) and neutrophils (*arrows*) obtained 72 h after proteose-peptone stimulation **B** or a 1 day **B** and 4 weeks **C**, **D** post-infection; also note an immature granulocyte in **D** (*inset*) with a doughnut-shaped nucleus and peripherally cytoplasmic FG and abolition of immunoreactivity **C** using RABU absorbed with bovine UB. ×1000

and the abolition of AEF activity in crude AEF by RABU (Fig. 5). These findings are in accord with our previous report on Alzheimer-brain-derived UB (Alizadeh-Khiavi and Ali-Khan 1990; Alizadeh-Khiavi et al. 1991). As shown in Table 2, regardless of whether the control mice were given 4 daily s.c. injections of 0.5 ml of 1 or 2% silver nitrate only or human haemoglobin, bovine serum albumin or azocasein in conjunction with 1% silver nitrate, splenic AA deposition did not occur in the test mice. These results show unambiguously that UB, purified by the ethanol-chloroform method (Matsumoto et al. 1984) and unexposed to heat treatment, contains potent AEF activity.

We next addressed an important question regarding the presumed heterogeneity of AEF: should there be one or more than one species of AEF? Previous studies, using size exclusion chromatography, predicted AEF to be a heterogeneous moiety ranging in molecular weight from 12 to more than 100 kDa (Ali-Khan et al. 1988; Alizadeh-Khiavi and Ali-Khan 1988; Axelrad et al. 1982; Niewold et al. 1987; Shirahama et al. 1990b; Yokota et al. 1989). This question was partly resolved by

immunoblotting and affinity purification methods (Figs. 2, 4). In addition, the affinity-purified large-molecular-weight UB adducts and monomeric UB tested positive for AEF activity (Fig. 4; Table 3). In another approach, in a functional assay similar to that used for blocking the protease-like activity of UB (Fried et al. 1987), RABU in a dose-dependent manner abolished AEF activity in the crude AEF (Fig. 5). In addition, both AEF and UB appear to share a number of common features. AEF is known to bind to particulates (Axelrad et al. 1982), AA microfibrils (Niewold et al. 1987) and nucleoproteins (Hardt and Hellung-Larsen 1972). Interestingly, UB also binds to murine AA (Chronopoulos et al. 1991a, b), Alzheimer neurofibrillary tangles, various fibrillar inclusion bodies in diverse neurodegenerative diseases (Mayer et al. 1991) and forms metabolically stable conjugates with histones and a variety of short-lived intracellular regulatory proteins (Haas and Bright 1985; Rechsteiner 1989). Taken together these results are at variance with the concept of AEF heterogeneity. If it were, the blocking effect of RABU against the crude AEF would have been partial. Thus, we suggest that the reported large-molecular-weight AEF may be the UB adducts. Additional supportive immunochemical and amino acid sequencing data will be required to resolve whether other large molecular proteins (Axelrad et al. 1982; Shirahama et al. 1990b; Yokota et al. 1989) besides UB adducts possess AEF activity.

PMN, macrophages and reticuloendothelial (RE) cells have been implicated in the formation of amyloid (Fuks and Zucker-Franklin 1985; Glenner 1980; Kisilevsky et al. 1977). These cells, during inflammatory stress conditions, contain high levels of UB (Fig. 6; also Chronopoulos et al. 1991b), or AEF (Abankwa and Ali-Khan 1988; Alizadeh-Khiavi and Ali-Khan 1988; Shirahama et al. 1990a). Recently, AEF-loaded macrophages were associated with the processing of SAA to AA (Shirahama et al. 1990a). Although, SAA is actively sequestered by UB-loaded murine PMN, macrophages and splenic RE cells (Chronopoulos et al. 1991b; Fig. 6) or other inflammatory leucocytes (Rosenthal and Sullivan 1979; Silverman et al. 1980) and both SAA (Meek et al. 1989) and UB (Alizadeh-Khiavi et al. 1991; Chronopoulos et al. 1991b) co-localize in relatively high concentrations during the pre-amyloidotic phase in the splenic perifollicular areas, we do not yet have a direct evidence whether SAA and UB interact with each other in a physiological sense. How can these findings be interpreted in the light that the splenic perifollicular area is an important site of SAA clearance and a major site of AA deposition (Kisilevsky 1983; Meek et al. 1989)? Given that UB has AEF activity, it binds to murine AA (Chronopoulos et al. 1991a) and both leucocytes and RE cells become "loaded" with UB prior to and during amyloidogenesis, a circumstance analogous to that of AEF-loaded macrophages (Shirahama et al. 1990a), it is reasonable to propose that UB may play a crucial role in amyloidogenesis. In effect, a physiological role has been suggested for UB bound to neurofibrillary tangles, and fibrillar inclusion bodies (Grundke-Iqbal et al. 1989; Ivy et al. 1989; Mayer et al. 1991). During conditions of cellular stress or as shown in the AHC-infected mice (Fig. 6; Chronopoulos et al. 1991b), the expression of UB increases significantly (Mayer et al. 1991; Rechsteiner 1989). Specificity of UB to bind and to proteolyse particularly short-lived proteins or structurally abnormal proteins has been well established (Rechsteiner 1989). Nonetheless, the precise role of UB in the biogenesis of ubiquitinated fibrillar inclusions or murine AA is unclear at this time, although evidence that UB may act as a protease is provocative (Fried et al. 1987). Besides UB, several other host components such as elastase (Skinner et al. 1986), α_1-antichymotrypsin (Abraham et al. 1988), amyloid P component (Axelrad et al. 1982) and sulphated glycoaminoglycans (Snow et al. 1987) also bind to amyloid. As yet none of these are known to possess AEF activity, although they have been implicated in amyloidogenesis.

In light of the current data, the concept of "fibril-AEF" (Niewold et al. 1987), which on passive transfer into mice behaves like AEF, is of considerable interest. It was postulated that fibril-AEF may act as nucleants in vivo in accelerating their own production. The ubiquitinated AA fibrils (Chronopoulos et al. 1991a, b) seem to correspond to such "nucleants", although phenylmethyl sulphonyl fluoride treatment of fibril-AEF, unlike that of crude AEF (Abankawa and Ali-Khan 1988; Alizadeh-Khiavi and Ali-Khan 1988; Ali-Khan et al. 1988) does not seem to abrogate its amyloidogenic properly (Niewold et al. 1991). The reason for this discrepancy is unclear.

Of further interest is the stimulatory role of calcium in UB-mediated amyloidogenesis (Table 1; Fig. 3) or proteolytic activity (Fried et al. 1987). High concentrations of calcium are found in AA amyloid deposits (Kula et al. 1977) and all the known SAA and AA sequences (McCubbin et al. 1988; Yamamoto and Migita 1985) contain a common tetrapeptide sequence $Gly^{48}-Pro^{49}-Gly^{50}-Gly^{51}$ which is homologous to the main calcium-binding site, $Gly^{30}-Xaa^{31}-Gly^{32}-Gly^{33}$, of bovine phospholipase A2 (Turnell et al. 1986). Recent studies on mouse SAA_1 and SAA_2 suggest that both SAA species bind calcium (McCubbin et al. 1988) and both UB and SAA are deposited in the SPA (Chronopoulos et al. 1991a, b). Thus it is likely that the added calcium (Table 1; Fig. 3) may play a role in amyloidogenesis when UB, requiring calcium for its catalytic/proteolytic activity (Fried et al. 1987) becomes sequestered in the extracellular matrix with a calcium-binding precursor protein of amyloid.

To summarize, the present study brings into focus the following: first the convergence, during acute inflammation, of two stress-related phenomena – the acute phase response and stress response; and second, the involvement of their respective expressed reactants, SAA and UB, in the induction of amyloidosis. Induction of stress response (i.e. increased level of UB; Fig. 6) during an acute phase response had not been recognized previously (Perlmutter 1988). Together they add a new dimension to the possible role of stress (Cowan and John-

son 1970; Hall et al. 1960; Page and Glenner 1972), that is to say a role for UB in AA amyloidogenesis. How the sentinel-like role of cytosolic UB is altered appears to be essential to the understanding of the pathogenesis of amyloidosis.

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