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Alveolar hydatid cyst induced amyloid enhancing factor (AEF): physicochemical properties and abolition of AEF activity by serine protease inhibitors

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Summary. Cell free supernatants prepared from amyloidotic liver, unfractionated and fractionated peritoneal and spleen cells from casein stimulated (16 h post-injection) or alveolar hydatid cyst-infected (7 or 12 weeks post-infection, p. i.) C57BL/6J mice were used to assess amyloid enhancing factor (AEF) activity and to determine its cell-source and physicochemical properties. Of the various supernatants used, the plastic adherent spleen cell lysate (95% macrophages) from 7 weeks p.i mice showed greater AEF activity, on a cell to cell basis, than the lysates prepared from whole spleen cells, peritoneal exudate cells or nonadherent (96% lymphocytes) spleen cells. Culture supernatants obtained from Con A, LPS or the parasite antigen stimulated amyloidotic spleen cells but not the normal mouse spleen cells contained AEF activity; the supernatant from unstimulated amyloidotic spleen cells was negative for AEF activity. AEF was precipitated with 25% and 50% saturation with $(NH_4)_2SO_4$ and after gel-filtration the low molecular weight fraction contained the AEF activity which on SDS-PAGE resolved into three peptides ranging between mol. wts 15 000 and 31 000. Of the various specific and nonspecific protease inhibitors tested, AEF activity was completely abolished by 30 min preincubation with 10 mM phenylmethylsulphonyl fluoride. Taken together, these results indicate that AEF may be a small molecular weight lysosomal neutral protease of neutrophil/macrophage origin.

Keywords: alveolar hydatid cyst, amyloid enhancing factor, macrophage, phenylmethylsulphonyl fluoride, gel filtration, SDS-PAGE

AA-type of amyloidosis can be induced experimentally in mice and other species by chronic inflammatory stimulation with casein, azocasein, silver nitrate (Cohen & Shirahama 1972; Kisilevsky 1983) or infection with protozoan or metazoan parasites (Andrade & Rocha 1979; Ali-Khan *et al.* 1983; Gonzalez *et al.* 1986; Alkarmi *et al.* 1986). The induction period varies from 5 days to several weeks depending on the amyloidogen employed. Just prior to, and during amyloid deposition, amyloid enhancing factor (AEF) appears in amyloidotic organs (Hardt 1971; Keizman *et al.* 1972;

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Axelrad *et al.* 1982; Kisilevsky 1983) and in the peripheral circulation (Janigan & Druet 1968; Flinn *et al.* 1986). AEF has the ability to reduce the amyloid induction period to 36–48 h, when administered in conjunction with an inflammatory stimulus. Spleen is the primary source of AEF, although it can be extracted from liver and other tissues (Axelrad *et al.* 1982; Kisilevsky 1983).

Efforts to characterize AEF have yielded a variety of conflicting results (Ranlov 1967; Cohen & Shirahama 1972; Keizman *et al.* 1972; Kedar *et al.* 1975; Hol *et al.* 1985; Varga *et al.* 1986). Neither the cell of origin nor its chemical nature has been unequivocally identified; polymorphonuclear leucocytes (PMNs), lymphocytes and reticuloendothelial cells have been implicated in its synthesis (Kedar & Ravid 1980; Kisilevsky 1983). AEF is believed to be a glycoprotein of high molecular weight although both low and high molecular weight AEF containing fractions have been shown to impart amyloid enhancing activity *in vivo* (Keizman *et al.* 1972; Kedar *et al.* 1975; Axelrad *et al.* 1982; Hol *et al.* 1985). Earlier in-vitro studies have shown that membrane-bound or secreted serine proteases from monocytoid cells and polymorphonuclear cells can degrade serum amyloid A protein (SAA) into an AA-like fragment (Skogen *et al.* 1980; Lavie *et al.* 1978; 1980; Silverman *et al.* 1982). Cells from normal hosts were able to degrade SAA completely, whereas those from amyloidotic individuals produced an abnormal degradation product similar to AA protein (Fuks & Zuker-Franklin 1985; Lavie *et al.* 1978). It is not known if the enzymes involved are related to AEF (Abankwa & Ali-Khan 1987).

In this study we have attempted to characterize the AEF generated during alveolar hydatidosis-induced amyloidogenesis (Alkarmi 1985; Abankwa & Ali-Khan 1987). The amyloidogenic activity of serum, spleen cell culture supernatants, splenic interstitial fluid, spleen cell subpopulations and inflammatory exudate cells from normal and alveolar hydatid cyst-infected amyloidotic mice was tested. The effect of a number of enzyme inhibitors on AEF activity was examined, and the factor was partially purified by ammonium sulphate precipitation and gel filtration techniques. The results are discussed in relation to the biochemical nature of alveolar hydatid cyst-induced AEF and complete blockage of its amyloidogenic activity by serine protease inhibitors. To our knowledge, this is the first time that AEF activity has been associated with neutral proteases.

Materials and methods

Mice. Six- to 16-week old male and female C57BL/6J (Jackson Laboratories, Bar Harbor, ME, USA) were used in these studies.

Infection. Animals were infected intraperitoneally with 100 ± 10 alveolar hydatid cysts (AHCs) according to the methods described previously (Ali-Khan 1978; Abankwa & Ali-Khan 1987).

Inflammatory stimulus. One of three amyloidogens was used: (1) 0.11 M aqueous $AgNO_3$ (Fisher Scientific Co., Montreal, Quebec); (2) 10% casein (Hammarsten quality, BDH Chemicals, Montreal, Quebec), w/v, in 2.5% $NaHCO_3$; (3) 10% casein/Complete Freund's adjuvant (Casein-CFA, 1:1, Difco Labs , Detroit MI). This last, in conjunction with AEF, most effectively induced amyloid deposition in the perifollicular areas of the spleen in 48 h and was used in all experiments except the initial cell lysate studies.

Buffers and tissue culture media. A phosphate buffered saline (PBS), 0.01 M containing 0.15 M NaCl, pH 7.4, was used in the preparation of AEF. PBS buffered at other pH activities, (pH 7·0, 7·5 and 8·0) was used to determine the effect of pH on AEF activity and its tendency to aggregate after freezing at −20°C (Abanakwa & Ali-Khan 1987). A modified PBS (PBS-mod), containing 0.5M NaCl, 0.01M NaN_3, pH 7.1 was used for the preparation a and dialysis of AEF precipitated

by $(NH_4)_2SO_4$, (25% and 50% saturation) as well as for gel column chromatography.

Eagles' minimal essential medium, pH 7.4 (MEM), or Hank's balanced salts solution, pH 7.4 (HBSS), was used for dissociation of spleens. MEM supplemented with 300 mg/l L-glutamine, 10 mM HEPES buffer, streptomycin (100 mg/ml) and penicillin (100 u/ml) and 0.1% bovine serum albumin (MEM -BSA) was used for spleen cell culture and separation of plastic adherent cells.

AEF preparation. Spleens from AHC-infected mice became amyloidotic at 6 weeks post-infection. AHC-infected amyloidotic and normal mice were killed and bled. Serum was separated and stored at −20° until used. Spleens and livers were used for the extraction of AEF as described previously (Abankwa & Ali-Khan 1987).

Preparation of peritoneal exudate cells (PECs). The peritoneal washings from AHC-infected amyloidotic mice (Devouge & Ali-Khan 1983; Treves & Ali-Khan 1984), or mice that had received a single 0.5 ml intraperitoneal injection of 10% casein 16 h previously, were collected (Tables 1 & 2). Peritoneal cavities were washed with HBSS containing 20 u/ml of heparin. Cells were washed twice in HBSS by centrifugation at 250 *g* for 15 min at 4°C, counted and resuspended in HBSS. Differential cell count was carried out on cyto-centrifuged cell preparations. Both Giemsa and nonspecific esterase staining were used for enumeration and identification of various cell types (Treves & Ali-Khan 1983; 1984; 1984a).

Interstial fluid and separation of spleen cells. Normal, or AHC-infected amyloidotic mice, 7 weeks p.i., were killed and spleens were aseptically removed, minced in cold MEM and single cell suspensions were prepared. The first spleen cell washing was harvested by centrifugation of the cells for 10 min at 250 *g* and stored at −20°C. The cell pellet was resuspended in MEM, washed twice by centrifugation and the cells were enumerated and their viability assessed by trypan blue exclusion. Cell preparations having a 95% or greater viability were used. The spleen cell suspension (7×10^6/ml) was layered over Ficoll-Paque (Pharmacia, Mon-

Table 1. Differential cell count (%) of peritoneal exudate cells (PECs), whole spleen cells or spleen cell subpopulations. Lysates from these cells were used for assessment of amyloid enhancing activity

	Percentage of leucocytes*		
Cell source	Lymphocytes†	Monocytoid cells‡	Neutrophils§
16 h casein-induced PECs	3.7	2.5	93.8
12 week p.i. PECs	13.3	9.4	77.3
7 week p.i. whole spleen cells	80.6	6.3	11.8
7 week p.i. adherent spleen cells	2.8	95.2	2.0
7 week p.i. non-adherent spleen cells	96.0	3.1	0.9

* Percentage counted from 300 Giemsa-stained, or α-naphthyl acetate esterase positive cells.

† Includes plasmacytoid, blast cells and lymphocytes.

‡ Includes macrophages and monocytes.

§ Includes both mature and immature neutrophils.

treal, Quebec) at a ratio of 4:3 in 12.5 ml polystyrene tubes, and centrifuged at 400 *g* for 35 min at 20°C. The mononuclear cells were removed from the interface, washed three times with MEM, resuspended in 10 ml of MEM-FCS, counted and their number adjusted to 10×10^6/ml. The cell suspension was incubated in plastic petri dishes (6 cm, 5×10^6 cells/dish; Falcon, Oxnard, CA) for 2 h at 37°C in a CO_2 incubator (5%, in air). The non-adherent cells were vigorously washed with warm (37°C) MEM-BSA and the adherent cells dislodged using a rubber policeman. Enumeration and identification of granulocytes, lymphocytes and macrophages in the unfractionated and fractionated spleen cell preparations were determined as described above. Using morphological and esterase positivity criteria, over 95 % of the adherent cells were identified as macrophages.

Preparation of cell lysate. Plastic adherent (mainly macrophages) and non -adherent cells (mainly lymphocytes) (Table 1) were lysed by repeated freezing at −20°C followed by thawing at 25°C. The preparations were then sonicated for 2 min on an ice bath and centrifuged at 27000 *g* for 1 h at 4°C. The supernatants were stored at −20°C until used.

Spleen cell culture supernatants. Single spleen cell suspensions from 7 weeks p.i. amyloidotic or normal mice, prepared as described above, were resuspended at a concentration of 50×10^6/ml in MEM-BSA under sterile

Table 2. Assessment of amyloid enhancing properties of cell free supernatants prepared from casein-stimulated or alveolar hydatid cyst (AHC) infected mouse peritoneal cells and unfractionated and fractionated spleen cells; peritoneal cells were obtained at 16 h and 12 weeks p.i. after injection of 0.5 ml of 10% casein or 100 ± 10 AHC, respectively. Spleen cells were obtained from normal mice and 7 weeks p.i. amyloidotic mice

Cell source	Inflammatory agent	Lysed cell supernatant* injected/mouse (equivalent to)	Incidence of amyloid
12 week p.i. PECs	$AgNO_3$	10^7	D
	$AgNO_3$	2×10^7	D
	$AgNO_3$	10^8	D
	$AgNO_3$	2×10^8	D
	$AgNO_3$	4×10^8	D
	casein	5×10^7	D
	casein	10^8	D
	casein	2×10^8	D
	casein/CFA	10^8	1/2†
16 h casein-induced PECs	$AgNO_3$	10^7	D
	$Hg\ NO_3$	5×10^7	D
	casein/CFA	5×10^7	D
7 weeks p.i. whole spleen cells	casein/CFA	10^7	3/3
7 weeks p.i. adherent spleen cells	casein/CFA	10^6	2/3
7 weeks p.i. non-adherent spleen cells	casein/CFA	8×10^6	1/3
	casein/CFA	4×10^7	3/3
Normal mouse spleen cells	casein/CFA	4×10^7	0/3

Test mice died within 12 h.
* Cell lysates adjusted to the number of cells.
† Number of mice positive for amyloid/number of mice tested.

conditions. Six hundred million cells were incubated in 250 ml tissue culture flasks at 37°C in a humidified atmosphere (5% CO_2 in air) with either: (a) Concanavalin A (Con A, 4 μg/ml, Pharmacia, Montreal, Quebec), (b) *Salmonella enteriditis* lipopolysaccharide (LPS, 25 μg/ml, Difco Labs, Detroit, MI), (c) AHC metabolic antigen (AHC-MET-Ag, 50 μg/ml, graciously supplied by Christiane Maroun, Department of Microbiology and Immunology, McGill University), or (d) medium alone. After 24 h, the cell cultures were vigorously shaken in order to dislodge weakly adherent cells. The supernatant was harvested by centrifugation for 15 min at 350 *g*. The non-adherent pelleted cells were washed twice by centrifugation (350 *g*) in 20 ml MEM, enumerated and their viability assessed by trypan blue exclusion. Approximately 64% of the cells were viable after 24 h. The supernatants were stored at −20°C until used.

The AHC-MET-Ag was prepared in HBSS by mincing solid-tumour-like AHCs with a pair of sharp scissors 1g/15 ml). The AHC-suspension was transfered to a urine jar and allowed to sediment for 45 min. The turbid supernatant was centrifuged at 5000 *g* for 30 min. The inflammatory cell- and cyst-free clear supernatant was placed in a boiling water bath for 60 min, centrifuged at 27 000 *g* for 60 min, concentrated (20-fold) in a dialysis bag (mol. wt 3500 cut off, Spectrum Medical Industries Inc., Long Beach, CA) against dry Carbowax PEG 20 000 (Fisher Scientific Co., Montreal, Quebec) and finally filtered through a Milipore filter (0·22 μm pore size). This antigen preparation gave four strong precipitin bands in Ouchterlony test against sera obtained from AHC-infected mice at 12 weeks p.i (unpublished data).

Ammonium sulphate precipitation. Seven grams of ultrapure $(NH_4)_2SO_4$ was slowly added to 50 ml AEF-containing liver extract at 25°C to make a 25% saturated solution (Dixon 1953). The pH was adjusted to 7.2 with 5% NH_4 OH. The lightly turbid solution

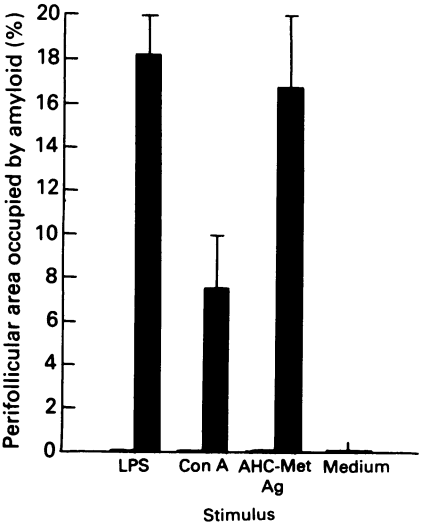

Fig. 1. Amyloid enhancing factor activity in normal mouse and amyloidotic alveolar hydatid cyst infected mouse spleen cell culture supernatants following 24 h incubation at 37°C (5% CO_2 in air) with lipopolysaccharide (LPS, 25 μg/ml), concanavalin A (Con A, 4 μg/ml) and metabolic alveolar hydatid cyst antigen (AHC-Met-Ag, 50 μg/ml) and medium alone. 0.5 ml of the supernatants were administered in mice in conjunction with 0.5 ml of a subcutaneous injection of 10% casein-complete Freunds adjuvant (1 : 1, v/v) and the degree of splenic perifollicular obliteration by amyloid was determined by computer assisted image analysis. ▦, Normal; ■, amyloidotic.

was stirred for 45 min at 4°C, centrifuged at 4000 *g* for 25 min and the resultant supernatnat was saturated to 50% by gradual addition of 7.7 g $(NH_4)_2SO_4$, while maintaining its pH at 7.2. This solution was processed as above to obtain the 50% pellet; the supernatant was dialysed and concentrated to assess residual AEF activity. Each pellet was homogenized in 25% or 50% saturated $(NH_4)_2SO_4$ solution, as appropriate, centrifuged and the resultant pellets were dissolved in 25 ml of PBS-mod. These were then dialysed extensively in dialysis tubes (mol. wt 3500 cut off) at 4°C against several changes of the same buffer.

Gel chromatography. Twenty millilitres of the 25%, and 8 ml of the 50% AEF-containing

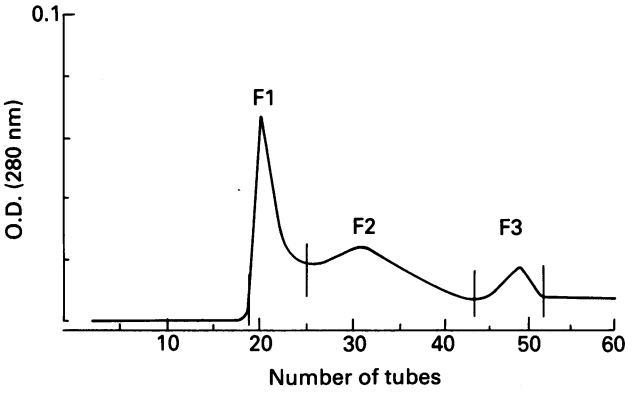

Fig. 2. Gel chromatography of a concentrated mixture of 25% and 50% ammonium sulphate precipitated alveolar hydatid cyst induced amyloid enhancing factor on Sephacryl S-300 column. Sample volume: 4 ml; Column dimensions: 2.5 × 88 cm; Buffer: 0.01 M phosphate buffer, 0.5 M NaCl, 0.01 M NaN_3, pH 7.1: flow rate: 11 ml/h; Tube volume; 3.65 ml.

solubilized $(NH_4)_2SO_4$ precipitates (pretested for AEF activity) were pooled and concentrated (see below) to 16 ml. Four samples, averaging 3.2 ml (total volume 13 ml), were chromatographed on a 2.5 × 88 cm Sephacryl S-300 gel column (Pharmacia, Montreal, Quebec) equilibrated with PBS-mod. The three separated peaks (F1, F2 and F3) were pooled, concentrated to 16, 16 and 19.5ml, respectively, and tested for AEF activity (Fig. 2). F3, which contained AEF activity, was subsequently fractionated on a 1.5 × 93 cm Sephadex, G-50 gel (superfine) column (Pharmacia, Montreal, Quebec), and the two resulting peaks (F1 and F2) were concentrated to 3 ml and 9 ml, respectively, and tested for AEF activity (Fig. 3). In a parallel experiment the crude AEF prepared in PBS-Mod was fractionated on a 1.5 × 93 cm Sephadex G-50 (superfine) gel column equilibrated with PBS-mod (Fig. 4). The sample resolved into four fractions. They were con-

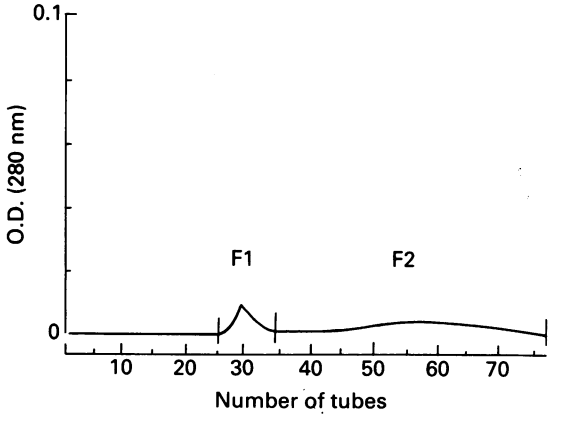

Fig. 3. Gel chromatography of F3 (Fig. 2) on a Sephadex G-50 superfine gel column. Sample volume: 2 ml concentrated F3 (from Sephacryl S-300); Column dimensions: 1.5 × 93 cm; Buffer: 0.01 M phosphate buffer, 0.5 M NaCl, 0.01 M NaN_3, pH 7.1; Flow rate: 7.35 ml/h; Tube volume: 2.45 ml.

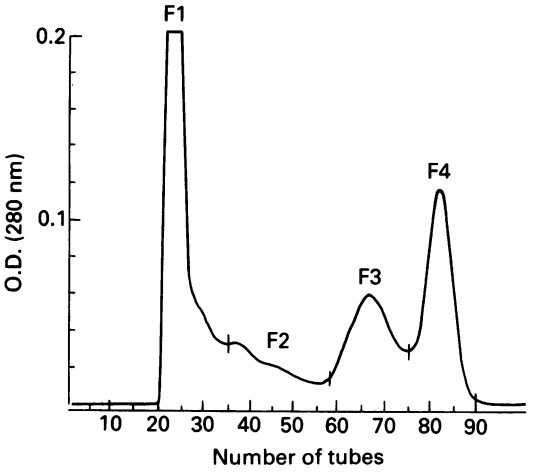

Fig. 4. Gel chromatography of crude alveolar hydatid cyst induced amyloid enhancing factor on a Sephadex G-50 superfine gel column. Sample volume: 2.6 ml; Column dimensions: 1.5 × 93 cm; Buffer: 0.01 M phosphate buffer, 0.5 M NaCl, 0.01 M NaN_3, pH 7.1; Flow rate: 6.5 ml/h; Tube volume: 2.2 ml.

centrated against PEG or Rexyn 1-300 (Biorad, Montreal Quebec), dialysed against PBS- mod for 24 h at 4°C and assayed for AEF activity. The precipitate formed during concentration or after dialysis was removed by a 1 h, 27 000 *g* centrifugation at 4°C.

Protein determination. Protein content was determined using the BIO-RAD protein assay kit.

Preparation of samples for SDS-PAGE. Concentrated chromatographed fractions were precipitated with 3 volumes of absolute ethanol at −20°C for 48 to 72 h. The samples were centrifuged (400 *g*, 10 min), the supernatant discarded and the 'pellet' air-dried before boiling in a water bath (5 min) in 50 μl Laemmli's sample buffer. Gels were prepared as per Laemmli (1970); the stacking and resolving gels contained 4% and 12% polyacrylamide, respectively. The gels were stained with 0·125% Coomassie Blue. Molecular weight standards (mol. wts 14 000 to 92 500) were run concurrently for molecular weight estimation.

Chemicals and enzyme inhibitors. Ethylenediaminetetraacetic acid (EDTA), dithiothreitol, iodoacetic acid, aprotinin, and α_1 anti-trypsin were obtained from Sigma Chemical Co., (St Louis, MO, USA). Phenylmethylsulphonyl fluoride (PMSF), and sodium dodecyl sulphate (SDS) were obtained from Schwarz/Mann Biotech, Cleveland, OH, USA, ε-Aminocaproic acid (E-ACA) was acquired from Aldrich Fine Chemicals (Milwaukee, WI, USA). A stable 100 mM stock solution of PMSF was prepared in dimethylsulphoxide (DMSO, Fisher Scientific Co., Montreal, Quebec). All other inhibitors were dissolved in PBS. In some experiments, PMSF was inactivated by incubation in 0.1 M Tris-HCl saline, pH 9.5 for 2 h at 25°C (James 1978) prior to incubation with AEF. Toxicity of various concentrations of the chemicals or inhibitors in conjunction with AEF were pretested by injecting them subcutaneously (s.c.) and intraperitoneally (i.p), respectively, in groups of mice. The concentrations of the reagents chosen for incubation with AEF and *in vivo* testing were harmless to the mice. AEF was incubated with varying concentrations of the chemicals/inhibitors for 30 min at 25°C before testing *in vivo*.

Treatment of recipient mice for determination of AEF activity. Groups of three mice were used

in each of these experiments. Each group received i.p. 0.5 ml of the following reagents: (1) chemical/inhibitor treated crude AEF, or (2) 25% or 50% $(NH_4)_2SO_4$-precipitable AEF-containing fractions, or (3) normal or amyloidotic spleen cell culture supernatants or splenic interstitial fluid from amyloidotic or normal mice, or (4) 1 ml serum (normal mouse, or AHC-infected mice at 8 weeks p.i.), or (5) varying dosages of cell lysates, or (6) concentrated chromatographed AEF-fractions. In addition, each test mouse received concomitantly a s.c. injection containing 0.5 ml of casein/CFA (1:1, v/v). Control animals (three mice per group) received: (1) 0.5 ml of PBS i.p. only; (2) untreated AEF i.p. only; (3) untreated AEF i.p. and a s.c. injection of an inhibitor; (4) 4 μg Con A, 25 μg LPS, or 50 μg AHC-Met-Ag in 0.5 ml of PBS i.p. or (5) AEF incubated with either DMSO or Tris Hcl buffer-inactivated PMSF. In addition each mouse received s.c., 0.5 ml of casein/CFA. Animals were killed 72 h after the treatments.

Histological assessment. The amyloidogenic potential of various AEF preparations with and without chemicals or protease inhibitors, culture supernatants or cell lysates was assessed on Congo red-stained sections of spleens. Spleens were embedded immediately in OCT compound (Miles Scientific, Naperville, IL, USA) and frozen at −15°C, sectioned 10 μm thick and fixed in absolute ethanol for 20–30 min before staining with Congo Red (Puchtler *et al.* 1962). Spleens from control mice were treated similarly. Amyloid was identified by its green dichroic birefringence under crossed polars, and quantified by computer-assisted image analysis (Abankwa & Ali-Khan 1987).

Results

The data are presented in three sections: assessment of amyloidogenic activity, physicochemical characteristics and purification of AEF.

Table 3. Effect of various chemicals/protease inhibitors on the activity of amyloid enhancing factor (AEF); the AEF activity was assessed as the percentage of splenic perifollicular areas (PFAs) obliterated by amyloid and is expressed as the percentage of AEF activity in the absence of an inhibitor/chemical

Chemical substance	Concentration	AEF activity (%)*
ε-Aminocaproic Acid (E-ACA)	0.05 g/ml	7.6
α_1-Antitrypsin	10 μg/ml	110
α_1-Antitrypsin	100 μg/ml	103
α_1-Antitrypsin	170 μg/ml	115
α_1-Antitrypsin	1 mg/ml	92
α_1-Antitrypsin	5 mg/ml	136
Aprotonin	5000 u	5.3
Dithiothreitol	0.001 M	101.7
	0.01 M	111.3
Ethylenediamine-tetra	0.001 M	98.7
acetic acid (EDTA, Na 111)	0.01 M	104.4
Iodoacetic acid	0.001 M	3.8
Phenylmethylsulphonylfluoride	0.001 M	11.5
	0.01 M	0.0
Sodium dodecyl sulphate (SDS)	0.1%	108.2

* Expressed as a percentage of AEF activity in the absence of inhibitor. Activity was assessed as the per cent of splenic PFA obliterated by amyloid.

Assessment of AEF activity

Table 1 presents the data on differential cell count of PECs (16 h post casein injection and 12 weeks p.i. mice) and spleen cells (unfractionated, adherent and nonadherent cells) from 7 weeks p.i. amyloidotic mice. Over 77% and 94% of the PECs were neutrophils from 12 weeks p.i. and casein-induced mice, respectively. The supernatants of lysed PECs and spleen cell subpopulations, splenic interstitial fluid and spleen cell culture supernatants were assessed for amyloidogenic potential in groups of three mice. Administration of high dosages of the PEC lysates prepared from either source resulted in the death of the recipient mice within 12 h (Table 2). Congo red-stained spleen sections prepared from these mice were negative for amyloid. However, one of two surviving recipients of the PEC lysate (equivalent to 10^8 cells from 12 week p.i. mice in conjunction with casein/CFA) showed splenic amyloid deposition at 72 h. As shown in Table 1, both these PEC preparations contained predominantly neutrophils.

Table 4. Comparison of amyloid enhancing activities of crude amyloid enhancing factor (AEF) and its various fractions obtained after $(NH_4)_2\ SO_4$ precipitation (25% and 50% saturation) or gel chromatography (Sephacryl S-300, Sephadex G-50) in 0.01 M phosphate buffer containing 0.5 M NaCl, 0.01 M NaN_3, pH 7.1 (details given in legends for Figs 2, 3 & 4); AEF activity was calculated as the percentage of splenic perifollicular areas (PFAs) occupied by amyloid

Material tested for AEF activity	Amount injected (ml)	Protein (mg)	AEF activity*
Crude AEF	0.5	2.38	23.9±3.5
$(NH_4)_2SO_4$ Precipitated AEF			
25% precipitate	0.5	0.43	10.3±2.8
50% precipitate	0.5	0.68	16.7±2.4
50% supernatant	0.5	1.13	13.4±1.9
Sephacryl S-300 chromatographed & $(NH_4)_2\ SO_4$ precipitated AEF (Fig. 2)			
F1	0.5	0.17	none
F2	0.5	0.55	none
F3	0.5	0.05	2.8±2.8
Sephadex G-50 chromatographed F3 from Fig. 2 (Fig. 3)			
F1	1.0	nd	none
F2	1.0	0.05	5.4±1.2
Sephadex G-50 chromatographed crude AEF (Fig. 4)			
F1	1.0	3.1	19.2±6.3
F2	1.0	0.76	0.8±0.06
F3	1.0	0.94	0.2±0.1
F4	1.0	0.24	0.1±0.1

* AEF activity was calculated as the percentage of the PFA occupied by amyloid.
nd Not done.

Over 80% of spleen cells from the 7 weeks p.i. mice were lymphocytes (Table 1). A minimum dosage of 10^7 whole spleen cell lysate/mouse was required to evoke an amyloidogenic response; three of three mice were positive for amyloid (Table 2). Data from whole spleen cell lysates ($1-5 \times 10^6$) which failed to induce accelerated amyloidosis in the recipient mice are not shown in Table 2. Non-adherent spleen cells, obtained from 7 weeks p.i. mice, were primarily lymphocytes (96%); they contained 3% and 1% monocytoid cells and neutrophils, respectively (Table 1). The lysate of 8×10^6 of these cells (300 μg protein in the supernatant) induced amyloid in one of three mice; increasing this dosage to 1.5 mg (equivalent to 4×10^7 cells) resulted in the deposition of amyloid in three of three recipients (Table 2). The adherent cells were primarily monocytoid cells (95%) (Table 1). The lysate of 10^6 of these cells evoked an amyloidogenic response and two of three mouse spleens were positive for amyloid (Table 2). The supernatant from normal mouse spleen cell lysate (4×10^7 cells) was nonamyloidogenic.

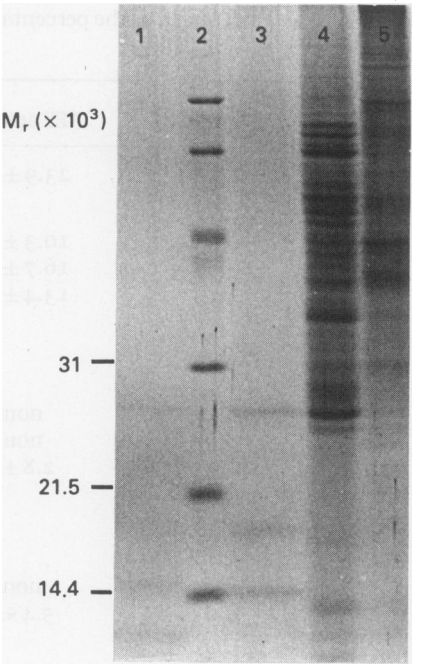

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Sephacryl S-300 (Fig. 2) and Sephadex G-50 (Fig. 3) chromatographed fractions. Lane 1, the trailing Sephadex G-50 fraction (Fig. 3); Lane 2, molecular weight markers 14 000 to 92 500; Lane 3, F3 fraction from Sephacryl S-300 (Fig. 2); Lane 4, F2 fraction from Sephacryl S-300 (Fig. 2); Lane 5, F1 fraction from Sephacryl S-300 (Fig. 2).

Intraperitoneal injection of concentrated interstitial fluid, obtained from the spleens of normal mice or 7 weeks p.i. amyloidotic mice in conjunction with casein/CFS, failed to provoke amyloidogenesis in the recipient mice. The volume administered corresponded to the interstitial fluid of a single donor amyloidotic or normal mouse spleen.

In order to determine whether whole spleen cells could secrete AEF into the culture medium, spleen cells from amyloidotic (7 weeks p.i.) or normal mice were incubated with three different stimulants (Con A, LPS and AHC-Met-Ag) or in medium alone. LPS, Con A and AHC-Met-Ag-stimulated spleen cell supernatants from amyloidotic mice contained AEF activity (Fig. 1). The supernatant from LPS-stimulated cells was most potent in this respect ($18.3 \pm 2.4\%$ of the PFA was obliterated by amyloid), followed closely by AHC-Met-Ag ($16.8 \pm 3.1\%$), and then Con A ($7.6 \pm 2.5\%$) (Fig. 1). Neither the supernatants from stimulated normal mouse spleen cells nor LPS, Con A and AHC-met-Ag diluted in PBS and injected in conjunction with casein/FCA were amyloidogenic. Similarly when the spleen cells from amyloidotic and normal mice were incubated in the absence of exogenous stimulants the supernatants lacked AEF activity.

One millilitre of 8 weeks p.i. serum from amyloidotic AHC-infected mice in conjunction with casein/CFA demonstrated AEF activity. No activity was detected in normal mouse serum at this dose.

Physicochemical characteristics

The AEF-containing tissue extract, when prepared in PBS, pH 7.4, and stored at

−20°C, exhibited a tendency to form aggregates when thawed. After centrifugation (27 000 *g*; 60 min at 4°C), all AEF activity was present in the pellet. In order to determine whether this aggregation is pH dependent, aliquots of AEF were dialysed against PBS of pH 7, 7.5 and 8; there was no detectable resolution of the precipitate after overnight dialysis at any of the three pHs. Similarly, the amount of amyloid deposited by mice receiving the dialysed AEF did not differ significantly between the three groups (data not shown). However, when AEF was prepared in PBS-mod (PBS containing 0.5 M NaCl, 0.01 M NaN_3, pH 7.1), this aggregation phenomenon did not occur.

To examine the possible enzymatic nature of AHC-induced AEF, a number of chemicals, some with anti-protease properties, were incubated with crude AEF. The concentration of various chemicals used for incubation with AEF were pretested and found not to be lethal to the mice. Their effect on AEF activity is presented in Table 3. PMSF at a concentration of 0.01 M completely abrogated the AEF activity; the activity was significantly decreased, but not entirely abolished by 0.001 M iodoacetic acid, 0.05 to 0.1 g E-ACA and 5000 U aprotinin. Conversely, SDS, α_1-antitrypsin, EDTA and dithiothreitol treatments appeared to enhance the AEF activity.

Purification and SDS-PAGE characterization of AEF

AEF was precipitated by saturation with 25% and 50% $(NH_4)_2$ SO_4. Both these fractions possessed AEF activity as well as the supernatant after 50% saturation with $(NH_4)_2$ SO_4. These fractions (25% and 50%) were mixed (2·34 mg/ml) and chromatographed on a Sephacryl S-300 gel column (Fig. 2). Only the third low molecular weight fraction possessed AEF activity; approximatley 50 μg of protein in the F3 induced accelerated amyloid deposition (Fig. 2, Table 4). F3 was pooled, concentrated and refractionated on a Sephadex G-50 gel column. Two fractions were eluted (Fig. 3); the second low trailing peak contained AEF activity and it was slightly more potent than the F3 (Table 4). In contrast the crude AEF resolved into four major fractions on a Sephadex G-50 gel column and AEF activity was detectable in all four fractions (Fig. 4, Table 4).

Further characterization of the AEF fractions was carried out by SDS-PAGE (Fig. 5). The AEF-containing F3 (Fig. 2) resolved into three major bands of approximate mol. wts 27 000, 18 000 and 15 000; a minor band of mol. wt less than 14 000 was also present (Fig. 5). When the F3 fraction (Fig. 2) was rechromatographed on Sephadex G-50 (Fig. 3), the AEF activity, as indicated above, was found to be confined to the trailing F2 and none in the F1 fraction (Table 4). On SDS-PAGE, the F2(G-50), showed a similar protein profile as Sephacryl F3, although the mol. wt 18 500 band was less distinct (Fig. 5). The AEF-negative F1 (G-50 lacked the mol. wt 15 000 peptide (Table 4). Our preliminary molecular weight screening of various fractions containing AEF activity suggests that most probably the peptide of mol. wt 15 000 which was prominently visible in all the active fractions, may indeed be AEF. Further studies are in progress to purify AEF to homogeneity.

Discussion

The accelerated model of experimental amyloidosis (Axelrad *et al.* 1982; Kisilevsky 1983; Varga *et al.* 1986) has done much to improve perspectives on the mechanisms of amyloidogenesis by providing a rapid, predictable and reproducible means of inducing the disease. However, a number of features, especially the biochemical and functional role of AEF in amyloidogenesis, remains unclear. We present here the results of studies designed to elucidate some of these characteristics.

Experimental evidence suggests that inflammatory cell or cells of the immune system are involved in the production of AEF; neutrophils, PECS, spleen cells, free and fixed

macrophages and T-lymphocytes have been implicated in the transfer of AEF activity in recipient mice (Janigan & Druet 1968; Kedar & Ravid 1980; Cathcart *et al.* 1972; Klisilevsky 1977; 1983). In this study, AEF activity was found in 8 weeks p.i. mouse serum, cell-free supernatants prepared from whole PECs and spleen cells, plastic-adherent (mainly monocytoid cells) and non-adherent (mainly lymphocytes) spleen cells obtained from AHC-infected amyloidotic mice and mice stimulated intraperitoneally with casein (Tables 1 & 2). Another interesting observation here is the presence of significantly potent AEF activity in the adherent cell lysate as compared to the whole spleen cell or lymphocyte lysates (Table 2). The latter two cell preparations required 8- to 10-fold greater cell numbers to manifest AEF activity as compared to that of the adherent cell preparation. It should also be noted that the amyloidogenic dosages prepared from 8×10^6 and 4×10^7 non-adherent spleen cells and 10^7 whole spleen cells contained 24×10^4, 12.4×10^6 and 6.3×10^5 macrophages, respectively (Tables 1 & 2). These findings point to the importance of adherent cells as one of the sources of AEF. Furthermore, the fact that AEF is found in measurable amounts in the serum (Flinn *et al.* 1986; Janigan & Druet 1968) and various soft organs during relevant inflammation may indicate that AEF might be an exocytosed or secretory product of activated phagocytic cells and/or lymphocytes. Colchicine, which blocks granule release by neutrophils (Zurier *et al.* 1973) also blocks the first phase of amyloidosis during which precursors and mediators of amyloidosis are presumably formed (Cohen *et al.* 1983; Kisilevsky *et al.* 1983; Brandwein *et al.* 1985). These observations support the theory that PMNs may be involved in the first phase of amyloidosis and also in the synthesis of AEF (Kedar & Ravid, 1980). The data presented in Table 2 are also suggestive of the contributory role of PMNs in the induction of accelerated amyloidosis; one of two surviving mice responded to amyloid deposition after receiving 10^8 PEC lysate which contained predominantly neutrophils.

AEF, though associated with cells, was not detectable in the splenic interstitial fluid of amyloidotic mice. This observation is contrary to the findings of an earlier report (Cohen *et al.* 1983). Our data indicate that AEF is not a freely diffusible cell constituent. It appears to be an intracellular component which is secreted on appropriate stimulation, as seen for lysosomal granules by PMNs or various proteases by monocytoid cells (Baehner *et al.* 1969; Zurier *et al.* 1973; Wahl *et al.* 1974; Gordon & Cohn 1978; Schnyder & Baggiolini 1978; Werb & Chin 1983; Baggiolini & Dequlad 1984). When normal and amyloidotic mouse spleen cells were stimulated *in vitro* with Con-A, LPS or AHC-Met-Ag, AEF activity was found in the culture supernatants of amyloidotic mouse spleen cells but not in those of similarly treated mice (Fig. 1). Furthermore, the unstimulated amyloidotic mouse spleen cell culture supernatant was negative for AEF activity. LPS and AHC-Met-Ag stimulated the production of similar amounts of AEF in the cell cultures. LPS is a well-known B-cell mitogen and inducer of IL-1 by macrophages which acts on hepatocytes to effect serum amyloid A protein (SAA) and other acute phase protein synthesis (McAdam & Sipe 1976; Sipe 1985). AHC-Met-Ag, on the other hand is highly phlogistic and amyloidogenic; it provokes a prompt acute inflammatory reaction and accelerated splenic amyloid deposition in mice (Alkarmi & Ali-Khan 1987). The observations that the mitogenic/antigen stimulated normal spleen cells and unstimulated amyloidotic spleen cells failed to secrete measureable amounts of AEF in the medium, strongly favour the involvement of a secretory process initiated by an exogenous stimulus in the induction of AEF by primed spleen cells only.

Serine proteases are, with increasing frequency, being implicated in the pathogenesis of amyloidosis. In-vitro experiments indicate

that these enzymes, present in serum and derived from monocytoid cells and PMNs, are responsible for proteolytic cleavage of SAA into an AA-like peptide (Skogen *et al.* 1980; Lavie *et al.* 1978, 1980; Silverman *et al.* 1982; Fuks & Zucker-Franklin 1985). Given the intense and persistent neutrophilic and histiocytic infiltration of AHC mass and peritonea of mice during alveolar hydatidosis (DeVouge & Ali-Khan 1983; Treves & Ali-Khan 1984; 1984*a*) it is not unreasonable to postulate that exocytosed lysosomal proteases released from activated and degenerating leucocytes *in vivo* (Wahl *et al.* 1974; Gordon & Cohn 1978; Schnyder *et al.* 1984; Fritz *et al.* 1986) might be functionally analogous to the above mentioned serine proteases *in vitro*. Since purified AEF is unavailable an indirect approach was adopted in the present experiments to determine whether AEF activity could be blocked by using various protease inhibitors (Table 3). If so, it might facilitate defining the biochemical and functional properties of AHC-induced AEF. Pretreatment of AEF with PMSF, a well established inhibitor of serine esterases (Gold 1965; James 1978) completely abolished the amyloidogenic activity of AEF in *in vivo*, suggesting that AEF might be a serine protease. Control mice which had received concomitantly, injection of 0.01M PMSF, casein-CFA and untreated AEF, were positive for amyloid after 72 h. These results show that only prior incubation of AEF with PMSF blocks its amyloidogenic potential. Studies are in progress to assess whether AEF activity is resistant or inhibited by specific inhibitors which selectively block the thiol group of proteases. Aprotinin and E-ACA, both non-specific protease inhibitors, as well as iodoacetic acid, which interferes with the thiol group in the enzyme molecule, significantly diminished the AEF activity, though not entirely. α_1-antitrypsin which inhibits the activity of chymotrypsin-like cationic proteins, however, had no inhibitory effect on the AEF activity (Table 3). The enzymes involved in SAA degradation *in vitro* range in molecular weight from 36 000 to 72 000 and are inhibited by a number of serine protease inhibitors, including α_1-antitrypsin (Lavie *et al.*, 1980; Skogen & Natvig 1981; Teppo *et al.* 1982). The AHC-induced AEF had a lower molecular weight and did not completely exhibit the same enzyme inhibitor profile (Table 3). Interestingly, a neutral proteinase of human spleen, an elastase, run under reducing conditions produced bands of mol. wts 27 000, 20 000, 15 000 and 13 000 to 10 000 (Starkey & Barrett 1976). Optimal extraction of this enzyme was effected with a buffer containing a high salt concentration and the isolated proteinase adsorbed to insoluble materials in the absence of salt. This extraction method and the behaviour of the enzyme resembles that of the AEF described by Axelrad *et al.* (1982). Coincidently, the molecular weight of the autolytic cleavage products of the spleen elastase are similar to those observed in the partially purified AHC-induced AEF (Fig. 5). Furthermore, Skinner *et al.* (1986) have recently reported an elastase (serine protease) which was found to be associated with AA fibrils extracted from human liver. They suggested that the fibril-bound elastase might be involved in physiological mechanisms of precursor protein degradation.

Finally, a number of attempts have been made to characterize AEF on the basis of molecular weight. Kedar *et al.* (1975) reported that in casein-treated mice, a dialysable factor of mol. wt $< 15\,000$ was responsible for the activity. A glycopeptide of similar molecular weight was implicated by Keizman *et al.* (1972). Janigan (1969) and Axelrad *et al.* (1982) reported that a high molecular weight component conferred the amyloid enhancing effect. Recently, Hol *et al.* (1985) reported both high and low molecular weight fractions possessed this activity. Self aggregation of AEF molecules under physiological conditions or their tendency to bind to particulate components or amyloid P component (Axelrad *et al.* 1982) may explain these conflicting results. Our results from the $(NH_4)_2SO_4$ precipitated and sequentially chromatographed AEF on Sephacryl S-

300 and Sephadex G-50 gel columns indicate that AEF activity is probably borne by a low molecular weight peptide of approximately 15 000. However, gel filtration of the crude AEF yielded results similar to those of Hol *et al.* (1985); the bulk of AEF activity was present in the high molecular weight volume and scant activity in the other lower molecular weight fractions (Fig. 4, Table 4). The suspicion that aggregation may be responsible for this phenomenon is strengthened by our finding that crude AEF extracted in PBS containing 0.15 M NaCl formed large aggregates when stored at −20°C and all AEF activity-pelleted on centrifugation (27 000 *g*, 1 h at, 4°C). The aggregates could not be solubilized once formed by dialysis against PBS of pH 7, 7.5 or 8, indicating that either aggregation is an irreversible process or that it is not pH dependent. AEF activity was not enhanced or reduced significantly be dialysis at either of the 3 pHs. When AEF was prepared in phosphate buffer containing 0.5 M NaCl, no visible turbidity appeared in the preparation but on chromatography the activity appeared in the void fraction as well as in the lower molecular weight fractions. The purification procedures reported here show that a combination of $(NH_4)_2SO_4$ (25–50% saturation) precipitation and Sephacryl S-300 gel fractionation eliminates most of the contaminants and yields a relatively pure AEF.

In our view, AEF seems to be a lysosomal neutral protease of PMN and/or macrophage origin. Further experiments are required not only to purify this entity, but also to define its functional role in amyloidogenesis *in vivo*.

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