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Induction of amyloid enhancing factor and its biological properties in murine alveolar hydatidosis

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Summary. The biological activity and time of appearance of alveolar hydatid cyst induced splenic amyloid enhancing factor (AEF) with respect to amyloid deposition in the spleens were determined in C57BL/6J mice. Mice were infected intraperitoneally with 100 alveolar hydatid cysts (AHC) and killed bi-weekly between 2 and 14 weeks postinfection (p.i.). AHCs and spleens were excised, weighed and a portion of each spleen was sectioned and stained for quantitation of amyloid deposits and histological studies. The remaining spleen pieces were sonicated separately in cold phosphate buffered saline, pH 7.4 (1 g/10 ml), centrifuged (27000 *g*, 60 min, 4°C) and the supernatant tested for AEF activity. Splenomegaly followed the progressive increase in the AHC biomass and AEF activity coincided with the appearance of amyloid deposits at 6 weeks p.i. A 2.5 mg intraperitoneal protein dosage of AEF in conjunction with a subcutaneous injection of 0.5 ml of a 0.11 M $AgNO_3$ solution in mice, induced the maximum amount of splenic amyloid deposition at 48 h; the amount of splenic amyloid deposits decreased by either increasing or decreasing the AEF dosage. *In vivo*, 70% of the AEF activity was abolished by day 4 post-injection of AEF and completly by 3 weeks. These findings indicate that AHC-induced AEF is functionally analogous to casein-induced AEF and its appearance in the spleen coincides with neutrophilia, histiocytosis and amyloid deposition.

Keywords: alveolar hydatid cyst, amyloid enhancing factor, amyloid, splenomegaly, silver nitrate, perifollicular area

Murine alveolar hydatidosis, which is caused by alveolar hydatid cyst (AHC), the larval stage of *Echinococcus multilocularis*, is characterized by a number of significant pathophysiological disorders in the host. Thymic atrophy, depletion of T-cells from peripheral lymphoid organs, depression of cell mediated immunity, plasmacytosis, lymphopenia, neutrophilia, macroglobulinaemia and generation of circulating immune complexes have been reported in mice as sequelae to the establishment of fulminant AHC-infection (Ali-Khan 1974; 1978*a*, *b*; Ali-Khan & Siboo 1982; 1983). Some of these immunological disorders have also been reported from patients with alveolar hydatid disease (Vuitton *et al.* 1984). In addition, inflammation-associated AA-amyloidosis, occurs as a complicating factor in both human and murine alveolar hydatid disease (Ozeretskovskaya *et*

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al. 1978; Ali-Khan *et al.* 1983; Alkarmi & Ali-Khan 1984; Alkarmi *et al.* 1986; Ali-Khan & Rausch 1987). Although the amyloidogenic potential of the AHC has been firmly established, the pathogenetic mechanisms responsible for amyloidogenesis remain unclear.

Extracts prepared from amyloidotic spleens of mice exposed to daily injections of casein or $AgNO_3$ for 3 to 4 weeks, and passively transferred to mice in conjunction with an inflammatory stimulus (e.g., casein or $AgNO_3$), have been shown to reduce to 48 h the period required for amyloidogenesis (Axelrad *et al.* 1982; Kisilevsky 1983). The factor bearing this activity has been designated as amyloid enhancing factor (AEF) and it is believed to be a crucial pathogenetic factor required for amyloid deposition in tissues; absence of AEF from target organs precludes amyloid deposition entirely (Axelrad *et al.* 1982; Kisilevsky 1983; Brandwein *et al.* 1985). A similar biological activity has recently been identified in amyloidotic spleen and liver extracts from AHC-infected mice and compared with casein-induced AEF (Alkarmi *et al.* 1985).

The aim of the present study was (a) to characterize the biological activity of the AHC-induced AEF (b) to quantify and correlate the amount of AEF activity with the size of the parasite biomass and the time of appearance and amount of amyloid deposited in splenic tissues and (c) to determine the cellular alterations in pre-, and post-amyloidotic splenic tissues. Two parameters were chosen to determine the biological activity of the AEF *in vivo*: first, the relationship between the dose of AEF administered and the amount of amyloid deposited, and secondly, the course of AEF catabolism.

Materials and methods

Experimental animals. Six- to twelve-week-old male or female C57BL/6J and Balb/c mice used in this investigation, were obtained from the Jackson Laboratories Bar Harbor, ME, USA), or bred from animals acquired from the same source in the animal facility in the Department of Microbiology and Immunology, McGill University. Mice were fed standard rat chow and watered *ad libitum*. Mice of the same sex and age were used in each experiment.

Infection of mice. Forty C57BL/6J mice were inoculated intraperitoneally with 100 ± 10 alveolar hydatid cysts. The methods for preparation of inocula and infection have been described previously (Ali-Khan 1974; 1978). Briefly, peritoneal AHCs from Balb/c mice, infected with 50 to 100 cysts, 3 months previously, were minced with a pair of scissors in 50 ml Hank's balanced salts solution, pH 7.2 (HBSS) and placed in a urine jar for sedimentation at room temperature. After 45 to 60 min the supernatant was discarded and fresh HBSS was added to the sedimented cysts and tissue pieces. This process was repeated five to six times until the supernatant was depleted of tissue debris and inflammatory cells. The cysts (2 to 3 mm in diameter) were separated from the heavier tissue pieces by decantation and the suspension was placed in 50 ml conical glass tubes. The cysts were further washed in HBSS (at least three times) by 15 min gravity sedimentation, counted and their number adjusted so that 0.5 to 1.0 ml of HBSS contained 100 ± 10 cysts.

AEF preparation. At 2, 4, 6, 8, 12 and 14 weeks p.i., the spleens and AHCs from five mice were removed and weighed; a portion of each spleen was fixed in 10% neutral formalin (pH 7) embedded in paraffin and sectioned (5 or 10 μm thick) for histological examination. Five uninfected mouse spleens were processed as above and used as controls. The remaining portions of spleens were pooled, weighed and used in the preparation of AEF. The spleens were homogenized in a Potter-Elvejhem tissue grinder (cold 0.01 M PBS, pH 7.4; 1g/10 ml), sonicated in an ice water bath for 2 min using a Sonifier cell disruptor (Branson Sonic Power Co., Rexdale, Ontario) at an output control setting of

7, and then centrifuged at 27 000 *g* (Sorvall Superspeed RC2-B, 60 min, 4°C). The supernatant was assessed for AEF activity in mice. Portions of the supernatant not immediatly used were frozen at −20°C until required.

Protein determination. Estimation of the protein content in the crude AEF preparation or normal spleen extract was determined employing the BIO-RAD protein assay kit.

Assessment of AEF activity. AEF activity from different AEF preparations (2 to 14 weeks p.i. spleens) was assessed in three or four C57BL/6J mice. AEF (0.5 ml; 2.5 mg protein) was injected intraperitoneally (i.p.) in conjunction with a subcutaneous (s.c.) injection of 0.5 ml of a 0.11 M $AgNO_3$ solution. In the dose–response experiments, mice in each group (three mice) received 0.05, 0.1, 0.25, 0.5 or 1.0 ml of AEF extracted from 12 weeks p.i. spleens, and $AgNO_3$ concomitantly. In the AEF catabolism experiments, eight groups of mice (four mice per group) were given 0.5 ml of AEF i.p. on day 0 and the inflammatory stimulus on day 0, 1, 2, 3, 4, 7, 14 or 21, respectively. Mice were killed 48 h later after the $AgNO_3$ injection by cervical dislocation, their spleens removed, embedded in either OCT compound for frozen sections or embedded in paraffin, following fixation in 10% neutral formalin. AEF activity was considered present if green birefringent congophilic amyloid deposits were detectable under a polarizing filter in the perifollicular areas.

Histology and quantification of amyloid. Spleen sections were stained in alkaline Congo red (Puchtler *et al.* 1962) or in haematoxylin and eosin.

Amyloid deposits were quantified using the McGill Image Calculator (Dr Harold Riml, Medical Physics Unit, McGill University). The microscopy images were captured by a NEWVICON camera (Panasonic WV 1550) digitized by a real time frame grabber (Coreco Oculus 200) and stored and analysed in an IBM PC AT. The software is capable of correcting for uneven illumination (shading correction) over the field of view, and of capturing images in colour by inserting colour filters in the iilluminator when appropriate.

Areas of amyloid deposition were evaluated by placing a region of interest (ROI) frame over appropriate areas of the image and counting the number of picture elements (pcls) occupied by each shade of grey (or colour, if appropriate) and displaying the result as a histogram. Amyloid-laden areas could then be evaluated by integrating the pcl versus greysacle (or colour, if appropriate) histogram over the range of grey (or colour) corresponding to the amyloid-laden areas in the image. The method is reproducible and is independent of the operator if pcls of amyloid-laden tissue (signal) and other structures (background) are separated by a minimum in the histogram (Fig. 1). The integral

$$A_1 = \int_0^x f(x)\,dx$$

would correspond to amyloid-laden areas and the integral

$$A_2 = \int_x^{xm} f(x)\,dx$$

to background. The software is highly interactive and thus allows for constant operator judgement of the biological validity of the results.

Spleen follicles were picked at random for amyloid quantification. On location of a follicle, the region of interest (ROI), that is the perifollicular area (PFA), was demarcated. Amyloid was detected by its optical characteristics and quantified as a percentage of the surface area of the PFA. Where amyloid deposits had extended into the red pulp or the follicle, the percentage of the total surface area obliterated by amyloid was measured. A minimum of 25 ROIs were quantified for each group which included three or more spleens. The mean and standard error of the mean were computed using an appropriately programmed Texas Instruments calculator, and expressed as the percentage of the PFA or the surface area obliterated by amyloid.

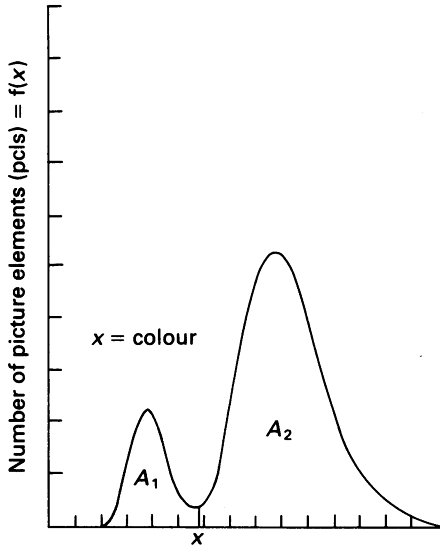

Fig. 1. Number of picture elements (pcls) versus colour.

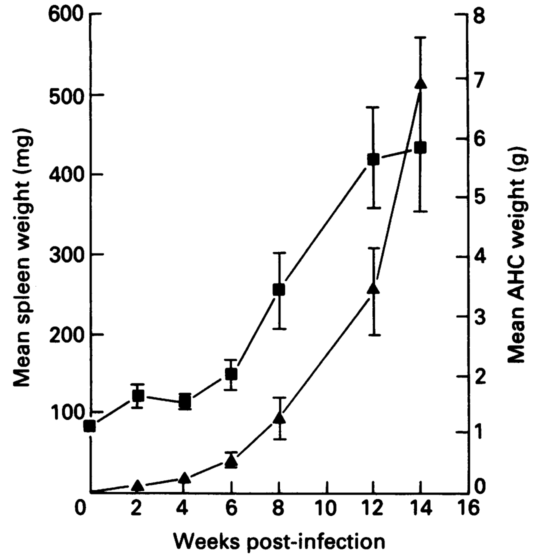

Fig. 2. Relationship between splenomegaly and alveolar hydatid cyst (AHC) growth during the course of murine alveolar hydatidosis. Points shown represent the mean of five animals ± s.e. ▲, AHC wt; ■, spleen wt.

Results

Splenomegaly and AHC growth

In order to examine the temporal relationship between splenomegaly and AHC biomass the spleens and tumour-like solid AHCs were removed and weighed at 2, 4, 6, 8, 12 and 14 weeks p.i. Figure 2 illustrates the correlation between the two parameters during the course of infection; splenomegaly followed the progressive increase in the AHC up to 12 weeks p.i. The mean spleen weight increased over 4-fold by 12 weeks p.i.; thereafter no significant increase was observed as atrophic changes began to occur in the spleen. The AHC mass increased steadily over the 14 week period. Mice with large AHCs began succumbing to the infection after 12 weeks.

Spleen histology

The pre- and post-amyloidotic histological changes in the spleens have been described previously (Ali-Khan *et al.* 1983; Alkarmi & Ali-Khan 1984). Briefly, in the pre-amyloidotic stage (2 and 4 weeks p.i.), the follicles appeared hyperplastic with prominent germinal centres; lymphocytes predominated in the perifollicular sinuses except that a few polymorphonuclear leucocytes (PMNs) and plasma cells were also visible. At 4 weeks p.i. the moderately expanded red pulp contained numerous megakaryocytes, foci of myeloid cells and lymphocytes with pycnotic nuclei. Isolated strands of amyloid fibrils (AA) appeared in the PFA at 6 weeks p.i. with a concomitant increase in PMNs and monocytoid cells. At 8 weeks p.i., due to increased deposition of AA and their fusion with each other, the PFA appeared as a hyaline ring depleted of cells; reduced numbers of lymphocytes and PMNs were present in a few intact, but relatively empty, marginal sinuses. Between 8 and 12 weeks p.i. AA deposits infiltered the red pulp as structureless hyaline sheets which often became confluent with the amyloid ring in the PFA. With increasing amounts of AA deposition within the follicles the majority of follicles appeared as acellular hyaline nodules. In some follicles a residual population of cells

were present around the central arterioles which contained flattened endothelial cells. Between 12 and 14 weeks p.i., well into the amyloidotic phase, significantly large areas of splenic tissues were obliterated by AA. Morphologically intact follicles were rare and the expanded red pulp contained foci of immature granulocytes (ring shaped nuclei) and lymphocytes with pycnotic nuclei.

Amyloidogenesis and AEF activity

AEF activity was defined as the ability of the amyloidotic spleen extract to induce AA deposition within 48 h of administration of an inflammatory substance ($AgNO_3$). The area of perifollicular obliteration by AA resulting from this treatment was taken as a measure of the amount of AEF present in the spleen extracts at 2, 4, 6, 8, 10, 12 and 14 weeks p.i. The data are presented in Fig. 3 along with the amounts of AA present in the spleens used for the extraction of AEF.

The thawed splenic extracts had the tendency to form aggregates. These aggregates were dispersed by homogenization before use. The timing and pattern of splenic AA deposition in the present experiments were similar to those described previously in AHC-infected C57BL/6 mice (Ali-Khan *et al.* 1983; Alkarmi & Ali-Khan 1984). The appearance of splenic AA at 6 weeks p.i. coincided temporally with the appearance of titratable AEF activity. Both the amount of AA deposits and AEF activity increased steadily with time. Amyloid deposits accounted for about 13% of the PFA at 6 weeks p.i., they increased to over 46% by 14 weeks p.i. Between these time intervals the AEF activity of amyloidotic spleen extracts increased fourfold. Normal spleen extracts or those prepared from 2 or 4 weeks p.i. amyloid-negative spleens were devoid of AEF activity.

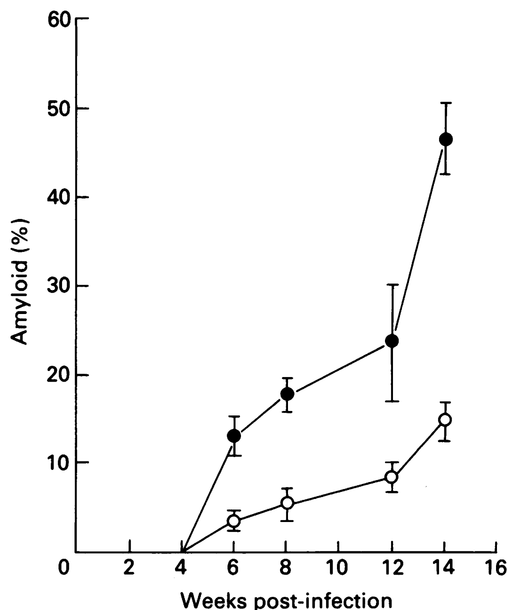

Fig. 3. Relationship between amyloidogenesis and amyloid enhancing factor (AEF) synthesis during the course of murine alveolar hydatidosis. At each time period postinfection portions of four spleens were sectioned, stained with Congo red and amounts of amyloid deposits (●) were assessed by computer-assisted images analysis. The remaining portions of spleens were used for the extraction of AEF and assessment of their amyloid enhancing potency (○) in the bioassay. The solid and open circles represent, at corresponding periods, the amounts of and amyloid deposit and AEF (mean ± s.e.) present in the donor spleens, respectively; the AEF titre was determined as described in 'Materials and methods', also described in Fig. 4. ●, Amyloid deposits; ○, AEF activity.

Dose-response relationship

Varying volumes (0.05 to 1 ml; 5 mg protein/ml) of AEF were injected i.p. along with a s.c. injection of $AgNO_3$ in five groups of mice. The amount of amyloid present in the spleen sections was quantified after 48 h for each dose administered. The data show a positive correlation between the amounts of splenic AA deposition and the optimal protein dosages of AEF administered; the maximum AA deposition was induced by the 2.5 mg dose (Fig. 4). With this dose the mean PFA displacement by AA was approximately 25%; this was more than 40% higher than when the protein content of AEF dosage was doubled (5 mg/mouse). The amount of AA

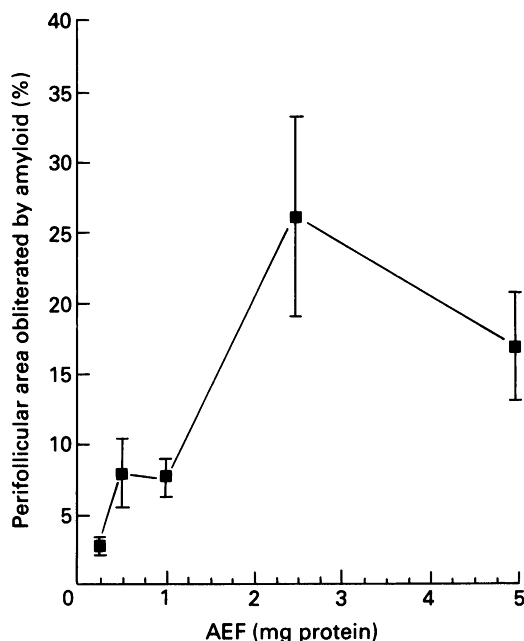

Fig. 4. Relationship between the dose of AEF administered and the amount of amyloid deposited in splenic tissues. Five groups of four mice received 0.25, 0.5, 1.0, 2.5, or 5.0 mg of AEF-containing spleen extract and 0.5 ml of 0.11 M $AgNO_3$ solution. Mice were killed 48 h after the treatment. Amyloid was quantified after Congo red staining by computer-assisted image analysis. Each point represents the mean amount of amyloid deposited ± s.e.

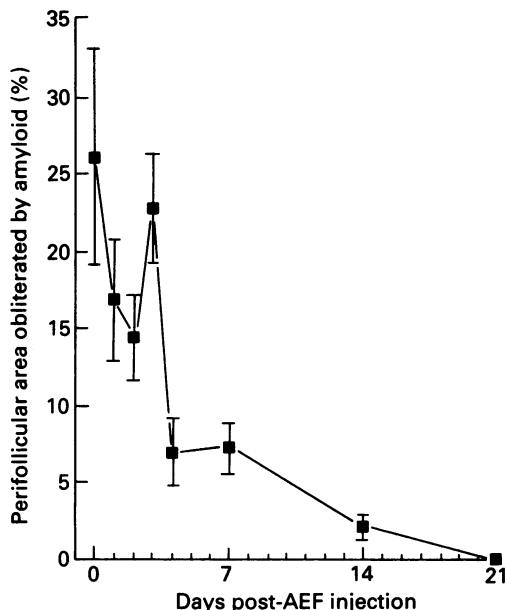

Fig. 5. Rate of AEF catabolism *in vivo*. Eight groups of three mice were given a single intraperitoneal dose of AEF on day 0. Each group then received a single subcutaneous injection of 0.5 ml of 0.11 M $AgNO_3$ solution on day 1, 2, 3, 4, 7, 14 or 21. Control mice received a concomitant dose of $AgNO_3$ on day 0. Mice were killed 48 h after receiving the $AgNO_3$. Amyloid was detected by its Congophilia and quantified by computerized image analysis. Each point represents the amount of amyloid deposited in the spleens in response to AEF administration after 1 to 4 days and on day 7, 14 and 21 postinjection. (mean ± s.e.).

deposition decreased by either increasing or decreasing the optimum 2.5 mg dosage.

In-vivo catabolism of AEF activity

The rate of AEF catabolism was examined by challenging AEF recipient mice with $AgNO_3$ at various periods post-injection. The AEF activity persisted relatively unchanged for 3 days (Fig. 5). By day 4, approximately 70% of its amyloidogenic potential had been abolished, as the average amount of amyloid occupying the PFA decreased from 22.8 to 6.9%. The activity dropped a further three-fold from day 4 to day 14. No AEF activity was detectable on day 21, as evidenced by the lack of AA in the PFA 48 h following $AgNO_3$ injection.

Discussion

Despite differences in the extraction method and route of injection between the two AEF induced either by AHC-infection, as in the present experiments, or casein/$AgNO_3$ (Kisilevsky 1983; Brandwein *et al.* 1985), the principal biological function of the AEF from both these sources appeared to be identical i.e., the transfer of AEF abolished the lag period and allowed rapid deposition of tissue AA in the recipient mice. Furthermore, similar to the casein-mouse model, only amyloidotic spleen extracts from the AHC-infected mice contained AEF activity (Fig 2). Axelrad *et al.* (1982) have shown that generation of

splenic AEF in the casein-mouse model precedes the appearance of tissue AA by at least 24 h and in fact tissue AA deposition is precluded entirely by the absence of AEF. Although we did not precisely determine these two events in the AHC-mouse model, titrable amounts of splenic AEF and histologically detectable splenic AA appeared concomitantly at 6 weeks p.i. In addition, the data presented in Figs 2 and 3 show a significant and corresponding increase in the AEF titre, amount of splenic AA deposition, parasite biomass and splenomegaly during the 14 week course of infection. The threshold value for amyloidogenesis with respect to the mean AHC weight in the present experiments (0.545 ± 0.91 g) was comparable to the value reported previously (0.53 ± 0.07 g) in C57BL/6 mice (Alkarmi & Ali Khan 1984). These observations indicate that with an infective inoculum varying between 50 and 100 cysts, splenic AA deposition occurs at 6 weeks p.i. and the AA induction period can be predicted on the basis of AHC weight. The minimal conclusions to be drawn from these experiments are: (a) that the AHC is intimately involved in the generation of AEF and AA; and (b) that AEF might also be an absolute requirement for amyloidogenesis in the AHC mouse model and its essential role analogous to that of casein-induced AEF.

Earlier studies on AEF-induced experimental amyloidosis indicated that the amount of splenic AA deposition is dependent on the nature of inflammatory agents used, severity of inflammation, extent of tissue necrosis and the amount of protease-like products released from the inflammatory infiltrates; the proteases were suggested to act as an amyloidogenic 'trigger' (Kisilevsky *et al.* 1977). Conversely, when the inflammatory stimulus was kept constant, the amount of splenic AA deposition was dependent on the dose of AEF administered (Axelrad & Kisilevsky 1980). Similarly, in the AHC-mouse model the amount of splenic AA deposits increased with a corresponding rise in the AEF titre and this suggested a causal relationship between these two events. It was established empirically by injecting constant volumes of the inflammatory stimulus ($AgNO_3$ solution) and splenic AEF obtained between 2 and 14 weeks p.i. (Fig. 3). However, the AEF preparations, in particular those from 12 and 14 weeks p.i. donor spleens (with heavy amyloid deposition), were unable to induce quantitatively similar amounts of splenic AA in the recipients. Lack of sustained inflammatory stimulation in the recipient mice as compared to the AHC-infected donors might explain this disparity.

As to the role of inflammatory cells in AHC-induced amyloidosis the data from our current (Abankwa & Ali-Khan 1987) and previous work corroborate the observations made by Kisilevsky *et al.* (1977) and Kedar & Ravid (1980). Briefly, in intraperitoneally growing AHCs, an intense leucocytic infiltration in the tumour-like AHC mass (2 to 5.4×10^6 leucocytes/100 mg of AHC) and mouse peritonea (5 to 36×10^6 mean number of leucocytes/mouse) characterized the entire 16 week course of infection. The predominant infiltrates at each of these sites were either neutrophils or macrophages and more than 50% of the intra-AHC leucocytes at each time period were nonviable and the rest contained phagocytosed immune complexes (Devouge & Ali-Khan 1984; Treves & Ali-Khan, 1984; 1984*a*). These events would indicate release of various chemical mediators and lysosomal proteases from both the activated and degenerating neutrophils and macrophages *in vivo* (Taichman *et al.* 1972; Turner *et al,* 1973; Wahl *et al.* 1974; Gordon & Cohn 1978; Schnyder & Baggiolini 1978; Ragsdale & Arend 1979; Werb & Chin 1983; Baggiolini & Deqlad 1984; Fritz *et al.* 1986). What then accounts for the absence of tissue amyloid during the first 5 weeks of infection that is the preamyloidotic phase when significantly elevated levels of serum amyloid A protein (SAA) (Ali-Khan *et al.* 1987), the presumed precursor of tissue AA, and significantly high numbers of viable and nonviable peritoneal and intra-AHC leukocytes are present in the infected mice? Simi-

larly a prolonged and persistent inflammatory phase and significantly elevated levels of SAA intervenes the preamyloidotic and amyloidotic phases in the casein-mouse model (Benson *et al.* 1977; Kisilevsky 1983; Brandwein *et al.* 1985). This may mean that in the preamyloidotic phase the interstitially deposited SAA is completely catabolized and cleared from the tissue by exocytosed proteases released from activated endothelial cells or immigrant leucocytes (Alkarmi & Ali-Khan 1984). Fuks and Zucker-Franklin (1985) and others have proposed that a defective and/or partial proteolysis of SAA by Kupffer cell serine proteases is essential for amyloidogenesis. Could this be that a relatively reduced but a critical level of these proteases is required to achieve partial proteolysis of SAA and to act as an amyloidogenic trigger? Conceivably, α-2-macroglobulin, α1-antichymotrypsin and α1-antitrypsin, the natural proteinase inhibitors could partially inhibit and thus reduce the proteolytic potential of macrophage and neutrophil elaborated proteases in the tissue (Fritz *et al.* 1986, review). Alternatively incessant and chronic exposure of fixed and free phagocytic host elements to AHC antigen/immune complexes in the AHC-mouse model (Ali-Khan & Siboo 1983; Treves & Ali-Khan 1984*a*) or to casein as shown by Fuks & Zucker-Franklin (1985) could also induce the necessary protease-associated degradation defect in the processing of SAA. However, the molecular mechanism of such a defect is presently unknown.

AEF is a natural tissue constituent and exists at low levels in normal tissues (Varga *et al.* 1986). Relatively high levels of AEF, which are detectable in the bioassay, are present in amyloidotic spleen and liver extracts (Kisilevsky 1983; Varga *et al.* 1986). Undoubtedly, multiple casein injections (Benson *et al.* 1977; Fuks & Zucker-Franklin 1985; Brandwein *et al.* 1985) or AHC-infection (Ali-Khan *et al.* 1983; Alkarmi & Ali-Khan 1984; Treves & Ali-Khan 1984; 1984*a*) has the potential to increase the number, enzyme content and secretory activity of splenic and hepatic endothelial cells and macrophages. An increase in some of these parameters have been demonstrated in experimental models of amyloidosis and others (Teilum 1967; Kazimierczak 1969; Wahl *et al.* 1974; Gordon & Cohn 1978; Schneider & Baggiolini 1978; Ragsdale & Arend 1979; Fuks & Zucker-Franklin 1985). These observations, taken together, strongly favour involvement of leucocyte proteases in the pathogenesis of amyloidosis and more importantly it raises the possibility that AEF might be a protease and/or a product of activated leucocytes. AEF is believed to be a glycoprotein and a secretory product of activated reticuloendothelial cells (Kisilevsky 1983). In addition, a number of investigators have shown the degradation of SAA to an AA-like peptide by enzymes derived from neutrophils and monocytoid cells (Lavie *et al.* 1978; 1980; Skogen *et al.* 1980; Silverman *et al.* 1982; Fuks & Zucker-Franklin 1985) and induction of accelerated amyloidosis in mice with neutrophil lysates (Kedar & Ravid 1980). Whether marked neutrophilia and histiocytosis at tissue sites prior to amyloid deposition (Alkarmi & Ali-Khan 1984) and as shown here in the splenic perifollicular areas account for the afore mentioned pathogenesis of AA can only be speculated at this time. The companion paper sheds further light on the cell soruce of AEF, its biochemical nature and complete inhibition of AEF activity with serine protease inhibitors (Abankwa & Ali-Khan 1987).

Finally, the rate of catabolism of AHC-induced AEF was examined *in vivo.* Axelrad *et al.* (1982) reported retention of casein-induced AEF activity up to 4 weeks post-injection in the recipient mice; whether total or partial AEF activity was observed at 4 weeks was not mentioned. In the present study, approximately 70% of the AHC-induced AEF activity had waned by day 4, and it was undetectable at 3 weeks post-injection (Fig. 5). The discrepancy between the two catabolic rates may also be explained by the difference in the routes of administration; an intravenous, as opposed to an intraperitoneal route may influence its activity and catabolism *in vivo* (Axelrad *et al.*

1982). As AEF is not available in pure form, the total protein content of spleen extracts fails to provide any precise information concerning AEF titre contained in any given preparation.

These investigations have shown the following: (a) AEF activity appears in the spleen approximately 6 weeks p.i., and coincides with the appearance of amyloid deposits, (b) the titre of AEF increases with time and corresponds to splenomegaly and the size of AHC biomass, (c) AHC-induced AEF is functionally analogous to casein-induced AEF (Alkarmi 1985) and finally increased neutrophil and histiocyte infiltration of splenic tissues during the course of infection may indicate a contributing role for the cells or their exocytosed components in the pathogenesis of AHD-induced amyloidosis.

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