THE ROLE OF AMYLOID ENHANCING FACTOR IN THE DEVELOPMENT OF AMYLOID A AMYLOIDOSIS

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Short Title of Thesis

Amyloid Enhancing Factor in Development of Amyloid A Amyloidosis

ABSTRACT

Secondary, or AA, amyloidosis is a systemic disease characterized by the extracellular tissue deposition of insoluble, fibrillar amyloid A protein. Aberrant metabolism of serum amyloid A protein by reticuloendothelial cells in the presence of amyloid enhancing factor (AEF) is thought to result in the accumulation of fibrils within the tissues. Treatment of mice with AEF, in conjunction with an inflammatory stimulus, induces amyloid deposition within 48-72 hours. *In vivo* examination of the effect of AEF on spleen macrophage activation-associated phenotype and functions reveal that AEF has no apparent effect on the ability of spleen and liver macrophages to phagocytose or kill *Listeria monocytogenes*. It did appear to block enhanced respiratory burst function as well as result in a decrease in the number of cells expressing the MHC Class II surface antigen. Preliminary electrophoretic purification of AEF from crude amyloidotic tissue extracts localized AEF activity to a region apparently consisting of 3 protein species with molecular weights ranging from 63.2 KDa to 47.9 KDa. *Ex vivo* studies of macrophage function [illegible] eluted from this "AEF-active" region did not indicate an inhibition of respiratory burst, suggesting that the action of AEF may be dependent on the presence of additional, possibly inflammation-associated, component(s).

ABSTRACT

L'amyloidose secondaire de type AA est une maladie systémique charactérisée par un dépôt tissulaire extracellulaire d'une protéine insoluble et fibrillaire, l'amyloide A. L'accumulation de ces fibrilles résulterait d'un métabolisme aberrant de la protéine précurseur, le "serum amyloid A", par les cellules du système réticuloendothélial en présence de "Amyloid Enhancing Factor" (AEF). Le traitement de souris avec l'AEF en parallèle avec l'induction d'une réponse inflammatoire, provoque l'apparition d'amyloidose en 48 à 72 heures. Nous avons étudié l'effet de l'AEF *in vivo* sur l'activation du macrophage tant au point de vue phénotypique qu'au point de vue fonctionnel. Les fonctions phagocytaire et bactéricide des macrophages spléniques et hépatiques ne sont pas affectées par l'AEF. Ce dernier, par contre, bloque l'induction du burst respiratoire et provoque une diminution de nombre de cellules exprimant l'antigène majeur d'histocompatibilité de type II à leur surface. La purification de l'AEF à partir de l'électrophorèse de l'extrait tissulaire sur gel de polyacrylamide a circonscrit l'activité AEF à une région comprise entre 63.2 et 47.9 Kd, obtenue par électroelution à partir du gel. Cette région montrant une activité AEF n'inhibe pas le burst respiratoire des macrophages *in vitro*, tel que démontré *in vivo*, suggérant que l'action AEF peut être dépendante de la présence d'autres facteurs possiblement associés au processus inflammatoire.

PREFACE

The experimental work presented in this thesis appears in chapters 2 , 3, and 4. The following option, as described in the "Guidelines Concerning Thesis Preparation" for the Faculty of Graduate Studies and Research at McGill University, has been used.

"Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory.

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

Additional material (procedural and design data, as well as descriptions of the equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscipts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph. D. Oral defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

As required by the Guidelines, Chapter 1 is a general introduction and review of literature relevant to the subject matter of the thesis.

Aberrant amyloid fibril precursor protein metabolism associated with macrophages at the site of amyloid deposition is believed to play an integral role in amyloid fibril formation. Amyloid enhancing factor has been identified in all types of amyloid tissue tested to date due to its ability to induce amyloid fibril deposition at an accelerated rate.

Though its precise chemical nature and mechanism of action have not yet been characterized, most work seems to suggest that it exerts its action at the level of the macrophage. Chapter 2 describes experimental data investigating the effects of amyloid enhancing factor on macrophage activation and function during amyloid enhancing factor-induced amyloidogenesis which has been published in the *Journal of Leukocyte Biology*, volume 53, July 1993.

The unavailability of definitive biochemical characterization of amyloid enhancing factor has hampered investigations regarding its seemingly universal role in amyloidogenesis in particular mechanism of action. Chapter 3 describes unpublished experimental data concerning the purification of amyloid enhancing factor.

Chapter 4 describes macrophage functional assays involving treatment with the purified amyloid enhancing factor prepared in chapter 3 in an attempt to link results obtained in chapter 2 using a more homogeneous preparation of amyloid enhancing factor.

Chapters 2, 3 and 4 are each comprised of an Introduction, Materials and Methods, Results, Discussion, and References. Additional co-authors of chapter 2 are Lise Hébert, Gina Pozzulo, and Francine Gervais. Lise Hébert provided the data concerning splenic macrophage O_2^- production (not including O_2^- production data described in chapter 4). Gina Pozzulo provided assistance in analysis of Ia surface expression FACScan data. Francine Gervais is my supervisor. Chapter 5 includes a general discussion summarizing the conclusions of the thesis, and lists claims to original research. Appendix I contains an actual copy of the manuscript published in the *Journal of Leukocyte Biology*, volume 53, July 1993 entitled Splenic Macrophage Activation and Functions in Amyloid Enhancing Factor-Induced Secondary Amyloidosis. Study of Phagocytosis, Killing, Respiratory Burst, and MHC Class II Surface Expression. AA amyloidosis is referred to as secondary reactive systemic amyloidosis, or simply secondary amyloidosis, throughout this manuscript.

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CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The term amyloidosis describes a heterogenous collection of systemic diseases characterized by the extracellular tissue distribution of proteinaceous amyloid fibrils of varied chemical diversity. Diseases involving amyloid deposition cover a diverse array including chronic renal dialysis, lipoprotein metabolism and artherosclerosis, inflammatory disorders, cancer, rheumatoid arthritis and Alzheimer's disease.

The mechanisms underlying the pathophysiological processing of normally innocuous, soluble precursor proteins of various amyloids are not well understood. Reticulo-endothelial cells are consistently found associated with amyloid deposits and are thought to play an integral role. In 1966, another key player, namely amyloid enhancing factor (AEF), was identified in amyloid tissue extracts (1). Recent studies demonstrate a causative role for AEF in amyloidogenesis and suggest that it is a common pathogenic link between the diverse forms of amyloidotic disorders (2-5). To date definitive biochemical characterization of AEF has remained elusive thereby limiting its identification to largely functional terms.

The work described here focuses on elucidating the biochemical nature of AEF as well as examining its possible cause and effect relationship to reticulo-endothelial cells found at the site of amyloid deposition.

AMYLOID AND AMYLOIDOSIS

Amyloid Deposits:

Amyloidosis arises when normally innocuous, soluble proteins polymerize to form insoluble fibrils. The resulting mass of amyloid fibrils is associated with plasma and extracellular matrix proteins as well as proteoglycans. As these amyloid deposits increase in size, they invade the extracellular tissue spaces of vital organs thereby destroying normal tissue architecture and function.

The term amyloid, meaning "starch-like", was first devised in the 1800's by Virchow based on the positive iodine staining reaction exhibited by amyloid deposits of diverse origins (6). Despite the fact that Friedreich and Kekulé later demonstrated that the major component of amyloid deposits is protein (7), the term "amyloid" has endured. It is likely that the carbohydrates, such as heparan sulfate proteoglycan, commonly found in association with amyloid deposits are responsible for the cellulose-like iodine staining reaction first observed by Virchow.

A major focus of amyloid research during the past decade has been the characterization of the individual proteins which form amyloid fibrils. To date at least 15

distinct amyloid peptides and their precursors have been identified to form deposits within specific clinical conditions associated with amyloidosis (8). All amyloid deposits, regardless of which of these proteins is the principal component, share certain definitive tinctorial, morphological, and ultrastructural properties. Early researchers identified amyloid deposits by sulfuric acid and iodine staining techniques (6), metachromatic staining with aniline dyes such as methyl violet and crystal violet (9), and eventually Congo red stain (10). When analyzed by light microscopy without polarizing optics, amyloid deposits in tissue sections appear homogenous, eosinophilic and amorphous (11) With polarizing optics, unstained amyloid deposits exhibit birefringence, indicating the presence of highly ordered structures (12). Upon staining with Congo red, this birefringence is increased intensely, with a characteristic apple green colour, in a manner not seen with other fibrillar structures (i.e. collagen) (12). Typically the presence of amyloid is detected using Congo red stain in conjunction with polarization microscopy.

Electron microscopic study of amyloid deposits have shown a fibrillar structure consisting of fibrils 7-10 nanometers in diameter (13, Fig. 1.1). Infrared and X-ray diffraction spectroscopy of isolated amyloid fibrils indicate that the proteins are arranged in a cross-ß-pleated sheet conformation (14) which is not normally present in non-pathogenic fibril polymers. The tinctorial and optical properties of amyloid fibrils (once stained with Congo red) as well as their physiological inertness within the tissues can be attributed to this ß-pleated sheet conformation (15, 13).

Classification of Amyloids and Amyloidosis

From Virchow's initial description of amyloid deposits until the early 1970's, popular theory held that all amyloid deposits were comprised of a common major substance. The classification of systemic amyloid syndromes therefore evolved based on the anatomic distribution of amyloid deposition and the associated clinical findings. Systemic amyloidotic disorders were usually grouped under either "secondary", "familial", or "primary" headings in addition to the rarer localized forms of amyloidosis. With the development of methods for dissolving and fractionating amyloid fibrils extracted from tissues (16, 17), a number of chemically distinct amyloid fibril proteins were identified. These findings made it apparent that grouping amyloidotic disorders according to their clinical nature alone is inappropriate.

At present it is the protein nature of the physiologically insoluble amyloid fibril component of the amyloid deposit which defines the type of amyloidosis and determines the pathogenesis of the disease which results from its deposition. The current nomenclature and classification of amyloids and amyloidosis (based on the chemical nature of the fibril

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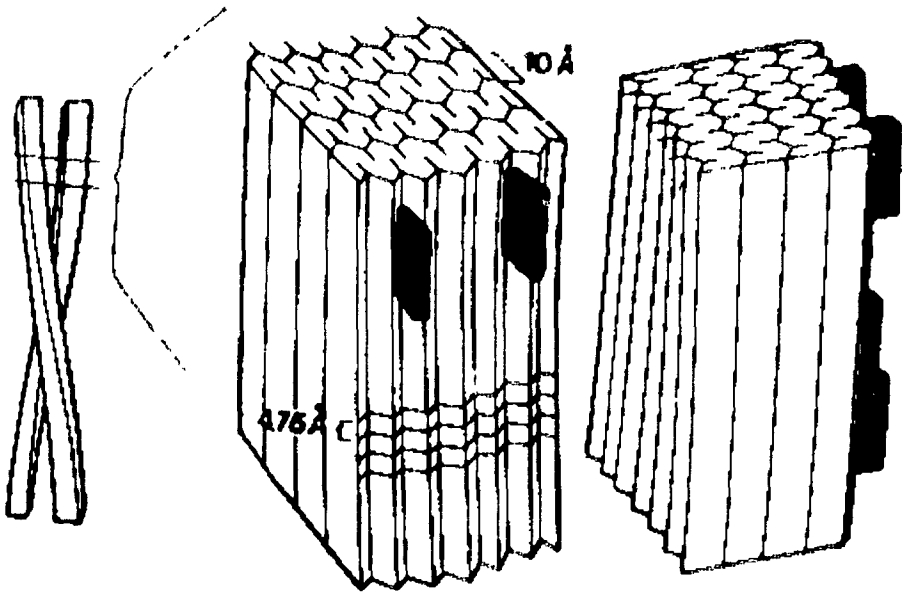

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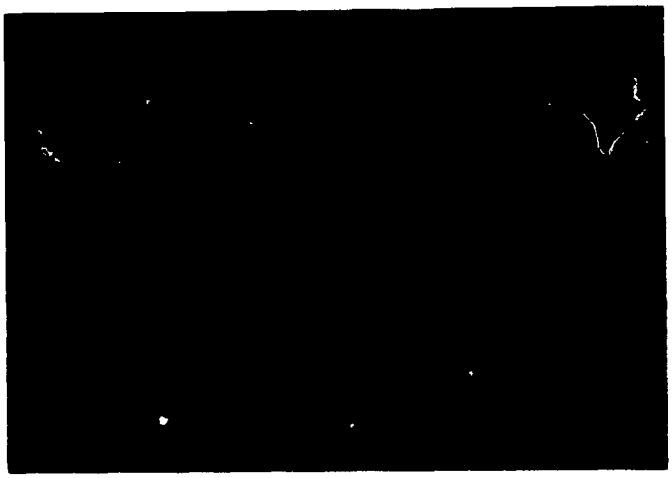


Fig. 1.1 **THE FIBRILLAR, ß-PLEATED SHEET CONFORMATION OF AMYLOID DEPOSITS.**

A Twisted ß-pleated anti-parrallel configuration of paired filaments of an amyloid fibril in AA amyloidosis Sites of binding of the planar Congo red dye are indicated by the stippled blocks. (Adapted from Glenner 1980).

B Congo red staining of a histiological section prepared from the spleen of a mouse with experimentally-induced AA amyloidosis (by repeated casein injections). Amyloid A deposits are identified by their characteristic birefringence under polarization microscopy.

protein), as devised at the VIth International Symposium on Amyloidosis in 1991, is summarized in Table 1.1.

Amyloid A (AA) Amyloidosis

Two observations have led to the theory that the nature of the pathogenic mechanism resulting in the formation of an amyloid deposit is the same regardless of the biochemical character of the deposit or the clinical setting in which it occurs (18). These observations are that a variety of proteins with fundamentally different amino acid sequences form tissue protein deposits with the same morphological, ultrastructural, and tinctorial properties, and that amyloid deposits with identical proteins occur in seemingly unrelated clinical settings. The elucidation of the pathological processes culminating in an amyloid deposit, regardless of its protein composition, is therefore pertinent to all disorders characterized by amyloid deposition. The studies described here are based on amyloid A, or AA, type amyloidosis experimentally induced in mice and are aimed at elucidating the mechanisms which result in amyloid deposition. The reported findings are therefore relevant to all types of amyloidosis as well as AA amyloidosis in animals and man.

AA amyloidosis (previously termed secondary reactive systemic amyloidosis) is characterized by the deposition of the AA protein usually consisting of 76 amino acids and a molecular weight of 8500 (19-21). The AA precursor molecule, termed serum amyloid A (SAA), circulates in the serum bound to high density lipoprotein (HDL). Although its exact physiological function is unknown, it behaves as an acute phase reactant exhibiting a significant increase in serum concentration levels in clinical situations which produce inflammation (22). It is not surprising then that AA amyloidosis occurs as a complication of chronic inflammatory disorders (such as rheumatoid arthritis), chronic infective disorders (i.e. leprosy, osteomyelitis, and tuberculosis), familial disorders characterized by recurrent inflammatory episodes, and certain malignancies.

Recently, Familial Mediterranean fever (FMF), an autosomal recessive disorder characterized by recurrent episodes of fever , peritonitis, arthritis, and pleuritis as well as a high incidence of AA amyloidosis, has been linked to the alpha-globulin locus on chromosome 16p (23). Other genes involved in the inflammatory process which map to this region include the integrins (a cluster of genes encoding the alpha-subunits of leukocyte surface adhesion receptors). Mutations of one of these genes (i.e. resulting in overadhesiveness) may explain the episodic inflammatory process in FMF. It is unlikely however, that AA amyloidosis associated with FMF is due directly to the identified chromosome 16p locus. Distinct ethnic groups exhibiting major differences in incidence

Table 1.1 Nomenclature and Classification of Amyloid and Amyloidosis

Amyloid Protein	Protein Precursor	Clinical Setting
AA	apoSAA	Reactive (secondary) Familial Mediterranean Fever Familial amyloid nephropathy with urticaria and deafness (Muckle-Wells' syndrome)
AL	kappa, lambda e.g. kIII	Idiopathic (primary), myeloma- or macroglobulinemia-associated
AH	IgG1	
ATTR	Transthyretin	Familial amyloid polyneuropathy, Portugese Familial amyloid cardiomyopathy, Danish Systemic senile amyloidosis
AApoA1	apoA1	Familial amyloid polyneuropathy, Iowa
AGel	Gelsolin	Familial amyloidosis, Finnish
ACys	Cystatin C	Hereditary cerebral hemmorhage with amyloidosis, Dutch
Aß	ß protein precursor e.g. ßPP 695	Alzheimer's disease Down's syndrome Hereditary cerebral hemmorrhage with amyloidosis, Dutch
Aß2M	ß2-microglobulin	Associated with chronic dialysis
AScr	Scrapie protein precursor 33-35, cellular form	Creuzfeld-Jacob disease etc. Gerstmann-Straüssler-Scheinker syndrome
ACal	Procalcitonin	In medullary carcinomas of the thyroid
AANF	Atrial natriuretic factor	Isolated atrial amyloid
AIAPP	Islet amyloid polypeptide	In islets of Langerhans Diabetes type II, insulinoma
AIns	Insulin	Islet amyloid in the degu (a rodent)
AApoII	apoAII (murine)	Amyloidosis in senescence accelerated mice

'Note: "Amyloid Protein" denotes the major protein component of the amyloid deposit.
Abbreviations-AA: amyloid A protein; SAA: serum amyloid A protein; apo: apolipoprotein; L: immunoglobulin light chain; H: immunoglobulin heavy chain.
ßPP 695 indicates the 695 amino acid residue protein.
Scrapie protein precursor 33-35 indicates the 33-35 kilodalton (KDa) forms.
Neither AIns nor AApoII are found in humans.

of AA amyloidosis demonstrate homogeneity, at least with respect to chromosomal location, of the locus responsible for FMF (23).

AA amyloidotic disorders are characterized by a common pattern of organ involvement. Amyloid A (AA) fibrils accumulate primarily within the spleen, liver, and kidneys, followed by possible deposition in the adrenal glands, gastrointestinal tract, lymph nodes, pancreas, prostate, thyroid, heart, and even the lungs (24, 25)

Experimental Induction of AA Amyloidosis

Most of the known forms of amyloidosis have only been identified in humans on whom research is limited for obvious ethical reasons. Though there are relatively few developed experimental models of amyloidosis the animal equivalent of amyloid A (AA) amyloidosis seen in association with inflammation is well established and has proven instrumental in the study of human AA amyloidosis. AA amyloidosis can be induced in a variety of species, with the exception of rats, using protocols involving chronic parasitic infection or multiple injections of inflammatory agents (26, 27). Since its original development in 1922 (28, 29), daily parenteral injections of mice with 10% casein in a 0.3 M sodium bicarbonate solution has provided a reliable and reproducible method of inducing AA amyloidosis and has been shown to involve the deposition of AA fibrils structurally similar to those involved in the human disease (30).

Marked genetic variation in resistance and susceptibility to induction has been observed between inbred strains of mice with CBA/J and C57Bl/6J strains among the most susceptible and A/J among the most resistant (31, 32). Furthermore, genetic analysis using segregating populations of mice derived from a cross between CBA/J and A/J mice has suggested that susceptibility to amyloidosis can be controlled by a single dominant gene (33).

In keeping with the contemporary theory that amyloid resulted from immunological derangement, the amyloid inducing-properties of casein were previously believed to be dependent upon its antigenicity (34). Current hypotheses recognize that a basic immunological disturbance could not account for the diversity of proteins seen as amyloids in the different diseases (35). It is likely that casein's amyloid-inducing capacity is related to its relatively large molecular weight (36) and ability to cause significant inflammation leading to increased SAA serum concentrations. It has been suggested that lipopolysaccharide (LPS) contamination may be the mechanism by which casein solutions promote amyloid deposition (37, 38). Analysis of the LPS content of the casein solution prepared in our laboratory however has revealed very low levels of contamination. Furthermore, it has been shown that orally ingested milk proteins enhance amyloid

deposition (28) even though LPS is degraded in the gut. Finally, C3H/HeJ mice (a low LPS responder strain) develop amyloid when treated with casein (32).

Serum Amyloid A Protein: The AA Fibril Precursor

SAA was originally identified in patients with and without amyloidosis as a serum protein sharing antigenic determinants with the fribrillar tissue AA protein (39). Subsequent comparative amino acid analysis of both AA and SAA proteins revealed identical amino acid sequences beginning at the the N-terminal ends, with the AA protein being shorter at the C-terminal end (40). These observations suggest that cleavage of an SAA precursor molecule at its carboxy-terminal end could result in the formation of the truncated AA protein. Indeed the transfer of human acute phase HDL into amyloidotic mice has resulted in human SAA being incorporated into mouse amyloid tissue (41).

As mentioned previously, the SAA protein has been identified in a variety of species as an acute phase response (APR) apolipoprotein which, like most APR proteins, is synthesized principally by the liver. During an APR, SAA associates with HDL, in particular the HDL_3 fraction, of which it can become a predominant apolipoprotein exceeding apolipoprotein A-I (apo-A-I) in molar ratio (42).

In all species studied to date, SAA is not a single protein but rather a family of several related proteins. The SAA genes reveal strong cross-species conservation among vertebrates and, in humans and mice, are clustered in small homologous regions on chromosomes 11p and 7 respectively (43, 44). In mice three genes (SAA1, 2, and 3) as well as a pseudogene (45), have been characterized. Although SAA1 and SAA2 show 96% homology, SAA3 is substantially divergent. All three genes are transcribed (46) however only SAA1 and SAA2 mRNA products give rise to dramatically elevated levels of hepatic mRNA and circulating, HDL-associated protein during APR. While SAA3 does contribute moderately to hepatic mRNA production, it yields no detectable, translated plasma or tissue product. The liver and, to a lesser extent, the gastrointestinal tract, produce the majority of SAA1 and SAA2 (47). It has been shown that tissues other than the liver and the gastrointestinal tract can, however, express SAA3 mRNA (48).

Three members of the human SAA gene family containing transcribable and translatable sequences have been identified (49, 50) as well as a fourth pseudogene (51). Three hepatic mRNA species code for the six major isoforms purified from the HDL_3 plasma fraction of individuals undergoing APR (52, 53). However, the gene family probably contains considerable polymorphism and, more recently, additional allelic and nonallelic variants have been reported (54, 55).

Much work has been done, in both humans and mice, showing that during an inflammatory reaction cytokines produced by activated macrophages, namely interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor, are responsible for regulating the transcription of SAA genes in the liver (56, 57, 58). The magnitude of apo-SAA synthesis appears to be controlled by cytokines acting synergistically (59, 60) involving both NF-ß and IL-6 response elements identified within the SAA promoter region (61). The mechanisms of synergistic stimulation of SAA production appears to involve enhanced mRNA transcription and subsequent SAA mRNA stabilization (62, 63). During disturbances in homeostasis plasma concentrations of SAA can increase from 100 to 1000 fold within 18-24 hours in mice (22) or 48-72 hours in man (56). During increased secretion SAA can comprise up to 20% of total murine HDL apoproteins (64).

Relatively little information is available regarding the biological significance of apo-SAA. It has been shown to modulate HDL lipid metabolism (65) during an APR, inhibit platelet function (66), exert immunosuppressive activity (67), and inhibit IL-1 and TNF-induced fever (68). In addition, rabbit synovial fibroblasts have been found to produce an SAA-like protein with capacity for autocrine modulation of collagenase production (69). A similar protein, GSAA1, has been described at the gene level in humans (70). It is possible that SAA functions physiologically as a regulatory protein inhibiting a variety of acute phase responses and/or regulating the removal of specific constituents from sites of tissue destruction.

Due to the amino acid sequence identity between murine apo-SAA2 and murine AA fibrils, apo-SAA2 is generally believed to the be the sole precursor of AA fibrils in mice (71). In the first 25 amino acid residues, murine SAA1 and SAA2 differ at only two positions (45, 71). It has been shown that synthetic peptides corresponding to the N-terminal 11 amino-acid-residue segment of SAA2 form amyloid-like fibrils *in vitro*, while the corresponding segment of SAA1 do not. This finding indicates that differences in the N-terminal portion of SAA determine the amyloidogenicity of murine SAA isoforms (72).

It has been suggested that SAA2 is rapidly and selectively removed from the circulating pool of SAA1 and SAA2 due to a dramatic decrease in plasma SAA2/SAA1 ratios (concurrent with a fairly constant SAA2/SAA1 mRNA ratio) during a 20-day course of murine amyloid induction using casein (73). Research done in our lab however did not indicate a selective decrease in serum apo-SAA2 before 38 days of casein injections, therefore well after amyloid deposition had been initiated (74). The relative time difference between these two studies may be due to the fact that the former study (73) measured SAA isotypes by isoelectric focusing versus SDS-PAGE followed by densitometry in the latter (74). It is possible that isoelectric focusing allows for better separation of the two SAA

isotypes leading to a better assessment of the ratio of each apoprotein. Furthermore, the apoSAA2 isotype is more easily displaced from its HDL carrier than apoSAA1 (75) and elements such as lipopolysaccharide and apoproteins AI and AII have been shown to compete with SAA for binding to the HDL carrier (75-77). Differential binding affinities between the two SAA isotypes as well as competition for binding with other factors may well influence apoSAA ratios as detected by different methods. For example, research was conducted in our lab using mice bred and maintained in specific pathogen free facilities. Reduced concentrations of circulatory lipopolysaccharide may well delay the selective removal of apoSAA2. Should this be the case, it would appear that amyloid deposition occurs regardless of whether apoSAA2 is selectively removed from the circulation.

Though some studies have reported that human SAA1 gene products account for the vast majority (80%-100%) of the AA protein isolated from any one individual (78), it is generally believed that preferential deposition of particular isoforms does not occur in humans. AA-proteins isolated from various individuals have been found to vary from 94 to 43 residues (positions 2-44) indicating that an amyloidogenic region is the N-terminal part of the molecule (79). No N-terminal variation is seen between the different human SAA isoforms possibly explaining why especially amyloidogenic human SAA variants have not yet been clearly indicated (72).

Process of AA Amyloid Deposition

The development of AA amyloidosis has been described as involving two phases: the pre-amyloid phase and the amyloid phase (80, 81). The first phase is comprised of sequential host responses to tissue injury and cell necrosis (i.e. an APR) including macrophage activation and cytokine production, transient hepatic SAA production and association of apo-SAA with HDL_3 ultimately resulting in elevated levels of circulating SAA. The proceeding amyloid phase is characterized by SAA precursor processing into AA molecules. Fibrils are assembled from filaments of AA molecules which, together with other constituents, accumulate as bundles of fibrils which constitute fibrous deposits within the tissues. Lateral aggregates of fibrils and filaments have been observed in proximity to cells, oriented perpendicularly to, and merging with the cell membrane. Amyloid fibrils are observed as bundles near cell membranes and more dispersed at greater distances from cells. Organ deposition in AA amyloidosis occurs primarily within the spleen, liver, and kidneys. The earliest site of deposition is the perifollicular area of the spleen in close proximity to phagocytic cells, around the follicles, and extending outwards to the red follicular pulp (82). Hepatic amyloid deposits are initially detectable around Kupffer cells

and parenchymal cells. In the kidney, deposition initiates within the mesangial region glomeruli then spreads to the basement membrane, endothelial cells, and renal medulla

In addition to the principal amyloid protein component of amyloid deposits other constituents have been found to be included in amyloid deposits of all varieties. At some point during the process of amyloid formation, these factors, most notably amyloid P protein, glycosaminoglycans, and AEF become involved resulting in their inclusion within the deposit. The importance of these components will be considered below

COMMON ELEMENTS OF AMYLOID DEPOSITS

Besides sharing common, definitive structural properties, all types of amyloid deposits tested to date have been found to include both amyloid P protein (AP), highly sulphated glycosaminoglycans (GAGs) and apolipoprotein E (apo E)

Serum Amyloid P Component:

During the identification of the various amyloid constituent proteins, an alpha-globulin was found associated with amyloid fibrils. Once isolated, this plasma component, termed amyloid P component (AP), was determined to be a pentagonally structured protein distinct from amyloid fibrils and identical to a normal circulating plasma constituent which was then called serum amyloid P component (SAP) (83). The terms AP and SAP therefore simply denote the different locations (either associated with amyloid tissues or circulating in the serum respectively) of the same protein. Though initially suspected to be absent from intracortical and intraneuronal amyloid deposits characteristic of Alzheimer's disease (AD) (84), AP immunoreactivity has now been detected in neurofibrillary pathology of AD and other neurodegenerative disorders characterized by amyloidotic plaques (85). AP has been detected in all other types of amyloid tested to date.

Studies done in AA amyloidosis in mice have given direct evidence that circulating serum SAP is indeed the precursor of AP within amyloid deposits. In short, when an exogenous source of either human or murine ^{125}I-labelled SAP is injected into AA amyloidotic mice, it is deposited within the liver, spleen and kidneys in a concentration directly proportional to the extent of AA fibril deposition (86). AP is stacked on AA deposits, therefore is not an integral part of the amyloid fibril, and may amount to 10-15% of the total AA fibril mass. Its deposition has been found to be coincidental with the appearance of AA fibrils (86).

SAP, human C-reactive protein (CRP) and other animal homologues such as Syrian hamster female protein (FP) are all members of a family of pentraxin proteins characterized by a discoid conformation of 5-10 noncovalently bound subunits arranged as two stacked pentraxins. Though a considerable amount of sequence homology exists between these

proteins they remain immunologically distinct SAP has been found in all vertebrate species investigated including the mouse, rabbit, pig, goat, sheep, donkey, human, cow, marine toad, plaice, flounder, and dogfish (87). Structurally, human AP consists of 10 identical alpha-glycoprotein subunits each of approximately 23 000 with a total molecular weight of 230 000 (88, 89). Murine SAP monomers migrate at a relative molecular weight of 31 000 (90) and possess approximately 10% carbohydrate (91).

Synthesis of SAP occurs in the liver. In man, the half-life of AP is 7.8-8.5 hours, suggesting a rapid continuous production to maintain a stable and significant serum level of 40-50 ug/ml (92). SAP is increased slightly in chronic active disorders including certain malignancies and rheumatoid arthritis (93).

In mice, SAP acts as an acute phase protein (90). There are marked, genetically determined differences in resting SAP levels within inbred strains of mice yet all strains appear to reach the same serum concentration (~170-240 ug/ml) within 24-48 hours after an inflammatory stimulus (94). *In vivo* and *in vitro* studies examining the effects of IL-1 (95), IL-6 (96), and TNF (97) have established that these molecules are involved in the mediation APR protein induction. Crude macrophage supernatants and purified IL-1 have been shown to regulate SAP synthesis specifically (98) and recombinant IL-6 and IL-1 are known to exhibit synergistic induction of hepatocyte SAP synthesis *in vitro* (99). Hepatocyte cultures exhibit sensitivity to cyclohexamide treatment with respect to SAP synthesis, yet relative resistance with respect to SAA synthesis, suggesting that different regulation by protein factors occurs in each case (100). Inhibition of SAP synthesis by actinomycin-D and colchicine indicates that regulation may occur both pre-translationally and during secretion (101).

While the biological function of SAP is not known, the stable evolutionary conservation of overall molecular structure and binding specificty within its family of pentraxin proteins suggests that it plays an important physiological role (56). AP has been reported to be a component of the microfibrils that are the structural units of elastin fibers in connective tissues (102) as well as a normal matrix glycoprotein of glomerular basement membrane (103). In addition, SAP has been shown to bind amyloid fibrils (104), fibronectin (105), heparan sulphate and dermatin sulphate (106), and C4-binding protein (105) in a Ca^{2+}-dependent manner and macrophages via their mannose 6-phosphate receptor (107). It possesses a high affinity, Ca^{2+}-dependent binding site for the 4,6-cyclic pyruvate acetal of the galactose residues present in agarose as the galactan homopolymer and in the cell walls of a few bacteria (108). SAP has also been reported to interact with immobilized single or double-stranded DNA in a Ca^{2+}-dependent manner (109). Furthermore, SAP is able to displace H-1 and interact with chromatin (110). It has been

proposed that SAP may solubilize extracellular DNA and help clear it from the circulatory system. In addition, SAP undergoes self-aggregation in the presence of calcium (111) Heparin and other glycosaminoglycans have been shown to inhibit this polymerization (106). It has therefore been suggested that heparin may influence the aforementioned interaction between C4-binding protein and SAP *in vivo*, thereby altering the distribution of SAP and C4-binding protein in the serum. In this case SAP may act as an important regulatory protein in the complement and/or coagulation systems (111, 112)

The role that SAP may play in the pathological process of amyloid deposition has yet to be elucidated. Studies of differences in SAP induction in amyloid susceptible and -resistant inbred strains of mice indicate that sustained high levels of SAP are consistent with susceptibility (113). It has been postulated that AP associated with microfibrils at the periphery of elastic fibers may bind amyloid fibril material and encourage its deposition at this site (102). Alternatively, SAP may influence amyloid deposition by binding to fibrils thereby protecting them from enzymatic degradation SAP has indeed been shown to inhibit the activity of enzymes including porcine pancreatic enzyme, and *Pseudomonas* and human elastase (114, 115).

Proteoglycans and Glycosaminoglycans .

Proteoglycans (PGs) are a diverse group of extracellular matrix proteins comprised of a glycosaminoglycan (GAG) attached to a protein core GAGs are sulphated carbohydrate moieties of which there are four types, namely heparin/heparan sulphate, chondroitin sulphate/dermatan sulphate, keratin sulphate, and hyaluronic acid (116) PGs are distinguished by their GAG content.

Highly sulphated GAGs have been demonstrated in all forms of amyloid examined to date (117). In AA amyloidosis, glycosaminoglycans and amyloid peptides have been shown to be deposited coincidentally (118) and in amounts directly proportional to the extent of amyloid deposition (119) This association between GAGs and AA amyloid deposits has been consistently observed regardless of the nature of inflammatory stimulus used to induce deposition, the organ site of deposition, or the length of time between induction and fibril deposition (118) Furthermore, studies have shown that the major type of proteoglycan occuring in AA deposits in experimentally induced amyloidosis is the basement membrane proteoglycan constituent heparan sulphate proteoglycan (HSPG) (120). Though cationic staining studies have been interpreted to show that both the protein core and the carbohydrate core are intimately associated with AA fibrils (121), alternative research comparing amyloid associated glycosaminoglycans before and after ß-elimination or enzymatic digestion seem to indicate that glycosaminoglycans associated with amyloid fibrils isolated from AA amyloidotic human liver do not appear as part of intact

proteoglycans (122). HSPG has been described in at least five forms of amyloid including AA, AL, the ß-amyloid of Alzheimer's disease, the prion amyloids in Creutzfeldt-Jacob disease, Gerstmann Straussler Syndrome, experimental scrapie, and the amyloid associated with type II diabetes (120, 123-125). In addition, the dermatan sulphate proteoglycan has been detected in AA amyloidosis and ß-amyloidosis in Alzheimer's disease (122,126).

The significance of consistent codeposition of GAGs with amyloid fibrils remains a mystery. As previously mentioned, the SAA2 isoform is the sole precursor to AA amyloid in mice. Studies with different GAGs and murine SAA1 or SAA2 showed that only SAA2 and heparan sulphate interacted so as to markedly increase ß-sheeting in SAA2 (127) The association of SAA2 with the GAG also protects the SAA2 from proteolytic digestion (128). This observation suggests that GAG or HSPG may play a role in both the pathogenesis and the structure of the amyloid fibrils. One potential way in which this might occur is through an ionic interaction between the amyloid peptide, or its precursor, and the sulphate moieties on the GAG. By sequence analysis, potential GAG binding sites have been identified on SAA (129).

Apolipoprotein E

More recently, apolipoprotein E (apo E) has been detected in association with a wide variety of amyloid fibrils including AA , ß-protein, scrapie protein, immunoglobulin light chain, and cystatin C amyloids (130, 131). Apo E is a 299 amino acid protein with a molecular weight of 34 000 (132). Besides its structural role in a number of lipoprotein particles, apo E is known to regulate lipoprotein metabolism. In addition apo E has been suggested to function as neurotrophic factor (133) as well as an immunoregulatory molecule (134). Apo E is produced by most organs with its greatest concentrations occuring in the liver and brain. A range of cell types, including macrophages, can produce apo E (132). Apo E is the principal lipoprotein in cerebrospinal fluid, where its levels have been observed to increase during certain central nervous system disorders (135). Apo E binds GAGs (136). It is possible that the consistent detection of apo E in amyloid deposits is simply the result of non-specific binding to GAGs.

At this point it is not clear whether the common presence of AP, GAGs, and apo E in such a variety of amyloids is the result of their integral role in the pathological process of amyloid deposition. Some investigators have felt that amyloid deposits represent a "sink" into which a variety of components including GAGs (137), and possibly SAP and apo E, are drawn. Indeed a diversity of proteins have been detected in some amyloids, for example vitronectin (138) or alpha-antichymotrypsin (139). To date, however, SAP, heparan sulphate, and apo E remain the sole non-amyloid protein constituents common to

all amyloid deposits examined, regardless of the type of amyloid deposit or the clinical situation in which the amyloid deposit occurs. It has been shown that amyloid fibrils can be formed *in vitro* in the absence of AP and GAGs (11) suggesting that a fibrillogenic protein and appropriate environmental conditions could be the exclusive requirements for the formation of amyloid deposits. It is possible however, that AP, GAGs and apo E function as 'pathological molecular chaperones' (130). 'Molecular chaperones' are a newly recognized family of proteins, found in all cell types, that interact with other polypeptides specifically so that they assume a functional tertiary structure, preventing them from undergoing incorrect interactions (141). 'Pathological chaperones' have therefore been defined as a group of unrelated proteins that mediate ß-pleated amyloid formation of polypeptide fragments without becoming integral components of the amyloid fibril (130). As such pathological chaperones AP, GAGs, and apo E may function by binding specifically to amyloidogenic protein surfaces that are formed during aberrant protein degradation thus preventing further proteolysis. Alternatively, they may be essential to establish or maintain conditions amenable to fibril formation or prevention of immediate resorption of fibrils before formation of bundles of fibrils and accumulation of amyloid deposits (129).

Much of the recent work concerned with elucidating the role of GAGs and SAP in amyloid deposition has focussed on the possible, fundamental involvement of the basement membrane. The basement membrane is a specialized form of extracellular matrix which provides an underlying structural support for epithelial cells as well as neuronal and mesenchymal elements (142). Basement membranes are composed of several major components including fibronectin, laminin, type IV collagen, and proteoglycans, each of which have been identified in AA amyloid deposits (143). Furthermore, AP is a normal constituent of basement membrane (103). Basement membrane disruption is observed in both AA amyloids and amyloid associated with Alzheimer's disease (144, 145). This basement membrane dysfunction, coupled with the inclusion of basement membrane components in amyloid deposits, has led to the postulation that amyloid peptides and/or their precursors could influence basement membrane organization or that basement membrane components could influence amyloid peptide organization into fibrils (146).

Amyloid Enhancing Factor (AEF)

It has become increasingly evident that a component isolated from amyloidotic tissue, termed amyloid enhancing factor (AEF), may also be included under the heading of "common elements of amyloid deposits". AEF was originally described in the context of AA amyloidosis (147), however its activity has now been identified in tissue extracts of at

least four forms of amyloid including AA, AL, ß-amyloid, and transthyretin amyloid (148, 149) implicating its probable relevance in all types of amyloid. It has been postulated that AEF may act as a common pathogenic link between the diverse forms of amyloid (150).

Though definitive biochemical characterization of AEF has not yet been accomplished, AEF can be identified by its ability to induce amyloid deposition 24 to 48 hours following coadministration with a single inflammatory stimulus (in animals undergoing sustained inflammation, amyloid deposition is not usually detected before 15 days) (150). Treatment of experimental animals with AEF alone (i.e. in the absence of an inflammatory stimulus) causes neither inflammation nor amyloidogenesis. AEF activity has been detected in cell suspensions from whole or partial sections of spleen or lymph node, and spleen, liver, kidney, heart, and bone marrow extracts from pre-amyloidotic and amyloidotic animals (151-154, 27, 4). It has been extremely difficult to separate AEF activity from the AA protein (155), however neither purified amyloid proteins nor commercially purified GAG preparations possess AEF activity (3, 119). Furthermore, when mice are treated with AEF of human origin in conjunction with an inflammatory stimulus, the AA deposited is of murine origin (150). Additionally, when mice are injected with murine AEF and human SAA, the amyloid fibrils are of human origin (41). These observations support the concept that AEF is an entity separate from the major amyloid fibril protein component or the GAG component of amyloid deposits.

Studies have shown that the concentration of AEF increases markedly during inflammation (5) and in doing so alters the metabolic processing of at least one acute phase reactant, apoSAA (156). Most research with regards to the origin of AEF suggests that it is derived from and exerts its action at the level of the macrophage. Indeed, it is likely derived from a cell common to both the liver and the spleen (i.e. reticuloendothelial cells) since splenectomized animals can generate AEF activity in their livers upon appropriate stimulation (157). AEF appears approximately 24-48 hours before the histological detection of amyloid fibrils (3). The kinetics of amyloid deposition in experimental animals using standard induction protocols or following the passive transfer of AEF, are identical with the exception of the lag period necessary for the appearance of AEF when using standard regimens (157). The lag phase for AEF induction is obviated during passive transfer. AEF activity has been indicated to be maintained for at least four weeks following intravenous injection, prior to clearance by reticuloendothelial cells (4).

The importance of AEF in amyloidosis has been clearly demonstrated in studies of amyloidosis susceptible inbred strains of mice. For example, work done in our lab was able to show that, following standard AA amyloidosis induction, genetically amyloid resistant A/J mice did not exhibit amyloid deposition nor did their spleens contain detectable

AEF activity. When these mice received AEF prepared from the spleens of amyloidotic, susceptible C57Bl/6 mice however, they developed amyloidosis at an accelerated rate. It was therefore deduced that resistance to amyloidosis in A/J mice can be attributed to their inability to produce sufficient AEF and that susceptibility to AA amyloidosis correlates with the increased concentration of AEF (158).

Methods of extraction of AEF activity vary from laboratory to laboratory. Isolation has been achieved by a variety of methods including homogenizing amyloidotic tissues in glycerol/TRIS-buffered solutions with further precipitation of AEF from the supernatant by dialysis against PBS (4), fractionation of the precipitated proteins by chromatographic methods (4, 154), precipitation of AEF activity from homogenized organs with ammonium sulphate followed by fractionation of activity with Sephadex, Biogel P-60 and benzamidine-Sepharose 6B affinity chromatography (5, 159), and primary extraction in high salt (KCl) concentrations (4). None of these methods have allowed for the complete purification of any specific component or components with AEF activity. The fact that AEF is relatively insoluble in physiological buffers has dramatically hindered the purification process. Work done in our lab has taken the purification of crude AEF extract prepared according to the first method described above one step further by solubilizing AEF activity in a 6% urea/ 1% ß-mercaptoethanol/ 1 mM EDTA buffer amenable to further purification procedures.

Most research examining the nature of AEF has assumed that it is a single, unique molecule. AEF has been assigned a number molecular sizes ranging from 5 .5 kDa (160) to 55.1 kDa (L. Hébert, unpublished observations) the smallest being attributed to ubiquitin (160). Accumulating evidence indicating different molecular sizes with respect to various AEF preparations suggests that the AEF activity detected in each type of amyloid deposit may not be attributable to a single, common molecule but rather to a group of molecules, perhaps with unrelated physiological functions, but sharing some exceptional characteristics which lead to their AEF activity in each type of amyloidosis.

Reports have claimed that a substance with AEF activity, detected in amyloid-laden tissue from both AA amyloidotic mice and patients with Alzheimer's disease, is actually ubiqutin (160, 161). Immunoblotting of crude AEF extracts, prepared in our lab from each of these sources, did not indicate the presence of ubiquitin (unpublished observations). Furthermore, when mice received an i.v. injection of ubiquitin in conjunction with a s.c. injection of silver nitrate as an inflammatory stimulus, we did not detect splenic AA fibril deposition within 72 hours. Differing AEF extraction protocols or AEF activity assessment regimes may explain apparent ubiquitin-associated AEF activity. For example, the studies confirming ubiquitin's AEF activity (160, 161) involved mice undergoing an inflammatory

response induced by s.c. silver nitate injections for 4 consecutive days. Animals were not sacrificed until 24 hours after receiving the final silver nitrate injection. Our AEF bioassay is conducted treating mice with a single s.c. injection of silver nitrate administered concomitantly to AEF injection. Animals are sacrificed 48 to 72 hours later. Continuous inflammatory stimulation of mice with multiple injections of silver nitrate may well result in the induction of some factor, such as endogenous AEF, which may use ubiquitin as a co-factor to produce accelerated AA amyloid deposition. Ubiquitin, when injected in this fashion, may then exhibit AEF-like bioactivity.

AEF has been proposed to promote amyloid deposition in a number of ways, none of which have been proven. As previously discussed, it is not clear whether selective removal of the AA amyloid precursor occurs during the development of amyloidosis in mice and does not appear to occur in humans. Data collected in our lab has shown there is no change in circulating serum apoSAA1/apoSAA2 ratios following accelerated amyloid induction by injection of AEF. If proven conclusively, this would suggest that AEF does not affect the SAA2 precursor at a stage proceeding the normal association of acute phase reactants with their HDL carriers (74). One hypothesis suggests that AEF could act as a nidus for fibril deposition (119) which disrupts local GAG synthesis or clearance thereby perpetuating amyloid deposition. AEF has also been suggested to alter the activity of lysosomal glycosidases (162) or may itself be a leukocyte-derived thiol/serine protease (163). The clarification of the role of AEF in the development of amyloidosis as well as the elucidation of its biochemical nature in AA amyloidosis are the focus of this thesis.

RETICULOENDOTHELIAL CELLS IN AMYLOIDOSIS

Fibril deposition in amyloidosis has been consistently observed in close association with reticuloendothelial cells (RE) *in situ* (164) suggesting that these cells play a significant role in fibril deposition. Both histological and experimental evidence points to the importance of phagocytic cells in amyloid fibril formation, particularly in inflammation-associated AA amyloidosis, in which mononuclear phagocytes are involved in the regulation of precursor production, formation of fibrils, and intraphagosomal digestion of fibrils (11, 165).

Normal peripheral blood monocytes are known to be capable of mediating SAA degradation *in vitro* through the action of cell surface enzymes. This degradative pathway is thought to involve initial proteolytic cleavage producing AA as an intermediate followed by complete proteolysis into smaller fragments (166, 167). Studies aimed at elucidating the exact relationship between RE cells and amyloid fibril deposition have led to the discovery that mononuclear leukocytes from healthy individuals are capable of mediating complete

SAA degradation whereas the cells of amyloidotic patients produce an intermediate protein fragment both the same size and immunologically cross reactive with AA (166). In addition, normal murine Kupffer cells have been found to degrade SAA completely, whereas Kupffer cells from amyloidotic animals produce the AA intermediate causing accumulation of the AA protein (168). These findings have led to the hypothesis that tissue amyloid deposition may be due to innappropriate or incomplete SAA degradation by cells of the monocyte/macrophage lineage, resulting in the accumulation of AA.

It has been suggested that a pathological modulation in macrophage-associated SAA degradation develops during the prolonged, pre-amyloid phase of standard AA-amyloid induction protocols. Induction of endogenous AEF is thought to occur during the pre-amyloid period (3). In keeping with this, treatment with an exogenous source of AEF in conjunction with an inflammatory stimulus would incur a more immediate macrophage modulation causing accelerated amyloid deposition. The theory that AEF may be the effector behind altered macrophage function ultimately resulting in AA deposition is supported by data comparing the kinetics of AA intermediate accumulation and the appearance of AEF activity (168, 3) in which detectable AEF activity necessarily precedes AA fibril formation. It is logical that a specific, pathological factor such as AEF must be present to cause incomplete SAA metabolism. If activated RE cells alone were responsible for the appearance of AA, then AA should be deposited in most inflammatory conditions and at the sites of inflammation (18). Amyloid deposition as a complication of inflammation, however, is rare and initial amyloid deposits, at least experimentally, are always found in precise anatomical locations within the spleen and liver but never at the sites of inflammation. In addition, both clinical and experimental observations show that serum SAA levels may be elevated for prolonged periods in the absence of amyloid deposition.

It has been established then that the mononuclear phagocyte system plays a central role in amyloidosis in that it provides cytokine signals that regulate expression of some amyloid precursors as well as the proteolytic enzymes that convert precursors to fibril proteins. It is therefore important to review the phenotypic and functional characteristics of the macrophage, particularly in the context of inflammation. Some of the work presented in this thesis involves the evaluation of the functional state of activated, splenic macrophages (including phagocytosis, bactericidal and respiratory burst activity, and MHC Class II expression) during AEF-induced AA amyloidosis Consequently, we will review some of the principles specifically related to those elements of tissue macrophage response

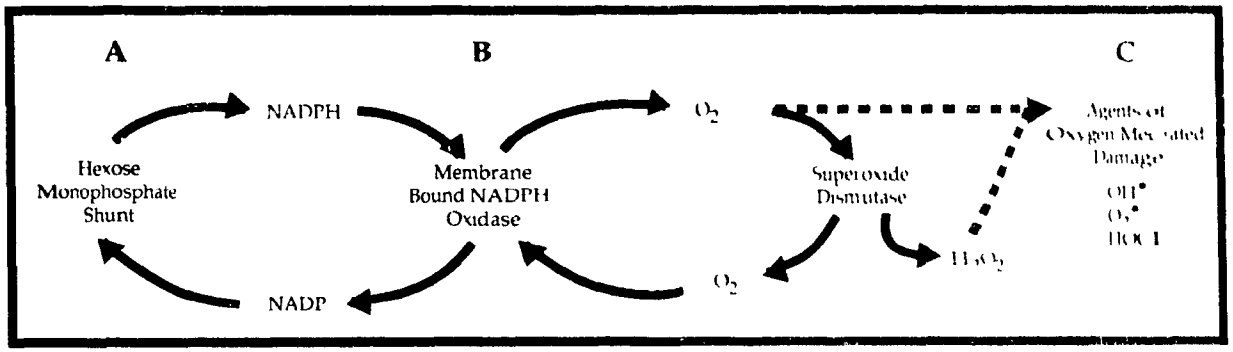
The mononuclear phagocyte (or reticuloendothelial cell) system represents a group of widely distributed, multifunctional, and morphologically heterogenous cells.

Originating from bone marrow stem cell precursors, all cells within this lineage pass through monoblastic then promonocytic developmental stages prior to release into the blood as monocytes. Following a short circulation period, monocytes migrate into tissues to form the macrophages of the lungs, liver, spleen, lymphoid organs, pleural and peritoneal cavities, bones and so on, where they undergo final, tissue-specific differentiation (169). In general, macrophages are key players in both the immune and inflammatory reponses. They respond to a variety of stimuli with aggregation, activation of movement, secretion, endocytosis, activation of respiration, and production of mediators including those that perpetuate inflammation, and are capable of phagocytosis and processing antigen (170, 171). During sustained inflammation macrophages become "activated", resulting in enhancement or initiation of certain functions including phagocytosis, bactericidal and respiratory burst activity, and antigen presentation (172-175). In the context of chronic inflammation, the activated macrophage is therefore relevant to the development of AA amyloidosis.

When mononuclear phagocytes encounter invading microorganisms or other appropriate stimuli, they undergo a "respiratory burst" in which they consume and convert oxygen into the potent microbicidal agents superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and singlet oxygen. These oxidants are reactive enough to destroy most biological molecules and are responsible for much of the damage inflicted by phagocytes on both microorganisms and surrounding tissues at sites of infection or inflammation. The metabolic pathway responsible for reactive oxidant production is initiated within a few seconds of plasma cell membrane exposure to any one of a large number of stimuli, among the most effective of which are several that are likely to be present at inflammatory sites including opsonized microorganisms, complement fragment C5a, leukotriene B_4, and N-formylated oligopeptides of bacterial origin. Activation of this membrane-associated pathway is characterized by an abrupt increase in oxygen uptake devoted to the production of a series of compounds including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and other oxygen-containing compounds, all of which are highly reactive. In addition, augmented oxidation of glucose occurs via the hexosemonophosphate shunt (HMPS) (176). The biochemical basis for the respiratory burst is described in in detail in Figure 1.2. Reactive oxidants are essential to the increased microbicidal capacity of activated macrophages (177).

In addition to its direct effector role in microbicidal destruction, the macrophage contributes to regulation of specific immune responses. This complex function includes

Fig. 1.2. THE BIOCHEMICAL BASIS OF THE RESPIRATORY BURST
Adapted from Babior *et al* (173).



A) The Hexose Monophosphate (HMP) Shunt. Glucose oxidation through the HMP shunt accelerates during the respiratory burst due to increased production of $NADP^+$, the availability of which limits the activity of the HMP shunt. $NADP^+$ is produced through the actions of (a) activated NADPH oxidase and (b) a glutathione dependent system that uses NADPH to detoxify H_2O_2 as follows:

(a) **$2\ O_2 + NADPH$** NADPH oxidase **$2\ O_2^- + NADP^+ + H^+$**

(b) **$NADPH + H_2O_2$** Glutathione peroxidase/reductase **$NADP + 2\ H_2O$**

Each glucose metabolized via the HMP shunt reduces two molecules of NADP to NADPH via the following set of reactions:

Glucose-6-P + NADP glucose-6-P dehydrogenase **6-phosphogluconate + NADPH**

6-phosphogluconate + NADP 6-phosphogluconate dehydrogenase **Ribulose-5-P + CO_2 + NADPH**

Thus NADPH necessary for the continued operation of the respiratory burst is replenished

B) The biochemical basis for the respiratory burst is the activation of NADPH oxidase dormant in resting cells, which catalyzes O_2 reduction at the expense of NADPH. Oxygen consumption and O_2^- production occuring during the respiratory burst are accounted for entirely by this reaction. H_2O_2 is then produced primarily via enzymatically catalyzed dismutation of O_2^- by superoxide dismutase.

C) The reactive oxidants responsible for most oxygen-mediated damage by phagocytes are generated from O_2^- and H_2O_2 produced in the respiratory burst. O_2^- and H_2O_2 are converted by a complex series of secondary reactions to a) oxidizing radicals and b) oxidized halogens. Examples of each of these are:

a) the $OH^{\bullet}$ (hydroxyl radical) produced primarily by the so called "modified Harber-Weiss" reaction:

$$O_2^- + Me^{n+1} \quad Me^n + O_2$$
$$\underline{Me^n + H_2O_2} \quad Me^{n+1} + OH^{\bullet} + OH$$
$$O_2^- + H_2O_2 \quad O_2 + OH^{\bullet} + OH^-$$

where Me^{n+1} represents the oxidized form of a trace metal.

b) oxidation of ubiquitous Cl^- ions to hypochlorous acid by H_2O_2 generated during the respiratory burst:

$$Cl^- + H_2O_2 \xrightarrow{\text{myeloperoxidase}} HOCL + H_2O$$

response, foreign antigens are internalized by macrophages and cleaved into peptide fragments which are returned to the cell surface as peptides bound to class II MHC molecules. Appropriate T lymphocytes expressing clonally derived receptors are then activated through recognition of the macrophage surface complex between the foreign peptide and the MHC molecule (178).

Class II MHC molecules are heterodimers of two highly polymorphic membrane glycoproteins of 33 to 35 kDa (the *a*-chain) and 27 to 29 kDa (the ß-chain). Each consists of two extracellular domains, a connecting peptide, a transmembrane domain, and a cytoplasmic tail. In the mouse there are two class II MHC, or Ia, molecules, A and E, which are encoded by a number of genes (A*a*, Aß, E*a*, and Eß respectively) residing in the MHC complex (179).

Macrophage Ia antigen expression is not constitutive, but is subject to regulation by cytokines. Research has shown that Ia expression is induced primarily by interferon (IFN)-gamma (180), however tumor necrosis factor-*a* (181), IL-4 (182), and granulocyte-macrophage colony-stimulating factor (183) also exhibit this capability. Furthermore, it appears that MHC class II expression in the macrophage is regulated mainly by transcription, either positively or negatively. Control of transcription has been directly linked to rapid transductional sequences initiated by IFN-gamma as well as to the binding of proteins to specific regions upstream of the promoter in macrophages (184). The enhanced expression of murine macrophage Ia antigens may be used as a marker for activation (179, 184).

Some of the studies described in this manuscript involve the quantification of spleen macrophages expressing Ia as a means of monitoring their activation state. To achieve this, a double antibody label system specific for Mac.1 and Ia surface antigens was used. Mac.1 is a differentiation antigen known to be involved in leukocyte adhesion reactions. It is a member of a family of structurally and functionally related leukocyte surface glycoproteins which also includes lymphocyte function-associated 1 (LFA-1) and p150,95 antigens. Each of these antigens are characterized by *a*1 and ß1 noncovalently associated subunits and are highly conserved in both mouse and man (185). Mac.1 is present on myeloid lineage cells but absent from lymphoid cells. In mice, it is expressed principally on macrophages, regardless of their activation state, but can also be detected on granulocytes and natural killer cells at a significantly lower surface density (186).

By labelling spleen cell isolates with Ia and Mac.1 specific antibodies prior to analysis by flow cytometry we were able to distinguish between macrophages expressing Ia antigen on their surface (Mac.1$^+$Ia$^+$), B cells (Mac.1$^-$Ia$^+$) and surface Ia negative macrophages (Mac.1$^+$Ia$^-$). As indicated previously, granulocytes and natural killer cells

express relatively small quantities of Mac.1 therefore do not stain intensely enough for detection as Mac.1$^+$ in our system.

CONCLUSION

To summarize, AA amyloidosis is common to a host of seemingly unrelated disorders. Clinically it can be a complication of long-standing inflammation, familial disorders, and various forms of malignancy and can be induced experimentally in animals through chronic inflammation. A common protein being deposited in such different clinical settings suggests there is a common pathogenic mechanism occurring in each case (81). The AA protein deposited in AA amyloidosis is thought to be derived from the N-terminal end of the naturally occuring circulating serum apolipoprotein SAA (40). ApoSAA is an acute phase serum protein of unknown function whose serum concentration increases both rapidly and substantially during any inflammatory process (22, 56). Activation of a variety of cells including polymorphonucleocytes and reticuloendothelial cells is an integral part of any inflammatory reaction. Upon activation, these cells release a range of inflammatory cytokines including IL-1, IL-6, and tumor necrosis factor, which are responsible for upregulating SAA gene transcription (56, 57, 58) such that apoSAA forms a substantial part of HDL during inflammation (64). The relative rarity of amyloidosis as a complication of inflammatory disease indicates however, that factors other than the availability of sufficient amounts of precursor protein are essential if amyloid deposition is to occur.

Little is known of the processing events which lead from the circulating apoprotein precursor to the AA fragment characteristic of the amyloid deposit. The *in situ* association between reticuloendothelial cells and amyloid fibrils is well known (164). Furthermore, both mononuclear leukocytes from healthy individuals and normal murine Kuppfer cells have been found to completely degrade SAA *in vitro* most likely through the action of cell surface enzymes. Cells from amyloidotic patients and mice, on the other hand, have been shown to produce an AA intermediate (166-168). These observations are among those supporting the popular hypothesis that tissue amyloid deposition is the result of innappropriate or incomplete SAA degradation by cells of the monocyte/macrophage lineage, resulting in the accumulation of AA.

The theory that AEF may be the effector behind altered macrophage function ultimately resulting in AA deposition is supported by data comparing the kinetics of AA intermediate accumulation and the appearance of AEF activity (168, 3) in which detectable AEF activity necessarily precedes AA fibril formation. Furthermore, AEF dramatically shortens the time necessary for the experimental induction of amyloidosis and can be

AEF activity necessarily precedes AA fibril formation. Furthermore, AEF dramatically shortens the time necessary for the experimental induction of amyloidosis and can be detected in tissues 24-48 hours before the appearance of amyloid (3). The kinetics of amyloid deposition using standard induction protocols, or following passive transfer of AEF, are identical with the exception of the lag phase necessary for the appearance of AEF (157).

It is clear that both augmented levels of precursor protein synthesis and generation of AEF activity, in the presence of macrophages, are necessary for fibrillogenic degradation of SAA into AA fibrils. It has yet to be determined why the AA peptides fold into the cross-ß-pleated sheet conformation (14) which is not normally present in non-pathogenic fibril polymers. The common presence of SAP, glycosaminoglycans, and apolipoprotein E in the variety amyloid deposits tested to date may well influence fibril formation. Figure 1.3 gives a general outline of the elements thought to be involved in the the pathogenesis amyloidosis.

Both reticuloendothelial cells and AEF are obviously integral to the development of amyloidosis, yet their exact roles and interactions have yet to be characterized. The lack of specific biochemical characterization of AEF has limited any research done in this area. The studies presented in this thesis have therefore been designed with two major objectives in mind: the purification of AEF to homogeneity and the characterization of the relationship between AEF, macrophages, and amyloid deposition using a solublilized AEF preparation produced in our lab. Studies of this nature are essential to our understanding of the pathogenesis of amyloidosis.

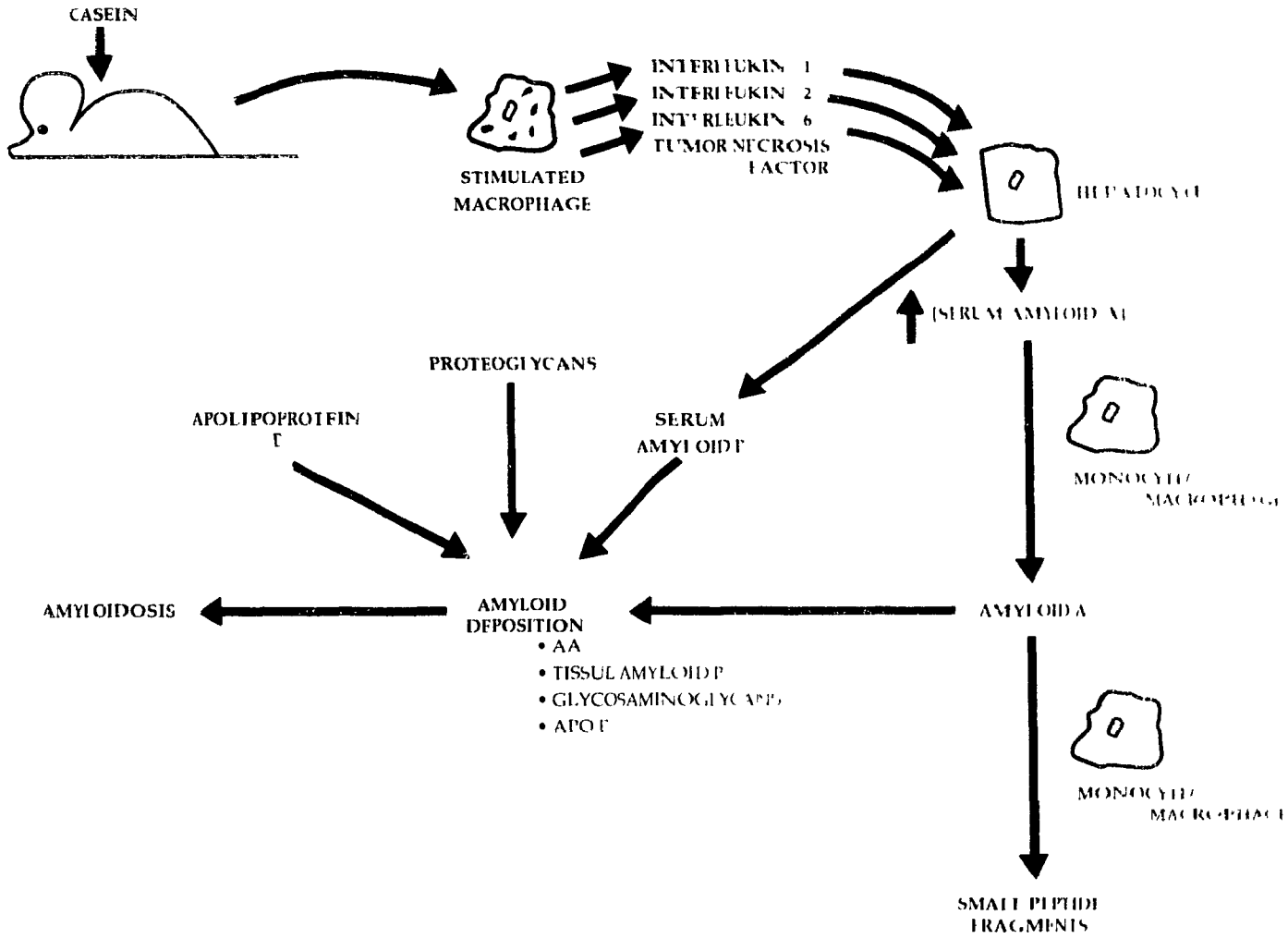


Fig. 1.3. The Pathogenesis of Amyloidosis

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CHAPTER 2

SPLENIC MACROPHAGE ACTIVATION AND FUNCTIONS IN AMYLOID ENHANCING FACTOR-INDUCED AA AMYLOIDOSIS

Study of Phagocytosis, Killing, Respiratory Burst, and MHC Class II Surface Expression

ABSTRACT

Secondary, or AA, amyloidosis is a systemic disease characterized by the extracellular tissue deposition of insoluble fibrillar amyloid A protein. Aberrant metabolism of serum amyloid A protein by reticuloendothelial cells is thought to result in the accumulation of fibrils within the tissue. Treatment of mice with amyloid enhancing factor (AEF) in conjunction with an inflammatory stimulus (i.e $AgNO_3$) induced amyloid deposition within 48-72 hours. The activation state of a macrophage largely defines its enzymatic capabilities. The following studies examine the effect of AEF on spleen macrophage activation using both functional and phenotypic assays. We found that while AEF, in the presence or absence of $AgNO_3$ has no apparent effect on the ability of spleen and liver macrophages to phagocytose or kill *L. monocytogenes*, it appears to block enhanced respiratory burst function (as measured by O_2^- production) observed with $AgNO_3$ alone. AEF therefore seems capable of inhibiting certain macrophage activation-associated functions while not affecting others. Our activation phenotype studies, using surface Ia expression, reveal that AEF blocks the increase in number of splenic macrophages expressing Ia seen with $AgNO_3$ alone. Treatment with IFN-gamma was found to restore decreased Ia expression in animals given AEF+ $AgNO_3$ but did not prevent amyloid A fibril deposition.

INTRODUCTION

Amyloidosis is a generic term for a diverse group of diseases characterized by the extracellular tissue deposition of insoluble protein fibrils, often resulting in failure of the involved organs. AA amyloidosis is typified by the deposition of amyloid A protein (AA) derived from the carboxy-terminal cleavage of its acute phase serum precursor: serum amyloid A (SAA) (1, 2). AA amyloidosis appears in conjunction with certain malignancies, chronic inflammatory disorders, or prolonged infection (3). Experimental AA amyloidosis is induced in laboratory animals by sustained inflammation or chronic bacterial or parasitic infections (4).

Amyloid enhancing factor (AEF) is believed to play an integral role in the pathogenesis of amyloidosis. Though its complete biochemical characterization remains elusive, AEF is routinely extracted from the tissue of amyloidotic animals where its activity is evident approximately 24-48 hours before the histological detection of amyloid fibrils (5). The hallmark characteristic of AEF is its ability to accelerate AA fibril deposition. While most experimental induction protocols require several weeks for the development of detectable amyloid deposits, administration of AEF in conjunction with an inflammatory stimulus results in fibril deposition within 24 to 72 hours (6). The ability of an exogenous source of AEF to shorten the induction period required for amyloid deposition suggests that it acts by precluding the lag time required for the endogenous production of AEF (7). Furthermore, genetic analysis of amyloid resistant and susceptible strains of mice suggests that susceptibility to the development of experimental AA amyloidosis is controlled by a single dominant gene (8). This observation, in conjunction with the finding that resistance in A/J mice can be overcome by i.v. injection of an exogenous source of AEF (9), further supports the role of AEF as a major determinant in the development of AA amyloidosis.

Reticuloendothelial cells have long been recognized in close association with amyloid deposits *in situ* (10). Normal peripheral blood monocytes are known to be capable of mediating SAA degradation *in vitro* through the action of cell surface enzymes (11, 12). Studies aimed at elucidating the exact relationship between macrophages or monocytes and the deposition of amyloid fibrils have led to the discovery that mononuclear leukocytes from healthy individuals are capable of mediating complete SAA degradation whereas the cells of amyloidotic patients produce an intermediate protein fragment, both the same size as AA and immunologically cross-reactive with AA (11). In addition, normal murine Kupffer cells have been found to degrade SAA completely, whereas Kupffer cells from

amyloid-induced animals produce the AA intermediate (13). This observation has led to the hypothesis that tissue amyloid deposition may be due to inappropriate or incomplete SAA degradation by cells of the monocyte/macrophage lineage, resulting in the accumulation of AA.

In light of the obvious importance of both AEF and reticuloendothelial cells in the formation of amyloid deposits, the aim of the present study was to elucidate the relationship between AEF and macrophages at the primary site of amyloid deposition in AA amyloidosis. Our working hypothesis was that an alteration in the normal processes of macrophage activation resulting from the production of endogenous AEF could cause aberrant SAA degradation by macrophages. Circumstantial evidence supporting our hypothesis is provided by the results of previous studies which showed that the appearance of AEF was correlated with the triggering of fibril accumulation *in vivo* (5). Furthermore, preliminary work done in our lab detected a decrease in splenic macrophage surface MHC Class II (Ia) expression which coincided with the appearance of endogenous AEF during induction of experimental AA amyloidosis by a chronic inflammation protocol (unpublished results). AEF could interfere with macrophage degradative processes by blocking activation resulting in the inability of the macrophage to completely degrade the SAA. The work presented here therefore examines macrophage activation on a functional level as well as on a phenotypic level following the induction of AA amyloidosis using AEF in combination with silver nitrate ($AgNO_3$) as an inflammatory stimulus.

Certain macrophage-specific activities such as phagocytosis, bactericidal function, and respiratory burst functions are enhanced during activation (14-16) and are therefore good functional indicators of macrophage activation status. Our experiments compare the *in vivo* ability of liver and spleen cells to phagocytose and kill *Listeria monocytogenes* as well as the *in vitro* oxidative burst response (as indicated by O_2^- production) of splenic macrophages between amyloidotic animals treated with $AgNO_3$ in conjunction with AEF and animals treated with $AgNO_3$ alone. In addition, we examined the differential expression of Ia antigen by macrophages isolated from the spleens of mice treated with AEF and $AgNO_3$ using flow cytometry and immunofluorescence analysis. The enhanced expression of MHC Class II molecules may be used as a marker of macrophage activation (17,18). Our aim was to determine whether or not treatment with AEF and silver nitrate causes a change in macrophage surface Ia expression over treatment with the inflammatory stimulus alone and, if so, could any such change reflect an alteration in the degradation pattern of the SAA precursor leading to AA amyloid fibril deposition.

MATERIALS AND METHODS

Animals

Specific pathogen free (SPF) C57Bl/6NHsd male mice, 8-12 weeks old, were obtained either from Harlan Sprague Dawley (NHsd; Indianapolis, IN) or bred in our own SPF facilities. Food and water were provided *ad libitum*. Animals were sacrificed by CO_2 narcosis followed by cervical dislocation.

Preparation of Amyloid Enhancing Factor and Induction of Amyloidosis

AEF was prepared from the spleens of C57BL/6 mice, subcutaneously injected for 21 days with 13% casein (United States Biochemical, Cleveland, Ohio). AEF was extracted from homogenized spleens in ice-cold 30% glycerol-10 mM Tris-HCl, pH 7.5 buffer as previously described (6). 0.5 mg of the lyophilized AEF product (an amount previously determined to induce significant amyloid deposition within 24-48 hours [9]) was resuspended in 0.2 ml sterile phosphate buffered saline (PBS) and administered via i.v. injection concomitant with 0.5 ml s.c. of 2% $AgNO_3$ solution.

Spleens from untreated, healthy mice were subjected to the same extraction protocol as above. The resulting product was used as a control, AEF-free extract. Control groups consisted of animals treated with AEF-free extract/$AgNO_3$ or with 0.5 ml 2% $AgNO_3$ solution or with 0.5 mg AEF alone. They were housed in the same facilities and were sacrificed after the same time periods as experimental mice.

Detection of AA Amyloid Deposits

At least a portion of each spleen was fixed in buffered 10% formalin, embedded in parrafin, sliced in 5 um sections, then stained with alkaline Congo Red (19). The presence of amyloid deposits was determined microscopically by the detection of green birefringence characteristic of AA fibrils under polarized light.

In Vivo Phagocytosis and Bactericidal Activity

Three days after the injection of AEF alone, $AgNO_3$ alone, or both AEF and $AgNO_3$, experimental animals were inoculated with 8×10^6 CFU *L. monocytogenes*. The livers and spleens of infected animals were tested for their ability to phagocytose and kill *L. monocytogenes* by determining the number of viable bacteria in individual organs 30 min. or 6 hours respectively post-infection (14). Spleens and livers were removed aseptically and homogenized in sterile saline for about 1 minute with a Tri-R Stir-R homogenizer (Tri-R instruments, Rockville Centre, NY.). Serial 10-fold dilutions of these homogenates were

plated on tryptose agar (1%; Bacto-agar; Difco Laboratories; Detroit, MN.) and colony counts were routinely executed following 18-24 hour incubations at 37 C. Five animals per group were tested.

Spleen Macrophage O_2^- Production

Responses were examined in splenic macrophages from naive mice as well as mice treated with AEF alone, $AgNO_3$ alone, or AEF/$AgNO_3$ 3 days prior. Internal experimental positive controls consisted of proteose peptone (PP)-elicited peritoneal macrophages.

Spleen cells were flushed from isolated spleens with ice-cold RPMI 1640 supplemented with 0.3 mg/ml glutamine and 50.0 ng/ml gentamicin. Red blood cells were lysed by hypotonic shock on ice. Cell differentiation was determined on cytospin slides (Shandon, Sewickley, PA.) stained with Giemsa (DiffQuik, Canlab, Montreal, Que.). Each cell suspension was adjusted to 1.5 x 10^6 macrophages /ml in RPMI 1640 +10% fetal calf serum. Macrophages were plated in 6- or 24-flat bottom well plates (Linbro, Flow Laboratories, Maclean, VA.) and purified by adherence overnight at 37 C, 5% CO_2. Each cell suspension was assayed in triplicate. In all instances, cell viability as established by trypan blue exclusion test was > 90-95%. The cell monolayers were > 90% macrophages as determined morphologically by Giemsa stain and phagocytosis of *L. monocytogenes*.

O_2^- production was measured following treatment with PMA (0.5 ug/10^6 cells) for 75 minutes by the method of Cohen et al. (20) The protein content of each well was then determined following cell lysis with 0.5 N NaOH using the Bio-Rad protein assay kit (21). Results are reported as nmoles O_2^-/mg protein/75 minutes $\pm$ SEM. Statistical analysis was done by a two-tailed Mann-Whitney U test with a present probability level of $a = 0.05$. Mean values represent data obtained from three experiments.

Ia Surface Expression

After aseptic removal, each spleen was flushed with ice-cold RPMI 1640 supplemented with glutamine and gentamicin. Following lymphoid cell purification on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario), the cells were washed by centrifugation (4 ºC) and resuspended in 10 ml ice-cold RPMI 1640. Enumeration of cells and determination of viability (by stain exclusion) was accomplished using a hemocytometer and Turks or trypan blue stain respectively. Slides for cell differential counts were prepared as previously described. Following resuspension at a final concentration of 1-2 x 10^6 cells/ml with 0.5% bovine serum albumin (BSA) in PBS, 1x 10^6 cells were aliquoted to 5 ml polystyrene round-bottom tubes (Becton Dickinson labware, Montreal, Que.) for immunofluorescent labelling. Following incubation with goat

IgG (Sigma, St. Louis, MO.) to reduce non-specific binding by blocking Fc receptors, cells were labelled successively with monoclonal anti-murine Mac.1 antibody (clone M1/70, Boehringer-Mannheim Biochemical, Laval, Que.), phycoerythrin-conjugated goat F(ab')2 anti-rat IgG (Caltag Laboratories, San Francisco), and fluorescein isothiocyanate-conjugated murine anti-Ia^k IgG (Cedarlane Laboratories). Each incubation with labelling antibody (at 1/20 dilution as determined optimal by serial titration) was carried out for 15 min. on ice. The appropriate control labellings were prepared to correct for autofluorescence, non-specific binding, and inhibition of binding due to steric hindrance for each experiment group. Labelled cells were fixed in ice-cold paraformaldehyde and kept at 4 ° C until analyzed on a FACScan flow cytometer with the Lysis II program (Becton Dickinson) the following day. When indicated, IFN-gamma treatment consisted of 5000 units of rat recombinant IFN-gamma (Amgen, Thousand Oaks, CA.) administered i.p. on days 0 and 2 of the experimental design.

RESULTS

In Vivo Phagocytosis and Bactericidal Activity During AEF-Induced AA Amyloidosis

In order to determine whether or not the ability of splenic and hepatic macrophages to phagocytose and kill *L. monocytogenes* is modified during AA fibril deposition, groups of mice were injected with AEF and $AgNO_3$ 3 days prior to i.v. inoculation with *L. monocytogenes*. Control groups consisted of naive mice and mice injected with AEF or $AgNO_3$ alone.

Phagocytosis by spleen and liver cells was measured 30 minutes following the i.v. injection of 2×10^8 bacteria *L. monocytogenes*. The development of amyloid deposits due to AEF/$AgNO_3$ treatment was not found to be associated with modified bacterial uptake as compared to naive animals or animals treated with AEF or $AgNO_3$ alone (Fig. 2.1). Approximatly 95% of the bacterial inoculum was taken up by the spleen and liver in the first 30 minutes following injection.

The bactericidal capacity of macrophages (measured 6 hours after the injection of *L. monocytogenes)* was not significantly modulated in the liver of AEF/$AgNO_3$ treated animals despite amyloid deposition after 3 days when compared to naive animals or animals treated with AEF or $AgNO_3$ alone (Fig. 2.1). All groups were shown to be capable of clearing approximatly 85-90% of the bacterial load within the first 6 hours following infection. No significant bactericidal activity was observed during the same time period in the spleen of both control and experimental animals.

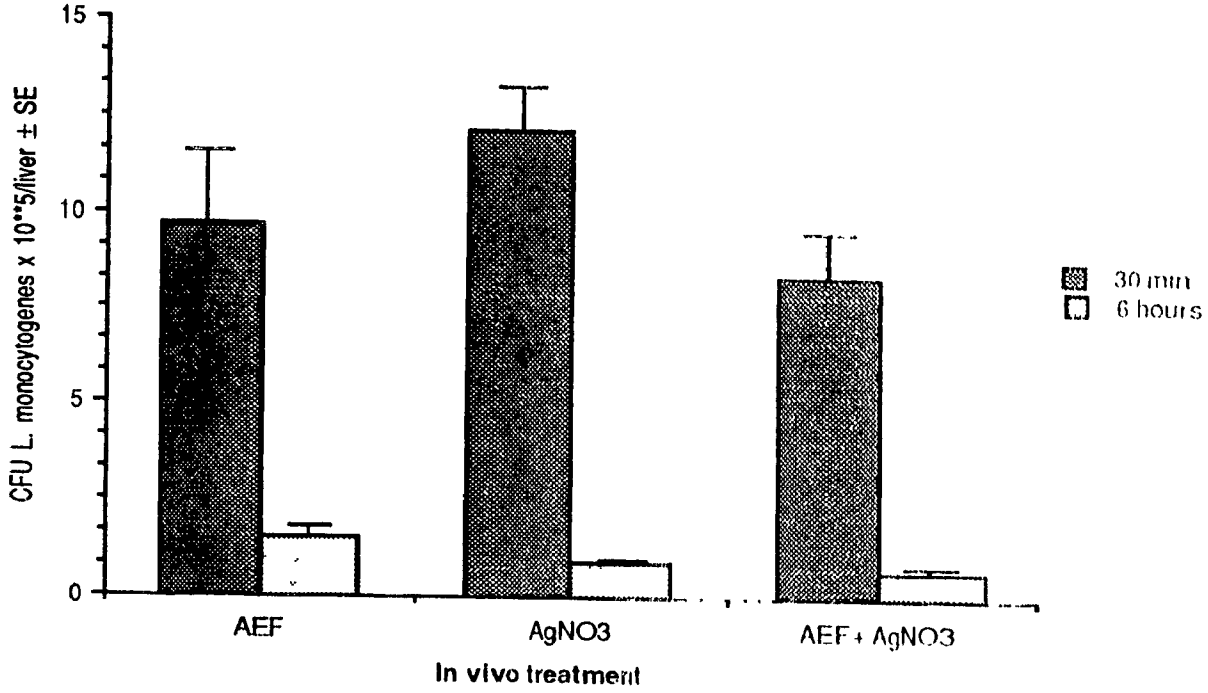

Fig. 2.1: ***In vivo*** **phagocytosis and bactericidal activity measured in the liver of mice treated with AEF/$AgNO_3$ as compared with mice treated with $AgNO_3$ or AEF alone**. Mice were treated with AEF and/or $AgNO_3$ three days prior being infected with 2 x 10^4 bacteria *L. monocytogenes*. The *in vivo* phagocytic ability of the liver cells was determined 30 min. following infection while the *in vivo* bactericidal activity was measured 6 hours later, as described in materials and methods. Normal mice displayed similar phagocytic and bactericidal activities observed in mice treated with AEF and/or $AgNO_3$.

O_2^- Production in AEF-Induced AA Amyloidosis

In order to determine whether the respiratory burst functions of splenic macrophages is altered during AA fibril deposition due to AEF, O_2^- production by cells isolated from mice 3 days following treatment with AEF/$AgNO_3$ or either agent alone was measured (Table 2.1). Splenic macrophages from mice injected with $AgNO_3$ alone (a non-amyloidogenic treatment) released a significantly higher concentration of O_2^- metabolite (following stimulation *in vitro* with PMA) than those obtained from the untreated control group. Remarkably, when animals were treated with both AEF and $AgNO_3$, the ability of their spleen macrophages to release O_2^- was significantly decreased as compared to cells isolated from the $AgNO_3$ treatment group. These results suggest that in an amyloid-inducing treatment, AEF partially blocks $AgNO_3$-induced O_2^- production by spleen macrophages.

This pattern of partial inhibition also occurred at the level of H_2O_2 production. Again, the amyloidogenic treatment was accompanied by a decrease in the $AgNO_3$ -induced release of the relevant metabolite. Data obtained for H_2O_2 release however, did not reach significance.

Ia Surface Expression During AEF-Induced AA Amyloidosis

The injection of a combination of AEF and $AgNO_3$ (as an inflammatory stimulus) is known to induce amyloid fibril deposition in mice within 24-48 hours (5). $AgNO_3$ alone was injected into mice in a parallel group in order to provide a basis of comparison of the level of activation of splenic macrophages harvested from animals subjected to an inflammatory stimulus in the absence of AEF. Using a double immunofluorescence protocol, we determined the level of surface Ia antigen expression on $Mac.1^+$ spleen cells by flow cytometry, as a means of monitoring the activation state of the macrophages, three days following treatment of mice *in vivo*. Results presented in Figure 2.2 reveal that maximal surface expression of Ia antigens by splenic macrophages occurred in response to the injection of $AgNO_3$ alone which does not produce amyloid deposits in the tissues. When this treatment was combined with an injection of amyloid-inducing AEF, however, there was a complete abrogation of the augmented macrophage Ia expression. Control preparations of AEF-free spleen extracts did not interfere with the inflammation-induced Ia expression suggesting that the observed down-regulation in surface Ia is a specific effect of AEF. AEF treatment without concomitant $AgNO_3$ treatment, caused a slight but insignificant increase in Ia expression relative to untreated animals (Fig. 2.2).

Table 2.1:

O2 Production by Macrophages in AEF Induced AA Amyloidosis

Treatment Group	O2- Production* (nmoles/mg protein/75 r ± SEM
Control	92 ± 9.20
AEF alone	111 ± 17.60
Silver Nitrate alone	507 ± 52.64
AEF and Silver Nitrate	218 ± 32.29**

*O2-Production was measured according to the method described by Cohen et al.(20)
Results obtained represent means ± SEM of 3 experiments (each done in triplicate)
**significantly less ($a<.05$) than value obtained with silver nitrate alone

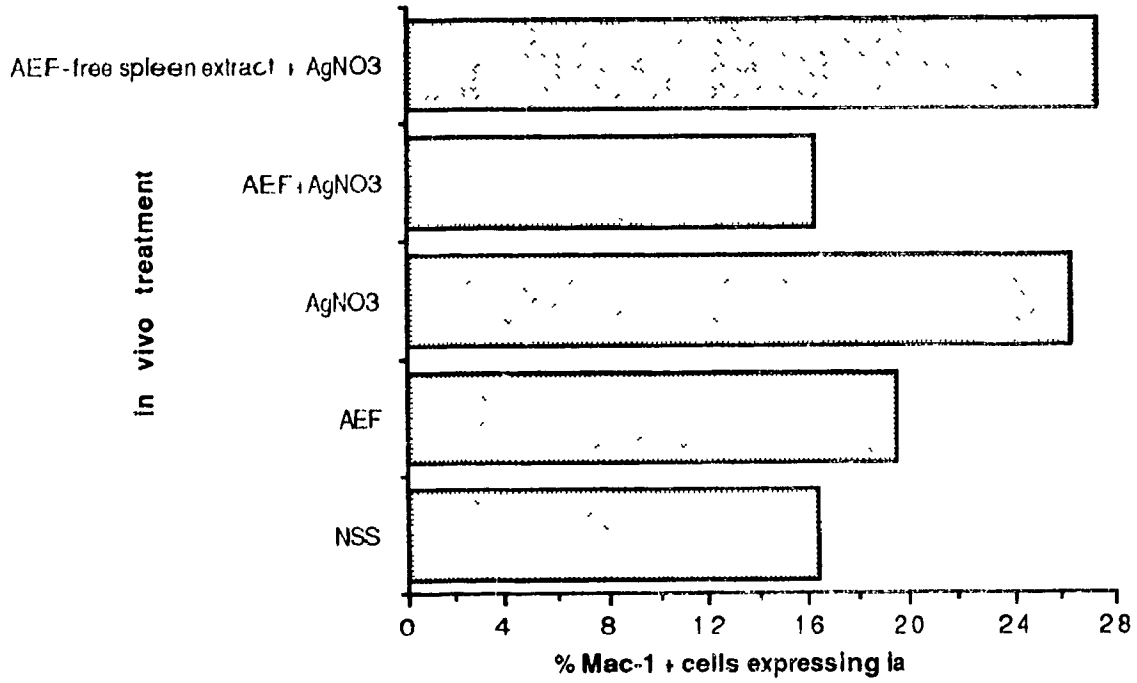

Fig. 2.2. **Modulation of spleen macrophage Ia expression from normal steady state in mice rendered amyloidotic by treatment with AEF/$AgNO_3$ as compared with mice treated with $AgNO_3$ or AEF alone, or a control, AEF-free spleen extract/$AgNO_3$.** Spleen cells were isolated from mice three days following the indicated treatments. Cells were labelled with Ia and Mac.1-specific antibodies, as described in Materials and Methods, prior to analysis by flow cytometry. This double antibody label system distinguishes macrophages expressing Ia antigen on their surface (Mac.1^+Ia^+) from B cells (Mac.1^-Ia^+)and surface Ia negative macrophages (Mac.1^+Ia^-). Granulocytes and natural killer cells, which express only small quantities of Mac.1 antigen, are weakly Mac.1^+ relative to macrophages (22) and therefore do not stain intensely enough to be included in Mac.1^+ determinations. Scatter gates were set to exclude dead cells and debris. Results are expressed as a percentage of Mac.1^+ cells which are Ia^+. As indicated on the x-axis, analysis of spleen cells obtained from untreated, control mice revealed that 16.5% of Mac.1^+ spleen cells are Ia^+ under normal steady state (NSS) conditions 1-3 groups (of at least 3 mice each) were tested for each treatment.

Having established that AEF may indeed downregulate the normal macrophage response to activating agents, at least at the Ia level, we examined the possibility that the observed downregulation may be related to a change in the degradation pattern of SAA leading to AA amyloid fibril deposition. By combining an injection of IFN-gamma (in a dose previously determined to be sufficient to induce increased Ia expression by splenic macrophages in C57Bl/6 mice [unpublished results]) with our AEF/$AgNO_3$ treatment protocol, we were able to restore Ia expression to levels seen with $AgNO_3$ alone (Fig. 2.3). Histological examination of portions of spleen retained from mice treated with all three agents however, reveal that despite the IFN-gamma-induced restoration of macrophage Ia expression in mice administered AEF and $AgNO_3$, splenic amyloid fibril deposition still occurred (Fig. 2.4, Table 2.2). This finding suggests that AEF-induced downregulation in splenic macrophage activation observed at the level of Ia expression is not related to aberrant SAA degradation leading to AA amyloid fibril deposition.

DISCUSSION

Based on studies reported in the literature (11, 13), we postulate that the degradation of amyloid precursors by macrophages is a critical event which prevents the deposition of amyloid fibrils in normal tissues. The process of macrophage activation involves a regulated series of changes in physiology which culminate in the ability to execute complex enzymatic functions (18, 22). The ability of a macrophage to perform certain specific functions, possibly including the degradation of SAA, may therefore be dependent on its activation state. In light of the fact that the appearance of endogenous AEF is directly correlated with the deposition of amyloid fibrils (5) and our preliminary observation that splenic macrophage surface Ia expression is decreased during the induction of amyloid fibril deposition (unpublished results), we suspected that AEF may indeed affect the ability of the macrophage to be appropriately activated for the degradation of the amyloid precursor, leading to the deposition of amyloid fibrils.

Since the mechanism by which SAA and macrophages interact remains unknown, we focussed our studies on functions known to be enhanced in the activated macrophage. Macrophage phagocytic and microbicidal activity was monitored by the *in vivo* uptake and killing of the facultative intracellular pathogen *L. monocytogenes* which has been shown to be processed by macrophages (14). Our results indicate that AEF, whether administered alone or in conjunction with $AgNO_3$, does not affect either capacity.

Primed and activated macrophages exhibit an increased release of reactive oxygen metabolites (23). Treatment of animals with an inflammatory agent, such as $AgNO_3$,

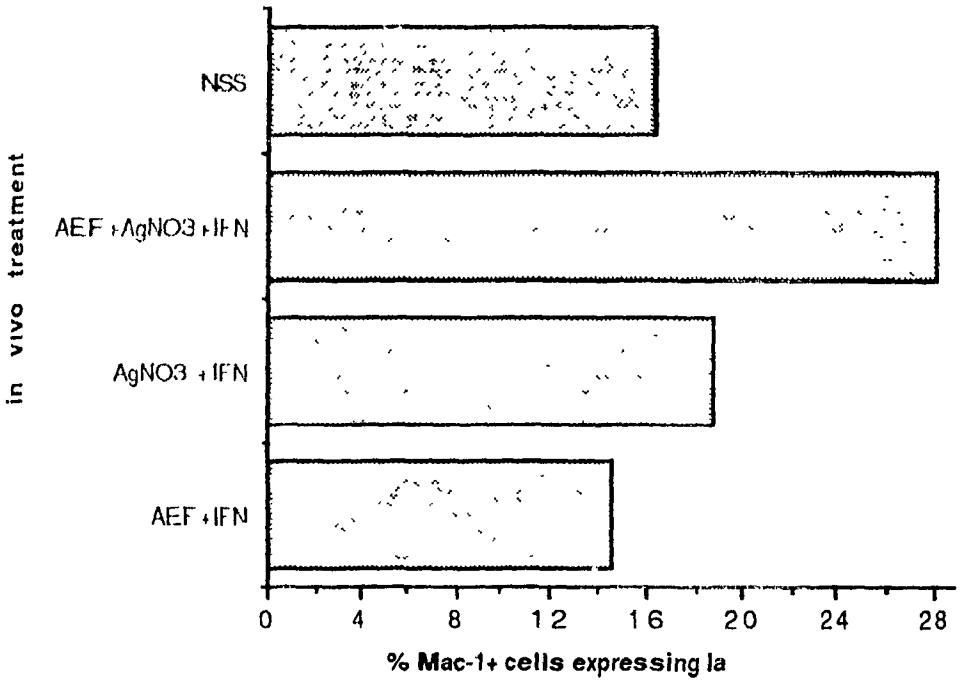

Fig. 2.3 **Restoration of spleen macrophage Ia expression in mice treated with AEF/AgNO3 by *in vivo* IFN-gamma treatment.** Spleen cells isolated from mice 3 days following *in vivo* treatments indicated in the figure (described in Materials and Methods) were prepared and analysed as described in Fig. 2.1. 1-3 groups (of at least three mice each) were tested for each treatment.

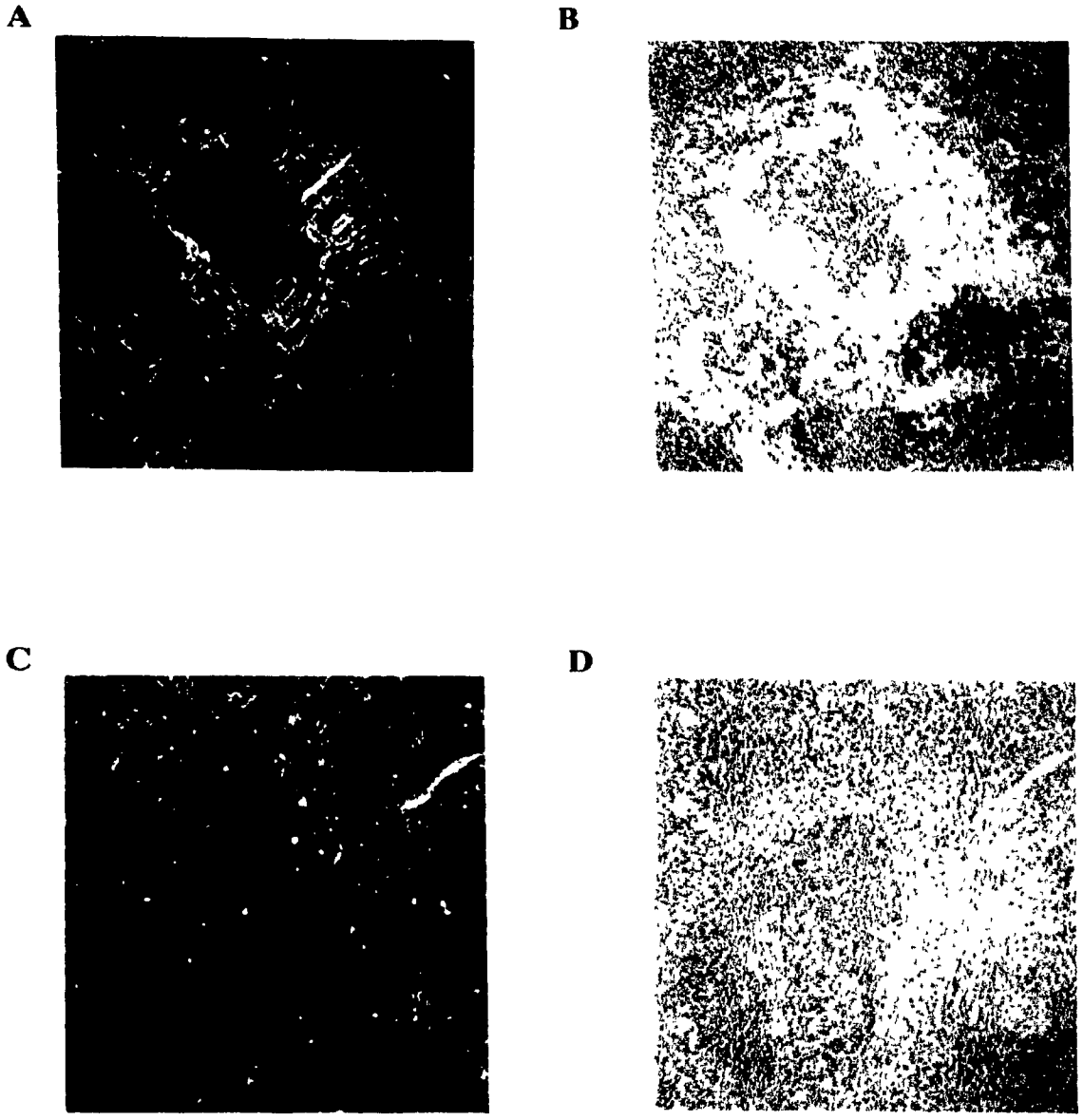

Fig. 2.4. **Detection of amyloid deposition by Congo Red stain.** Amyloid fibril deposits within spleen sections obtained from an AEF/$AgNO_3$ treated animal exhibit characteristic green birefringence under polarized light (panel A) once stained with Congo Red . Spleen sections from normal animals do not exhibit birefringence under polarized light (Panel C). Panels B and D show Congo Red stained spleen sections from amyloidotic and untreated mice respectively, as observed under a normal microscope. Spleen sections were prepared as described in Materials and Methods.

Table 2.2

Incidence of Amyloid Deposition in Spleen Sections from Mice Treated with AEF, Silver Nitrate, and IFN-gamma

Treatment Group*	Amyloid Deposition**
AEF alone	0/3
Silver nitrate	0/3
AEF/silver nitrate	9/9
Control AEF-free extract	0/3
AEF/IFN-gamma	0/3
Silver nitrate/IFN-gamma	0/6
AEF/silver nitrate/IFN-gamma	6/6

*Treatment groups as described in Materials and Methods.
**sections were stained with Congo Red as described in Materials and Methods. Amyloid fibril deposition was detected by characteristic green birefringence under polarized light.

results in an enhanced macrophage oxidative metabolism (15). Our experiments measuring reactive oxygen metabolite production by splenic macrophages reveal that AEF dampens O_2^- production by $AgNO_3$-induced macrophages and may similarly affect H_2O_2 production. At this point, one can only speculate as to the mechanism by which AEF modulates O_2^- production. It is conceivable, however, that AEF-inhibition occurs at the level of membrane components of the phagocyte superoxide generating system, for example by interfering with NADPH oxireductase cytochrome C activity. When AEF was administered in the absence of $AgNO_3$ no significant modification in metabolite release was observed as compared to steady state, suggesting that the down-regulatory action of AEF is dependent upon a concomitant inflammatory stimuli. It is probable that AEF either acts in concert with other factors induced or released during an inflammatory response or, exerts an inhibitory effect only on macrophages rendered responsive through inflammatory stimuli. This would explain why AEF triggers AA fibril deposition only when administered in conjunction with an inflammatory stimulus.

Having thus determined that AEF may selectively affect some macrophage activation-associated functions but not others we chose to examine the effect of AEF on macrophage activation at the phenotypic level. Increased MHC class II (Ia) surface expression is considered a marker of macrophage activation (18,24,25) and is therefore useful for monitoring macrophage activation status. We first compared surface Ia expression by splenic macrophages harvested from mice subjected to amyloid deposit-inducing and non-inducing treatment protocols. Our data indicate that $AgNO_3$ treatment alone, ineffective in causing amyloidosis, results in splenic macrophage activation as reflected by enhanced Ia expression over normal steady state. This increased expression is dampened or blocked in the presence of AEF. The absence of any such "block" in Ia expression in mice treated with AEF-free spleen extract (obtained from control, untreated mice) in conjunction with $AgNO_3$ suggests that decreased Ia expression is attributable to AEF and not some contaminant within our AEF preparation. Furthermore, it is interesting to note that AEF treatment alone revealed a slight increase in Ia expression levels over normal steady state. As indicated by our respiratory burst studies, AEF seems to exert an inhibitory effect only in the event of a concomitant inflammatory stimulus. Again, this observation may be related to the fact that AEF is amyloidogenic only when administered in conjunction with an inflammatory stimulus.

Having thus linked decreased splenic macrophage Ia expression to AEF, we looked at whether the observed suppression of macrophage Ia expression in the presence of AEF played an integral role in altered SAA degradation leading to AA fibril deposition. By counteracting AEF-induced down-regulation of splenic macrophage Ia expression with

IFN-gamma, we were able to show that the effect of AEF on macrophage activation status during the induction of amyloidosis at the level of Ia expression is not necessary for the production and deposition of AA fibrils in the spleen. It is possible that IFN-gamma increases Ia expression via a different pathway than that which may be blocked by AEF. Studies on the regulation of macrophage surface Ia expression suggest at least four activation-associated transductional signal cascades (i.e. cAMP cascade, Ca^{++} and protein kinase C cascades) may be important for its regulation (18,26). It is therefore conceivable that Ia expression could be restored by IFN-gamma despite continued inhibition of an alternative cascade by AEF. Should this alternative transduction cascade also mediate SAA degradation (i.e. by transcriptional regulation of SAA metabolic enzyme genes), the end result could be that AEF blockage of one macrophage activation pathway (causing aberrant SAA degradation) could persist despite replenished Ia expression due to IFN-gamma.

These studies have established that, in the presence of an inflammatory stimulus, AEF selectively modifies certain aspects of the activated splenic macrophage. It is probable that AEF induces amyloid fibril deposition by altering the macrophage function responsible for normal or aberrant degradation of SAA. Studies such as those described here will prove most revealing once the exact nature of this function has been characterized.

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CHAPTER 3
PURIFICATION OF AMYLOID ENHANCING FACTOR

INTRODUCTION

Though first detected in amyloid tissue extracts in 1966 (1), the biochemical nature of AEF remains a mystery. At present AEF is defined most clearly in functional terms as a non-inflammatory, non-amyloidogenic substance that, when co-administered with a single inflammatory stimulus, shortens the pre-amyloid phase of amyloidosis to result in splenic amyloid deposition within 24 to 48 hours. Under conditions of sustained inflammation in animals, deposition takes approximately 15 days (2). The consistent detection of AEF in several forms of amyloidosis including AA, AL, ß-amyloid, and transthyretin amyloid (3,4) suggests that AEF activity may be a common link between the diverse forms of amyloid (2).

The purification of AEF is essential to the progression of research into the pathogenic mechanisms leading to the formation of amyloid deposits. A significant portion of my work has concentrated on identifying the particular protein responsible for AEF activity. Positive identification of the exact protein obviously preceeds complete biochemical characterization of AEF including amino acid sequencing or preparation of AEF-specific antibodies. Besides opening the door to endless research possibilities such as the development of tissue culture systems as *ex-vivo* models of AA amyloid generation or methods for monitoring pathological conditions associated with the increased risk for amyloidosis, discoveries of this nature are essential to the study of AEF production kinetics, potency, and sites of action.

To provide sufficient raw material, AA amyloidosis was induced in large batches of C57Bl/6 mice. Mass amounts of a relatively crude preparation of AEF was then extracted from the amyloidotic spleen of these mice and separated on multiple acrylamide-SDS preparative gels. Protein electroeluted from specific regions of these gels was then assayed for AEF activity allowing us to define the specific area of the gels containing AEF activity. Two dimensional (2-D) electrophoretic analysis of protein contained within this "AEF-active" area has identified 3 proteins of interest not expressed in "AEF-free" controls.

MATERIALS AND METHODS

Animals

C57Bl/6J male mice were obtained from Jackson Laboratories (Bar Harbor, ME) or bred in our own facilities. Food and water were provided *ad libitum*. Animals were sacrificed by CO_2 narcosis followed by cervical dislocation.

Induction of AA Amyloidosis

AA amyloidosis was induced in C57Bl/6 mice by subcutaneous injection with 0.5 ml 13% casein (United States Biochemical, Cleveland, Oh.) prepared in sterile 0.3 M $NaHCO_3$, pH 7.0 (Gibco Laboratories, Grand Island, NY) for 21 consecutive days. The $NaHCO_3$ and the casein solutions contained less than 0.02 ng lipopolysaccharide/ml as measured by the E-toxate (Limulus ameobocyte lysate) detection and semi-quantitation test (Sigma, St. Louis, MO). Spleens were harvested on the 22nd day and frozen at -70 C in 30% glycerol-10 mM Tris (Fisher Scientific, Fairlawn, NJ) buffer at pH 7.5.

Detection of Amyloid Deposits

Whole or partial spleen were fixed in buffered 10% formalin then immersed in parrafin. Five um histiological sections prepared from the wax-embedded organs were then stained with alkaline Congo Red (6). The presence of amyloid deposits was then determined microscopically by the detection of green birefringence characteristic of AA fibrils under polarized light.

Preparation of AEF Extract

AEF was extracted from the spleen of amyloidotic C57Bl/6J mice as previously described (5). Spleens were homogenized in ice-cold 30% glycerol-10 mM Tris buffer at pH 7.5 with a Tri-R Stir-R homogenizer (Tri-R Instruments, Rockville Centre, NY). Following sonication for 1 min. with a Sonic Dismembrator (Artek Systems Corporation, Farmingdale, NY), the preparation was mixed at 4 C for 1 hour then centrifuged at 250 000 x g, 4 C, for 3 hours. The supernatant was then dialysed against five changes of an 80-fold volume of 0.01 M phosphate buffered saline (PBS) pH 7 in SpectraPor 3 (m. wt. cutoff 3 500 Da) dialysis bags (Spectrum Medical Industries Inc., Los Angeles, CA). The precipitate was recovered by centrifugation at 17 300 x g, 4 C, for 30 min. Following resuspension of the pellets in small volumes of PBS, sterile solubilisation buffer composed of 6% urea/1% ß mercaptoethanol/1 mM EDTA was added to the preparation in a 1 to 1 volume ratio. Mixing was achieved overnight at room temperature on a Roto-Torque rotator (Cole Parmer Instrument Company, Chicago, Il) at 60 Hz. Following centrifugation at 10 000 x g and 10 C for 10 min. to remove unsolubilised particulates, the supernatant was dialyzed against five changes of 0.01 M PBS pH 7 in SpectraPor 3 (Spectrum Medical Industries) dialysis bags. Immediately following dialysis the preparation was frozen at -70 C then lyophilized with a Virtis lyophilizer (Gardiner, NY). This dry AEF preparation was then stored at -70 C until future use.

The AEF activity of all AEF preparations was ensured by their ability to induce amyloid deposition at an accelerated rate. Mice were injected intravenously with 100-300

ug dried AEF extract resuspended in PBS, in conjunction with a subcutaneous injection of 0.5 ml 2% $AgNO_3$ (Fisher Scientific) solution in deionized water. Spleen sections were then tested for amyloid deposition within 48-72 hours.

Spleen extract containing no AEF was prepared according to the extraction protocol described above but using spleen from uninduced, healthy mice. The resulting "AEF-free" product was used as a control.

Preparative Gel Electrophoresis

Separation of 3-5 mg of the lyophilized AEF extract described above was achieved by 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (7). Using Bio-Rad Protean II preparative gel apparatus (Bio-Rad, Richmond, CA), 1.5 mm thick SDS-polyacrylamide gels were prepared and polymerized at room temperature then stored overnight. Complete gels consisted of a 4.5% stacking gel and a 12.5% resolving gel. Both stacking and resolving gel solutions were thoroughly vacuum degassed prior to initiation of polymerization with 0.05% ammonium persulfate and 0.05% TEMED. Gels were pre-electrophoresed at a constant voltage of 250 V and approximate current of 175 mA. The running buffer consisted of 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS, pH 8.3. Following addition of 1 ml each of double distilled H_2O (ddH_2O) and sample buffer, AEF preparations were boiled for 4 min. then applied to the sample well. The sample buffer was composed of 0.063 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 0.001% bromophenol blue. Rainbow molecular weight markers (Amersham International, Amersham, UK) were run concomitantly in a single small well for reference. These consisted of myosin (200 000 Da), phosphorylase b (97 400 Da), bovine serum albumin (69 000 Da) , ovalbumin (46 000 Da), carbonic anhydrase (30 000 Da), trypsin inhibitor (21 500 Da) and lysozyme (14 300 Da). The markers were prepared in a 1:1 dilution with sample buffer and boiled as was the experimental sample. During protein migration through the stacking gel, electrophoresis was run at 250 V and 25 mA per gel then at 375 V and 35 mA per gel through the resolving gel. The entire run took from 4 to 5 hours and the current was held constant throughout.

After completion of electrophoresis, vertical strips of 1.0-1.5 cm width were removed from the left and right sides of the gel and stained separately in Coomassie blue R-250 overnight. Small "tabs" were incorporated while cutting these peripheral strips to allow for proper realignment with the main, untreated gel piece following destaining and rehydration of the strips in ddH_2O. Staining of the sides of the gel in this manner allowed for the visualization of separate protein bands. Using these as reference, distinct bands or regions were then excised from the entire gel. Protein contained within the major,

untreated portion of the gel was therefore not subjected to any unnecessary chemical modification caused by staining. Excised portions of the unstained gel were stored at -20 C until future use.

Electroelution

Separated proteins were isolated from the preparative gels using the S&S Elutrap® electro-separation system (Schleicher & Schuell, Keene, NH) designed for rapid isolation and purification of macromolecular samples from fractionating gels. In brief, the system consists of a sample chamber limited at each end by an inert membrane with a dense matrix through which buffer ions and molecules less than 3-5 kDa can pass under the influence of an electric field. A second, microporous membrane placed at one end of the chamber, on the inner side of the first membrane, acts as a prefilter that prevents acrylamide and other particulates from entering the purified sample. Together these two membranes form a trap into which the sample migrates. The electric field acts as the driving force filtering the molecules through the membranes; when the voltage is switched off, the membranes seal the trap, preventing the diffusion of the sample out of the trap. Electroelution was carried out in CAPS buffer (10 mM CAPS in 10% methanol pH 11.0 titrated with 2 N NaOH) suitable for high protein transfer efficiency from acrylamide gels (8). After the thawed gel slices were cut into small pieces and placed in the buffer-filled chambers, a 50 V/1 A upper limit current was run through the system for at least 18 hours. Eluted proteins, in final volumes of 400 ul, were then collected individually from the membrane-bound traps. Protein was then precipitated from the CAPS buffer solution at -20 C overnight with a 1 to 4 volume of 1:1 ice-cold acetone:methanol solution. Alternatively, the CAPS-protein solution was dialyzed 3 times against ddH_2O in SpectraPor 3 dialysis bags (Spectrum Medical Industries) then frozen overnight at -70 C for lyophilization. Proteins recovered from various regions of the preparative gels in this manner were then either injected into mice (as described above for our initial AEF preparation) to test for AEF activity, analysed by 2-D electrophoresis, or used in O_2^- production assays.

Two Dimensional (2-D) Gel Electrophoresis

Isoelectric focusing (IEF), the first dimension: IEF was conducted using components of the Multiphor II electrophoresis system apparatus (Pharmacia LKB Biotech., Uppsala, Sweden) on gels consisting of an immobilized pH gradient, ranging from pH 5 to pH 8, obtained by the copolymerization of acrylamide, piperazine diacrylamide (PDA) as a crosslinker, and a series of Immobiline® buffering acrylamide derivitaves.

Prior to IEF the dehydrated gels were incubated for approximately 6 hours in rehydration buffer composed of 8 M urea, and 2.5% Pharmalyte® 3-10 (Pharmacia) as the

carrier ampholyte. Lyophilized AEF samples were dissolved in this same buffer with an additional 2% ß-mercaptoethanol and 2% Triton X-100. All solutions were filtered before use with 0.2 um filters. ß-mercaptoethanol was omitted from the rehydration step since it produced mercaptan bands at 50 and 70 kDa which were detectable upon silver staining. Samples were centrifuged at 13 600 x g for 15 min. and the supernatants loaded into sample cups. IEF was carried out under a protective layer of light mineral oil to avoid gradient drift caused by atmospheric CO_2. The system was run at 300 V, 1 mA, 5 W for 3 hours (900 Vh) and 2 500 V, 1 mA, 5 W for 14 hours (35 000 Vh) for a total of 35 900 Vh. IEF gels were equilibrated twice for 10 min. in 125 mM Tris-HCl (pH 6.8) with 1% SDS and 10% glycerol prior to SDS-PAGE.

SDS-PAGE, the second dimension: Vertical, 1.5 mm thick, polyacrylamide/PDA (T=30%, C=2.6%) gradient slab gels were generated using a gradient maker (Hoefer Scientific Instruments, San Francisco, CA) with a dense solution consisting of 0.5 M Tris HCl (pH 8.8), 18% PA, 0.1% SDS, and 10% glycerol (mixing chamber) and a light solution consisting of 0.5 M Tris-HCl (pH 8.8), 12% PA, and 0.1% SDS (resevoir). Polymerization was initiated with the addition of 6 ul TEMED (0.0003% final concentration) to each chamber and 30 ul and 15 ul ammonium persulfate to the light and dense solutions respectively ($5x10^{-5}$ final concentration). Gels were poured using a 3-channel peristaltic pump and the Bio-Rad Protean II apparatus (Bio-Rad). Complete gels included a 4% stacking gel as well as the resolving portion of the gel. IEF gels were applied flat onto the surface of the stacking gel. Electrophoresis was carried out at 25 mA/gel through the stacking gel, then at 50 mA/gel through the separating gel. Migration through the separating gel required approximately 3 hours. Rainbow molecular weight markers (Amersham International) included in the second dimension were diluted with 10 parts SDS-PAGE sample buffer (4 mM Tris-HCl pH 6.8, 4% SDS, 16 % glycerol) with 10% ß-mercaptoethanol and 0.006% bromophenol blue, then boiled for 3 min. prior to loading.

Once electrophoresis in the second dimension was completed the gel was silver stained according to the procedure devised by Bürk *et al* (9).

RESULTS

SDS-PAGE Analysis of AEF Extract

When denatured by heating in the presence of excess SDS, most polypeptides bind SDS in a constant weight ratio such that they have essentially identical charge densities and migrate in polyacrylamide gels according to their size (10). A plot of $\log_{10}$ polypeptide

molecular mass versus relative mobility (R_f) reveals a straight line relationship. Using the migration distances of our marker polypeptides [myosin, 200 kilodaltons (kDa); phosphorylase b, 97.4 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa], a standard curve was constructed and used to calculate the apparent molecular mass of distinguishable bands resolved by electrophoretic analysis of our AEF extract. Separation of AEF extracted from amyloidotic spleen on 12.5% SDS-polyacrylamide gels revealed a relatively constant banding pattern comprised of 7 significant bands (Fig. 3.1). Some minor variabilty in staining intensity and inter-band distances did occur between gels most likely due to minor differences in electrophoretic conditions or between AEF samples prepared in different extraction batches. For this reason molecular weight estimations calculated from each individual prep gel were used to determine the average values reported in figure 3.1. The migration distances of proteins were measured relative to that of the dye front since some differences in protein migration (probably due to differences in temperature over the gel) occurred horizontally across the gels.

The Mr's of bands 1 to 7 (as indicated in Fig. 3.1) are 81.0 kDa ± 7.7, 73.3 kDa ± 6.7, 63.2 kDa ± 5.8 , 54.6 kDa ± 7.4, 47.9 kDa ± 4.4, 15.6 kDa ± 6.4, and 12.4 kDa ± 7.0 respectively. A group of indistinguishable bands were detected in the region relative to the 30 kDa molecular weight marker. Previous studies conducted in our lab have shown that protein electroeluted from this 30 kDa molecular weight region do not exibit AEF activity.

Automated amino acid sequence analysis of band 7 has revealed an amino terminal end sequence identical to that of murine histone 3 protein of either H3.1 or H3.2 subtype (L. Hébert, unpublished data) (11, 12). Neither band 6 nor band 7 were found to exhibit AEF activity.

Electrophoretic analysis of "AEF-free" control extract prepared from the spleen of healthy animals revealed a similar banding pattern with the exception of band 4 which was not detected (Fig. 3.1).

Isolation of Region Containing AEF Activity

As described in Materials and Methods, protein was electroeluted from specific regions of each preparative gel. Horizontal variability in protein migration and indistinct band edges prevented individual excision of bands 3, 4, and 5 in some cases. These three bands were therefore isolated collectively. I.v. injection of mice with 300 ug protein corresponding to bands 3, 4, and 5, (in conjunction with an injection of $AgNO_3$, s.c., as an inflammatory stimulus) resulted in spleen amyloid deposition within 72 hours (Table 3.1). Animals treated with $AgNO_3$ and 300 ug protein electroeluted from either bands 1 or 2 did not

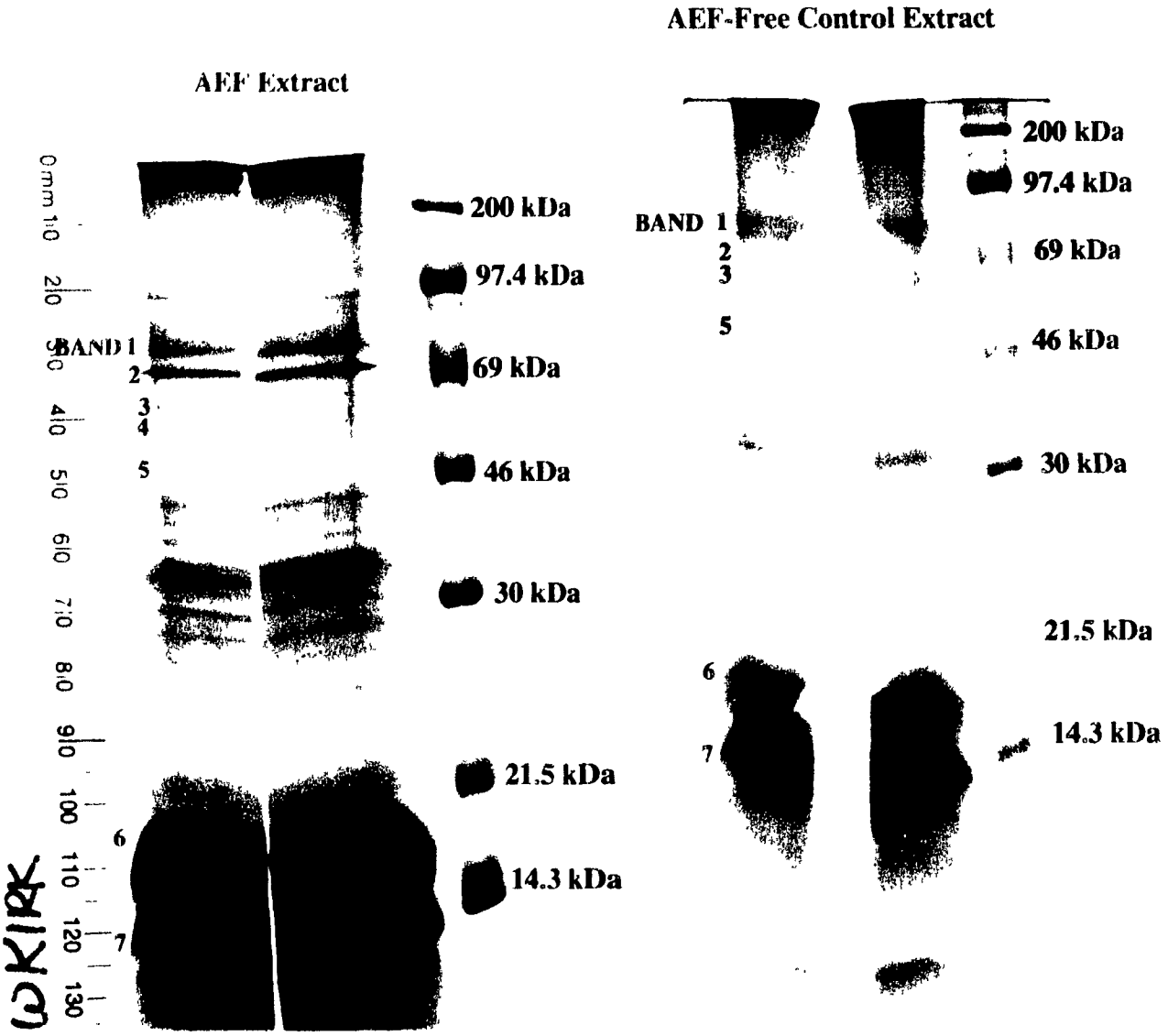

Figure 3.1 **Separation of AEF extract on 12.5% polyacrylamide preparative gels.** Strips excised from the vertical edges of preparative gels were stained with Coomassie blue R-250 (refer to Materials and Methods). Molecular weight markers were run in the small lanes appearing to the outermost left of each photograph. Bands 1 to 7 (as described in Results), as well as the region determined to contain protein responsible for AEF activity, are indicated. **A.** Electrophoretic separation of AEF extract prepared from the spleen of amyloidotic mice. **B.** Separation of "AEF-free" control extract prepared from the spleen of healthy animals. Note the absence of a detectable band 4 from the "AEF active" region in this case.

Table 3.1 Incidence of AEF Activity Indicated by Amyloid Deposition in Spleen Sections from Mice Treated with Protein Electroeluted from Preparative Gels

Treatment Group	Amyloid Deposition
Band 1	0/3
Band 2	0/3
Bands 3, 4, and 5	3/3
Band 6	0/3
Band 7	0/3

Note: Each treatment group received 300 ug of the indicated protein as described in Materials and Methods.

Refer to Fig. 3.1 for protein band designations.

Amyloid deposition was indicated on Congo Red stained spleen sections by characteristic green birefringence under polarized light (as described in Materials and Methods).

reveal amyloid deposition within 72 hours. These results indicate that the amyloid enhancing activity of our spleen extract is attributable to either bands 3, 4, and/or 5. We have labelled the region bound by bands 3 and 5 the "AEF-active" region of our electrophoretically separated sample (refer to Fig. 3.1).

2-D Gel Electrophoretic Resolution of the AEF-active Region

Following elution from preparative SDS gels, AEF active region proteins were analysed by two dimensional gel electrophoresis. This technique separates proteins according to two independent parameters, namely isoelectric point (pI) and molecular weight (13). Isoelectric focusing is carried out in the first dimension followed by SDS-PAGE in the second dimension. The similarity in protein pattern detected on both gels shown in Fig. 3.2 is indicative of the reproducibility of our results.

2-D analysis of protein electroeluted from the "AEF-active" region of multiple SDS-polyacrylamide preparative gels loaded with AEF extract revealed a protein pattern consisting of 13 distinguishable polypeptides (Fig. 3.2 **A**). The apparent pI of each polypeptide spot was estimated from migration distances based on the assumption of linear progression within the pH gradient in the first dimension which ranged from pH 5 to pH 8. They are, from spot 1 to spot 13 respectively: 6.2, 6.2, 6.9, 6.7, 5.6, 7.5, 7.1, 7.5, 7.1, 5.6, $\leq$ 5.0, 5.3, and $\leq$ 5.0.

2-D separation of protein eluted from the area corresponding to the AEF-active region (as determined relative to molecular weight markers) of SDS polyacrylamide preparative gels loaded with AEF-free extract is shown in fig. 3.2 **B**. Comparison by eye between **A** and **B** revealed that 3 distinct proteins (labelled 2, 3, and 4 in fig. 3.2) are detected in the AEF-active preparation only. All three proteins exhibit pI's just below 7.0 (6.2, 6.9, and 6.7 respectively) indicating that all three are slightly acidic. Their relative mobilities during SDS-polyacrylamide electrophoresis in the second dimension indicate their Mr's to be 12.0 kDa, 31.6 kDa and < 14.3 kDa respectively. Immunoblotting of both preparative gels and 2-D gels with anti-SAA antibodies (data not shown) showed no reaction thus eliminating the possibility of SAA contamination.

A number of spots (i.e. 1, 5, 6, 9, 10, 11, and 12) appear with greater intensity in the AEF-free control versus the AEF-active sample in Fig. 3.2. This is may be due to differences in the amount of material loaded and/or the quality of staining in each case. The large spot appearing in the lower right corner of each gel is likely an artifact of cathodic drift in the presence of heterogenous low M.W. protein products generated during electroelution and freeze/thawing of samples. Cathodic drift, which occurs due to the continuous passage of cathodic end components of the gel buffer into the resevoir buffer

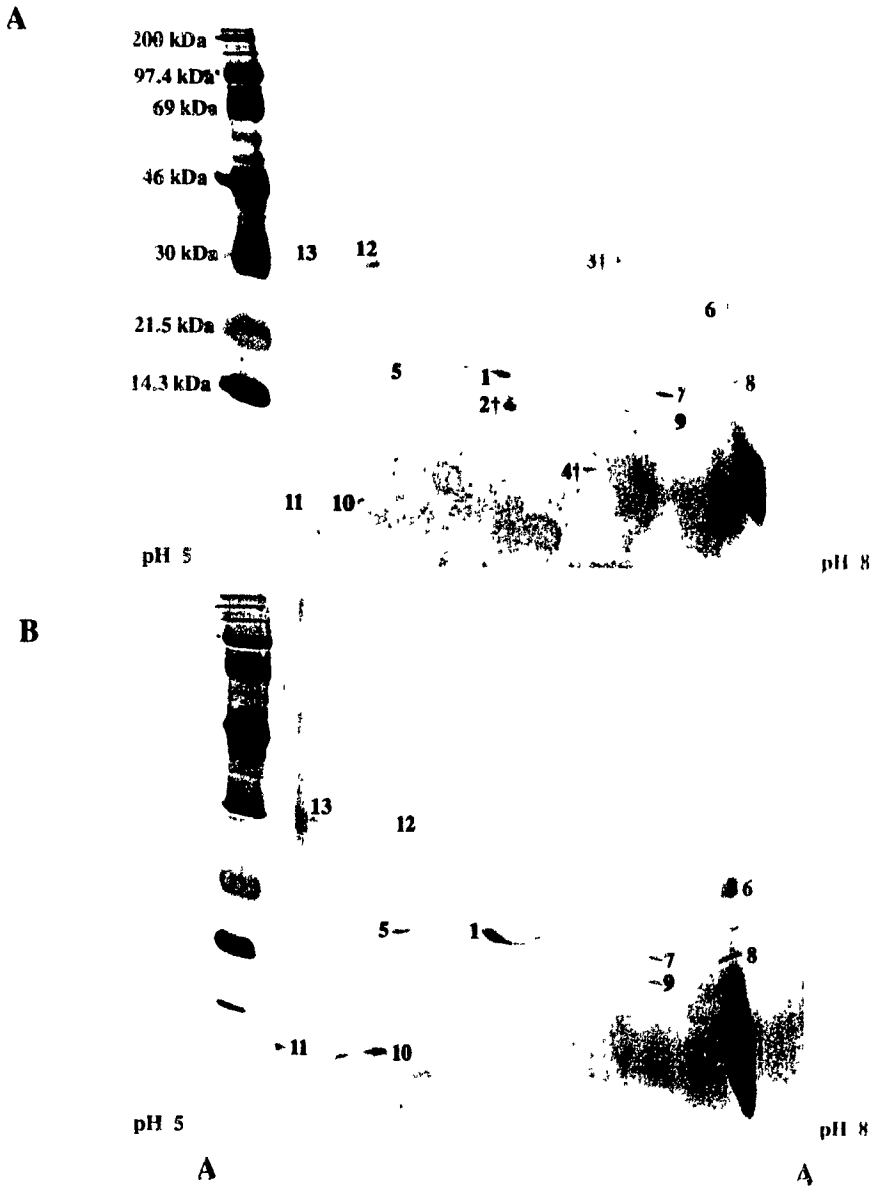

Fig. 3.2 **Two-dimensional gel electrophoretic resolution of the AEF-active region.** Proteins are separated according to pI by isoelectric focusing in the first dimension and according to molecular weight by SDS PAGE in the second dimension. Gels were loaded with: **A)** protein electroeluted from the "AEF active" region of prep gels and **B)** protein electroeluted from the corresponding region of AEF-free controls. Refer to Results for description of each sample. † indicates protein spots which are detected in the AEF-active preparation (**A**) but not in the control preparation (**B**).

and electroosmosis, can cause major distortions during isoelectric focusing resulting in the loss of resolution of basic proteins (14).

DISCUSSION

Despite the devotion of considerable research on the subject, the precise biological role of AEF in the pathogenesis of AA amyloidosis has yet to be determined. The elucidation of its amyloid-associated mechanism has been hindered to the greatest extent by the unavailability of a purified AEF preparation. Complete biochemical characterization of AEF in AA amyloidosis would not only indicate possible modes of action by comparison with known proteins, but would allow for the determination of whether AEF activity observed across the spectrum of amyloidotic disorders is attributable to a single factor, or rather a group of factors with similar function. The availability of purified AEF could also facilitate the development of *ex vivo* models of AA amyloid generation using tissue culture systems. Furthermore, should AEF appear to be an enzyme involved in amyloid fibril formation, studies examining its potency, kinetics, and active site may allow for therapeutic intervention with amyloid deposition. In addition, the production of AEF-specific antibodies could be useful for monitoring patients with pathological conditions which put them at risk of developing amyloidosis. Obviously, the possibilities for future research dependant on the determination of the nature of AEF are endless.

Electrophoretic analysis of our active AEF tissue extract under denaturing conditions revealed a relatively consistent composition of 7 significant bands. The estimated molecular weights of these bands (as indicated in Fig. 3.1) are contingent upon a constant SDS to protein binding ratio of 1.4 to 1 (15). Previous periodic acid Schiff's base staining and ionic-exchange HPLC analysis of our extract (Lise Hébert, unpublished observations) however, did not indicate the presence of sugars which are most likely to cause SDS binding modifications (16).

Histones are phylogenetically conserved, basic, chromosomal proteins found associated with nonhistone proteins and nucleic acids within eukaryotic nuclear chromatin (11). The inclusion of murine histone 3 (H3) protein (band 7) in our AEF extract, despite solubilization with urea and ß-mercaptoethanol, is most likely the result of its strong aggregative affinity for other proteins. The identity of band 7 as murine H3 is confirmed by the consistency of its Mr with the published 12.4 kDa molecular weight value for H3 (17).

With exception of band 4, electrophoretic analysis of "AEF-free" control extract revealed the same banding pattern as observed with tissue extracts containing AEF. The

absence of this 54.6 kDa band from the control prep indicates that it may be responsible for AEF activity. The remaining bands, namely 1, 2, 3, 5, 6, and 7, are common to both pathological and non-pathological tissue environments suggesting their presence does not necessarily result in amyloid fibril deposition The proteins occuring in these latter bands however, may well act as cofactors to the process of amyloid deposition.

The preceeding conclusions are supported by our examinations of AEF activity of specific protein bands eluted from SDS-polyacrylamide preparative gels Only protein eluted from the region of the gels containing bands 3, 4, and 5 revealed AEF activity. Though it is likely that band 4 is responsible for AEF activity, it would be interesting to determine whether the presence of one or both bands 3 and 5 (i.e. as cofactors) are necessary for amyloid deposition to occur.

2-D analysis of protein eluted from the "AEF-active" region (refer to results section) of multiple prep gels revealed a more complex polypeptide composition than expected. The detection of 13 distinct polypeptides from a starting material consisting of only 3 apparent protein bands is due, in part, to the greater resolving power of 2-dimensional separation techniques over electrophoresis within a single dimension. Individual protein species with similar molecular weights are not distinguishable by SDS-PAGE analysis alone. However, if such species differ in inherent pI, a combined IEF/SDS-PAGE procedure can separate them. Furthermore, the SDS-PAGE dimension of our 2-D analysis consisted of a polyacrylamide gradient which has a higher resolving power than SDS-PAGE with constant polyacrylamide composition.

Additionally, protein detection on our 2-D gels is enhanced by the chosen staining method. Silver stain, though innappropriate for preparative gels due to large protein loads, was used. Silver staining methods have been reported to have a 100-fold increased sensitivity over Coomassie Blue methods (18). It is probable that some protein species detected in our 2-D studies were indiscernable on our Coomassie Blue stained preparative gels.

The decrease in Mr general to all protein species as detected on our 2-D gels (recall the "AEF-active" region of preparative gels spans 63.2 kDa to 47.9 kDa) is probably the result of partial degradation during electroelution (i.e. by base-catalyzed proteolysis in CAPS buffer) and lyophilization.

It is unlikely that the increase in number of detected protein species in our 2-D analysis is due to the dissassociation of multimeric proteins into their respective subunits since ß-mercaptoethanol (i.e. as a reducing agent) was included in sample buffers used for preparative gel electrophoresis.

Horizontal and vertical streaking observed on these gels may be the result of electroendosmotic effects produced by grafting of the immobilized pH gradient used in isoelectrofocusing, into the SDS gel matrix. Electroendosmotic alterations can hinder protein transfer from the first to the second dimension ultimately causing streaks (19).

The detection of spots 2, 3, and 4 in AEF-active preparations, but not AEF-free controls, suggests any one of these may be the protein, or a polypeptide derivative of the protein, responsible for AEF activity. 2-D gels, prepared according to the methods described here, are easily amenable to microsequencing via transfer to polyvinylidene difluoride membranes. Proteins blotted onto these chemically inert membranes can be sequenced directly without additional manipulations (20, 21). Microsequencing of the polypeptides of interest has already been initiated. Whole or partial protein sequences generated in this manner will be used to search for protein identity (by comparison with available sequences stored in data banks) as well as for preparing specific DNA probes for cloning as yet uncharacterized proteins.

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Chapter 4

Macrophage Respiratory Burst Function in the Presence of Purified Amyloid Enhancing Factor

INTRODUCTION

In chapter 2 we considered the effects of AEF on macrophage activation and function during amyloidogenesis. These experiments were conducted using a relatively crude extract with AEF activity. The studies described in chapter 3 concentrated on purifying AEF contained within this extract. This fourth and final chapter examines the effects of *ex vivo* AEF treatment of peritoneal macrophages as a continuum of data collected in chapter 2, using the purified AEF preparation devised in chapter 3.

Our experiments measuring reactive oxygen metabolite production by splenic macrophages revealed that AEF dampens O_2^- production by $AgNO_3$-induced macrophages and may similarly affect H_2O_2 production. We speculated that AEF either acts in concert with factors induced or released during an inflammatory response or is able to exert an inhibitory effect only on those macrophages rendered responsive through inflammatory stimuli. In addition, we suggest that AEF may modulate O_2^- production by acting at the level of membrane components of the phagocyte superoxide generating system, for example by interfering with NADPH oxireductase cytochrome C activity.

By investigating the effect of AEF on isolated proteose peptone-elicited macrophages we hoped to clarify the nature of the interaction between AEF and the macrophage indeed to determine whether the modulatory effects of AEF are dependant only upon the presence of AEF and the inflammation-induced macrophage.

MATERIALS AND METHODS

Four treatment groups were included in each assay of oxygen radical production. These groups were treated with either AEF extract, with protein electroeluted from the"AEF-active" region of multiple SDS-PAGE preparative gels (as described in Chapter 2), or with a control, AEF-free preparation derived from the spleen of healthy mice. The fourth control group received no treatment.

Animals

C57Bl/6J male mice were obtained from Jackson Laboratories (Bar Harbor, ME.) or bred in our own facilities. Food and water were provided *ad libitum*. Animals were sacrificed by CO_2 narcosis followed by cervical dislocation.

Cells

Peritoneal exudate cells were harvested from mice by peritoneal lavage with 10 ml cold RPMI supplemented with glutamine and gentamycin 3-4 days following

intraperitoneal injection with 1 ml 10 % proteose-peptone (Difco, Detroit, MN.). The proteose peptone had been previously determined to contain <0.1 ng lipopolysaccharide/ml as measured by a chromogenic *Limulus* assay (M.A. Bioproducts). Cells from at least 8 mice were pooled for each assay. The cells were washed twice in ice-cold media then adjusted to 1.5 x 10^6 macrophages/ml in RPMI 1640 + 10% fetal calf serum. Cell differentiation was determined on cytospin slides (Shandon, Sewickley, PA) stained with Giemsa (DiffQuik, Canlab, Montreal, Qué.). Cells were plated on 24-flat bottom well plates (Becton Dickinson Labware, Montreal, Qué.) in 1 ml aliquots then purified by adherance at 37 C for 90 minutes. In each case, cell viability was determined by trypan blue stain exclusion to be > 90-95 %.

Measurement of Superoxide (O_2^{-}) Production

O_2^- production was monitored using the method described by Cohen et al (1). In brief, adherant macrophages were washed with Hanks balanced salt solution (HBSS) then incubated in 2% fetal calf serum in RPMI 1640 with 5 to 20 ug lyophilized AEF sample for 4 hours. Dried AEF samples (described in chapter 2) consisted of solubilised AEF extract, protein electroeluted from the "AEF-active" region of multiple SDS-PAGE preparative gels, or a control, AEF-free preparation derived from the spleen of healthy mice. Following treatment with AEF, cells were washed, and incubated with fresh reactive media (0.02% glucose, 0.1% cytochrome C in HBSS) and PMA (phorbol myristate, Sigma, 0.5 ug/10^6cells) for 60-90 minutes. The absorbance change at 550 nm was recorded from 1 ml aliquots of spent reactive media from each well. The protein content of each well was then determined following cell lysis with 0.5 N NaOH using the Bio-Rad protein assay kit (2). Results were recorded as nmoles O_2^-/mg protein/75 minutes.

RESULTS

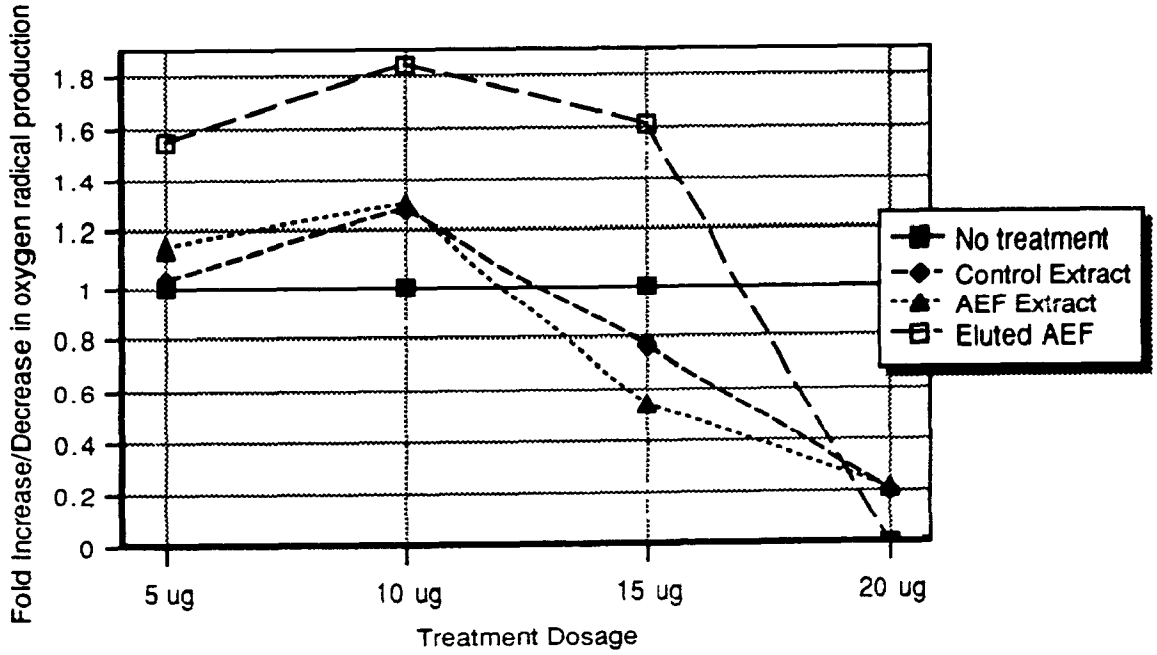
Results shown in Table 4.1 and figure 4.1 reveal that treatment with 5 or 10 ug of either our AEF extract or our AEF-free control sample had little effect on measurable O_2^- production by PMA-triggered macrophages. Notably, treatment of cells with similar doses of AEF protein eluted from the active region of our SDS preparative gels resulted in increases in O_2^- production as compared to control macrophages not treated prior to PMA triggering.

Table 4.1 Oxygen Radical Production by Peritoneal Macrophages Treated with either AEF Extract or Protein Eluted from the AEF-Active Region

5 ug	84.59	(7.17)	89.15	(9.45)	100.70	(5.08)	107.63	(8.44)
	60.65	(9.16)	61.13	(13.91)	65.26	(9.58)	117.74	(6.74)
10 ug	215.10	(38.02)	258.76	(29.11)	260.87	(4.86)	228.04	(57.85)
	61.54	(25.06)	94.65	(22.45)	98.43	(7.96)	281.38	(108.46)
15 ug	370.68	(32.64)	293.79	(55.26)	172.37	(58.65)	568.09	(17.99)
	239.51	(17.82)	177.5	(31.54)	157.94	(13.20)	413.63	(80.59)
20 ug	101.73	(13.79)	37.11	(11.32)	37.82	(2.36)	0	
	79.59	(40.00)	0		0		0	

Note: Mean values and standard deviations calculated from at least 3 assay samples.
For descriptions of both control and AEF extract as well as protein eluted from the AEF active region refer to the Materials and Methods section of this chapter.

Figure 4.1. Oxygen Radical Production by Peritoneal Macrophages Treated with either AEF Extract or Protein Eluted from the AEF-Active Region



Note: Fold increase/decrease values were obtained by comparing mean values calculated from at least 6 assay samples in each test group with untreated controls
For descriptions of both control and AEF extract as well as protein eluted from the AEF active region refer to the Materials and Methods section of this chapter.

With treatment doses of 15 ug, both the AEF extract and the AEF-free sample inhibited detectable O_2^- production. Cells treated with 15 ug eluted AEF active region protein however, again exhibited augmented O_2^- production.

When treated with 20 ug protein, cells in all groups produced little or no detectable O_2^-.

DISCUSSION

Cells of the macrophage lineage are considered the most likely cellular candidate responsible for degradation of SAA preventing the accumulation of amyloid fibrils within normal tissue (3, 4). During sustained inflammation macrophages become "activated", resulting in enhancement or initiation of certain function including phagocytosis, bactericidal and respiratory burst activity, and antigen presentation (5-8). In the context of chronic inflammation, the activated macrophage is therefore relevant to the development of AA amyloidosis.

The processs of macrophage activation involves a regulated series of changes in physiology which culminate in the ability to execute complex enzymatic functions (5-9). The ability of a macrophage to perform certain specific functions, possibly including the degradation of SAA, may therefore be dependant on its activation state. In light of the fact that the appearance of endogenous AEF is directly correlated with the deposition of amyloid fibrils (10), we suspect that AEF may indeed affect the ability of the macrophage to be appropriately activated for the degradation of the amyloid precursor, ultimately leading to the deposition of amyloid fibrils.

Our previous work (Chp. 2, 11) has shown that spleen macrophages derived from animals treated with an exogenous source of AEF, in conjunction with an inflammatory stimulus, exhibit decreased reactive oxygen metabolite production in comparison to production by spleen macrophages derived from animals treated with the inflammatory stimulus alone. In addition, we found that when AEF was administered in the absence of $AgNO_3$, (the inflammatory stimulus) no significant modification in metabolite release occured relative to steady state. The fact that the down-regulatory action of AEF is dependent upon a concomitant inflammatory stimuli suggests that AEF may act in concert with other factors induced or released during an inflammatory response or may exert an inhibitory effect only on macrophages rendered responsive through inflammatory stimuli. This would explain why AEF triggers AA fibril deposition only when administered in conjunction with an inflammatory stimulus. By exposing proteose-peptone elicited macrophages to AEF *ex vivo* we hoped to determine whether AEF-induced-modulation of

O_2^- production is dependant only upon the presence of AEF and the inflammation-induced macrophage.

Our observation of significantly decreased O_2^- production in the presence of higher doses of our AEF samples, including the control AEF-free preparation, suggests that inhibition of metabolite production observed at 20 ug and 15 ug may be caused by toxicity of the samples to macrophages. Trypan blue exclusion studies performed post-incubation with treatment media revealed only a slight decrease in viability at these concentrations (data not shown), however the ability of the macrophages to produce O_2^- may have been impaired.

Furthermore, it is possible that the observed stimulation of reactive oxygen metabolite production in the presence of protein prepared from the "AEF-active" region of SDS preparative gels is due to SDS contamination. This is suggested by the fact that treatment with non-toxic doses of simple AEF extract, which contains active AEF but has not undergone final purification procedures involving SDS-preparative gels, does not result in enhanced O_2^- production. The triggering of the respiratory burst in macrophages involves a series of molecular events which take place at the level of the plasma membrane (12). Macrophages are known to respond to a variety of stimuli including particles subject to phagocytosis and soluble agents capable of interacting with the cell membrane (13) SDS is an ionic detergent that can denature and solubilize membrane proteins when present at high concentrations. It is conceivable then that contaminant SDS may affect the conformation of cell surface proteins involved in O_2^- production thereby triggering the respiratory burst. Despite extensive dialysis of samples following elution in CAPS-methanol buffer, it is probable that a small quantity of SDS remained. Using acetone:methanol precipitation to recover protein following electroelution eliminates contaminatant SDS from the protein quite effectively (14). This procedure however, further denatures the already extensively processed AEF preparation and was deemed innappropriate for the preparation of a biologically active sample A possibility for further investigations into this question would be to conduct the O_2^- production assay on cells treated with protein electroeluted from the region of SDS gels of control, AEF-free sample corressponding to the "AEF-active" region of AEF sample-run gels. Preparation of such a control sample is greatly hindered by the fact that relatively little protein is contained within the appropriate region of the preparative gel. Inordinate amounts of healthy murine spleen are therefore required as starting material to produce the necessary microgram quantities of electroeluted, AEF-free end sample.

It is possible that the absence of AEF-associated down-regulation in O_2^- production observed in the experiments presented here is due to the fact that peritoneal macrophages

(versus spleen macrophages examined in chapter 2) were used. The ability of macrophages to produce oxygen metabolites can vary according to their site of isolation and extent of differentiation (15). Modifications within the oxygen metabolite production pathway occuring during site-specific macrophage differentiation could conceivably alter cellular susceptibility to the effects of AEF. Studies have shown however that coincubation of amyloidotic peritoneal macrophages and SAA results in amyloid fibril formation (16). We have therefore assumed that peritoneal macrophage function is in fact vulnerable to AEF-induced modulations associated with amyloid deposition.

Overall, data obtained during these experiments does not resemble data obtained with respect to the *in vivo* model of macrophage reactive oxygen metabolite production during AEF-induced amyloidogenesis described in chapter 2. This may indicate that elements other than the inflammation-associated macrophage and AEF are required to incur dampening of cellular O_2^- production or that active AEF is not present in sufficient concentration in our treatment preparations. In future, the complete characterization of AEF, allowing for its purification to homogeneity, will eliminate the possibility of intereference by unknown components included in current AEF preparations. Further oppurtunity for study lies in the investigation of the effects of adding specific factors known to be induced or released during an inflammatory response (i.e. acute phase proteins such as SAA or cytokines such as the interleukins 1 and 6 or tumor necrosis factor) to our *in vitro* assay system. These types of experiments may well lead to the elucidation of the elements involved in, as well as the mechanism characteristic of AEF-induced modulation of macrophage function.

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CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

DISCUSSION

Amyloidosis describes a heterogenous collection of diseases characterized by the extracellular deposition of proteinaceous amyloid fibrils of great chemical diversity. Nevertheless, the shared tinctorial and structural characteristics of all amyloid deposits suggest that amyloidosis occurs due to a single, common pathophysiological process. The consistent detection of AEF in a variety of amyloidotic tissue and its apparent causative role in amyloidogenesis suggests that AEF is an integral component of this common pathogenic process (1-5). Physiological processing of precursor proteins of various amyloids by activated macrophages, RE cells, or microglia has also been consistently implicated in amyloidogenesis (4, 6-9).

Our *in vivo* studies focusing on the relationship between AEF and reticuloendothelial cells found at the site of amyloid deposition have shown that AEF selectively affects some macrophage activation-associated functions. Examination of functions known to be enhanced in the activated macrophage revealed that while AEF does not affect spleen macrophage phagocytic or microbicidal activity whether administered alone or in conjunction with an inflammatory stimulus, it does dampen reactive oxygen metabolite production as measured by O_2^- production. When AEF was administered in the absence of an inflammatory stimulus no significant modulation in metabolite release was observed. The apparent dependence of AEF activity on a concominant inflammatory stimulus suggests that AEF either acts in concert with factors induced or released during an inflammatory response, or exerts an inhibitory effect only on macrophages rendered responsive through inflammatory stimuli. This would explain why AEF triggers AA fibril deposition only when administered in conjunction with an inflammatory simulus.

The acute phase state, characterized by the presence of particular mediators (i.e. IL-6), has in fact been associated with amyloidotic tissue environments in non-AA amyloidosis (10, 11). Future investigations elucidating the specific aspects of the APR necessary for amyloid fibril formation in the presence of AEF may reveal oppurtune areas for therapeutic intervention in amyloidogenesis, for example through the use of anti-inflammatory medications.

Further studies examining the effect of AEF on Ia surface expression (classically used as a marker of RE cell activation status [12-14]) revealed that increased Ia expression indicating inflammation-associated macrophage activation is blocked in the presence of AEF. As indicated by our respiratory burst studies, AEF seems to exert an inhibitory effect only in the event of a concomitant inflammatory stimulus. Taking these investigations a step further we then examined whether the observed suppression in macrophage Ia

expression played an integral part in altered SAA degradation leading to AA fibril deposition in the spleen of experimental animals. Our results show that restoration of surface Ia expression with IFN-gamma did not prevent production and deposition of AA fibrils in the spleen indicating that the mechanisms responsible for MHC antigen expression, though affected by AEF, are not directly involved in amyloidogenesis.

Our studies examining the hitherto elusive biochemical nature of AEF are on the brink of realization. Thus far SDS-PAGE analysis of AA amyloidotic spleen extracts coupled with concomitant AEF activity assays have pinpointed AEF activity to occur within a molecular weight range between 63.2 kDa and 47.9 kDa consisting of three distinct bands. Significantly, a single protein band with an Mr of 54.6 kDa was detected in AEF-active samples but not in AEF-free control preparations suggesting it as a possible AEF candidate.

2-D electrophoretic resolution of the region containing AEF activity revealed 13 distinct polypeptide spots suggesting that the three protein bands detected by in one dimensional SDS-PAGE analysis are comprised of greater than one protein species and that degradation events producing consistent polypeptide products has occurred. Three spots in particular have been identified to occur in active AEF samples but not AEF-free controls. Sequence characterization of these 3 spots, regardless of whether they represent whole or partial protein species, will be used to search for protein identity (by comparison with available sequences stored in databanks) as well as for preparing specific DNA probes for cloning as yet uncharacterized proteins. Both human and mouse cDNA libraries constructed from mRNA isolated from tissue known to produce AEF (i.e. the liver) are currently available. Obtaining cDNA clones corresponding to cellular transcripts of the AEF gene will be useful for obtaining the complete amino acid sequence of the protein, predicting specific structural or functional features of the protein by computer analysis, functional analysis of the protein in transfection experiments and site-directed mutagenesis, and elucidation of the control of the expression of this gene in normal and pathological situations.

Recent studies in our lab have led to the successful characterization of AEF isolated from the brain tissue of patients with Alzheimer's disease (unpublished results) and found it to correspond to myelin basic protein (MBP). Though our AA amyloid-associated preparation did not react with anti-MBP antibodies (data not shown), any similarities in sequence and/or function between MBP and AA amyloid-associated AEF (once characterized) will be highly indicative of specific features which attribute AEF activity to a protein.

The synthesis and action of AEF is a key element in the pathogenesis of amyloidosis. Elevated AEF levels have been shown to necessarily accompany amyloid deposition in the murine model of AA amyloidosis (3) Determining both the mode of action of AEF as well as its biochemical character are essential to the elucidation of the amyloidogenic process common to all forms of amyloidosis Once these objectives have been achieved it will be possible to investigate ways of inhibiting AEF synthesis or its action in triggering the process of fibril formation.

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CLAIMS TO ORIGINAL RESEARCH

1. I have established that AEF selectively affects certain activation-associated macrophage functions. Our *in vivo* studies show that while AEF, in the presence or absence of silver nitrate, has no apparent effect on the ability of spleen and liver macrophages to phagocytose or kill *L. monocytogenes*, it blocks enhanced respiratory burst function (as measured by O_2^- production).
2. I have demonstrated that treatment with AEF in conjunction with an inflammatory stimulus ($AgNO_3$) blocks the increase in number of splenic macrophages expressing Ia seen with the inflammatory stimulus alone. Furthermore, treatment with IFN-gamma was shown to restore decreased Ia expression in animals given AEF and $AgNO_3$. I have also shown that this restoration of Ia expression did not prevent amyloid A fibril deposition.
3. I have identified three candidate proteins (or polypeptide derivatives of these proteins, present in AEF-active preparations only, which may be responsible for AEF activity. I have presented these proteins on 2-D gels easily amenable to future microsequencing via transfer to polyvinylidene difluoride membranes.

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Finally, I could not finish without thanking my sister Lucy, without whom this work would never have reached the printed page.

APPENDIX 1

Manuscript of Paper Entitled:

Splenic Macrophage Activation and Functions In Amyloid Enhancing Factor-Induced Secondary Amyloidosis

Study of Phagocytosis, Killing, Respiratory Burst, and MHC II Surface Expression

Splenic macrophage activation and functions in amyloid enhancing factor-induced secondary amyloidosis Study of phagocytosis, killing, respiratory burst, and MHC class II surface expression

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Abstract Secondary amyloidosis is a systemic disease characterized by the extracellular tissue deposition of insoluble fibrillar amyloid A protein. Aberrant metabolism of serum amyloid A protein by reticuloendothelial cells is thought to result in the accumulation of fibrils within the tissue. Treatment of mice with amyloid-enhancing factor (AEF) in conjunction with an inflammatory stimulus (i.e., $AgNO_3$) induced amyloid deposition within 48–72 h. The activation state of a macrophage largely defines its cytomatic capabilities. In the studies reported here, we examined the effect of AEF on spleen macrophage activation using both functional and phenotypic assays. We found that while AEF in the presence or absence of $AgNO_3$ has no apparent effect on the ability of spleen and liver macrophages to phagocytose or kill *Listeria monocytogenes*, it appears to block enhanced respiratory burst function (as measured by O_2^- production) observed with $AgNO_3$ alone. AEF therefore seems capable of inhibiting certain macrophage activation-associated functions while not affecting others. Our activation phenotype studies, using surface Ia expression, reveal that AEF blocks the increase in number of splenic macrophages expressing Ia seen with $AgNO_3$ alone. Treatment with interferon-γ was found to restore decreased Ia expression in animals given AEF + $AgNO_3$ but did not prevent amyloid A fibril deposition. *J. Leukoc. Biol.* 53: 651–657, 1993.

Key Words. *reticuloendothelial cells · Ia · bactericidal action · reactive oxygen metabolites · FACS analysis*

INTRODUCTION

Amyloidosis is a generic term for a diverse group of diseases characterized by the extracellular tissue deposition of insoluble protein fibrils, often resulting in failure of the involved organs. Secondary amyloidosis is typified by the deposition of amyloid A protein (AA) derived from the carboxyl-terminal cleavage of its acute-phase serum precursor, serum amyloid A (SAA) [1, 2]. Secondary amyloidosis appears in conjunction with certain malignancies, chronic inflammatory disorders, or prolonged infection [3]. Experimental secondary amyloidosis is induced in laboratory animals by sustained inflammation or chronic bacterial or parasitic infections [4].

Amyloid-enhancing factor (AEF) is believed to play an integral role in the pathogenesis of amyloidosis. Although its complete biochemical characterization remains elusive, AEF is routinely extracted from the tissue of amyloidotic animals, where its activity is evident approximately 24 h before the histological detection of amyloid fibril [5]. The hallmark characteristic of AEF is its ability to accelerate AA fibril deposition. Although most experimental induction protocols require several weeks for the development of detectable amyloid deposits, administration of AEF in conjunction with an inflammatory stimulus results in fibril deposition within 24 to 72 h [6]. The ability of an exogenous source of AEF to shorten the induction period required for amyloid deposition suggests that it acts by precluding the lag time required for the endogenous production of AEF [7]. Furthermore, genetic analysis of amyloid recurrent and nonrecurrent strains of mice suggests that susceptibility to the development of experimental secondary amyloidosis is controlled by a single dominant gene [8]. This observation in conjunction with the finding that resistance in A/J mice can be overcome by intravenous (i.v.) injection of an exogenous source of AEF [9] further supports the role of AEF as a major determinant in the development of secondary amyloidosis.

Reticuloendothelial cells have long been recognized in close association with amyloid deposition [10]. Normal peripheral blood monocytes are known to be capable of mediating SAA degradation in vitro through the action of cell surface enzymes [11, 12]. Studies aimed at elucidating the exact relationship between macrophages or monocytes and the deposition of amyloid fibril have led to the discovery that mononuclear leukocytes from healthy individuals are capable of mediating complete SAA degradation, whereas the cells of amyloidotic patients produce an intermediate protein fragment, both the same size as AA and immunologically cross-reactive with AA [11]. In addition, normal murine Kupffer cells have been found to degrade SAA completely, whereas Kupffer cells from amyloid-induced animals produce the AA intermediate [13]. This observation has led to the hypothesis that tissue amyloid deposition may be due to inappropriate or incomplete SAA degradation by cells of the monocyte/macrophage lineage, resulting in the accumulation of AA.

In light of the obvious importance of both AEF and reticuloendothelial cells in the formation of amyloid deposits

Abbreviations: AA, amyloid A protein; AEF, amyloid enhancing factor; BSA, bovine serum albumin; IFN-γ, interferon-γ; IgG, immunoglobulin G; MHC, major histocompatibility complex; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; sAA, serum amyloid A; SPF, specific pathogen free.

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macrophage activation to arise from the production of endogenous AEF could cause aberrant SAA degradation by macrophages. Circumstantial evidence supporting our hypothesis is provided by the result of previous studies which showed that the appearance of AEF was correlated with the occurrence of fibril accumulation in vivo [9]. Further experiments carried out in our laboratory detected a decrease in splenic macrophage surface major histocompatibility complex (MHC) class II (I-A^b) expression that coincided with the appearance of endogenous AEF during induction of experimental secondary amyloidosis by extreme inflammation protocol (unpublished results). AEF could interfere with macrophage degradative processes by blocking activation pathways, rendering the ability of the macrophage to degrade the SAA complex. We therefore examined macrophage activation on a functional level as well as on a phenotypic level following the induction of secondary amyloidosis using AEF in combination with silver nitrate ($AgNO_3$) as an inflammatory stimulus.

Certain macrophage properties such as phagocytosis, bactericidal function, and respiratory burst functions are enhanced during activation [11–15] and are therefore good functional indicators of macrophage activation status. Our experiment compare the in vivo ability of liver and spleen cells to phagocytose and kill *Listeria monocytogenes* as well as the in vitro oxidative burst response (as indicated by O_2^- production) of splenic macrophages between amyloidotic animals treated with $AgNO_3$ in conjunction with AEF and animals treated with $AgNO_3$ alone. In addition, we examined the differential expression of Ia antigen by macrophages isolated from the spleens of mice treated with AEF and $AgNO_3$ by using flow cytometry and immunofluorescence analysis. The enhanced expression of MHC class II molecules may be used as a marker of macrophage activation [12, 18]. Our aim was to determine whether treatment with AEF and silver nitrate causes a change in macrophage surface Ia expression compared with treatment with the inflammatory stimulus alone and if so, whether any such change could reflect an alteration in the degradation pattern of the SAA precursor leading to AA amyloid fibril deposition.

MATERIALS AND METHODS

Animals

Specific pathogen-free (SPF) C57Bl/6NHsd male mice, 8–12 weeks old, were either obtained from Harlan Sprague Dawley (NHsd, Indianapolis, IN) or bred in our own SPF facilities. Food and water were provided ad libitum. Animals were sacrificed by CO_2 narcosis followed by cervical dislocation.

Preparation of amyloid-enhancing factor and induction of amyloidosis

AEF was prepared from the spleens of C57BL/6 mice, injected subcutaneously (s.c.) for 21 days with 13% casein (United States Biochemical, Cleveland, OH). AEF was extracted from homogenized spleens in ice-cold 30% glycerol–10 mM Tris HCl, pH 7.0, buffer as previously described [6]. A 0.5-mg portion of the lyophilized AEF product (an amount previously determined to induce significant amyloid deposition within 24–48 h [9]) was resuspended in 0.2 ml of sterile phosphate buffered saline (PBS) and administered via i.v. injection concomitant with 0.5 ml s.c. of 2% $AgNO_3$ solution. 2 ml of 2% $AgNO_3$ solution or 0.5 mg of AEF alone. They were housed in the same facilities and were sacrificed after the same time periods as experimental mice.

Detection of secondary amyloid deposits

At least a portion of each spleen was fixed in buffered 10% formalin, embedded in paraffin, sliced in 5-μm sections, and then stained with alkaline Congo Red [19]. The presence of amyloid deposits was determined microscopically by the detection of green birefringence characteristic of AA fibrils under polarized light.

In vivo phagocytosis and bactericidal activity

Three days after the injection of AEF alone, $AgNO_3$ alone, or both AEF and $AgNO_3$, experimental animals were inoculated with 6×10^6 colony-forming units of *L. monocytogenes*. The livers and spleens of infected animals were tested for their ability to phagocytose and kill *L. monocytogenes* by determining the number of viable bacteria in individual organs 30 min or 6 h, respectively, after infection [14]. Spleens and livers were removed aseptically and homogenized in sterile saline for about 1 min with a Tri-R Stir-R homogenizer (Tri-R instruments, Rockville Centre, NY). Serial 10-fold dilutions of these homogenates were plated on tryptose agar (1% Bacto-agar, Difco Laboratories, Detroit, MI) and colony counts were routinely executed after 18–24-h incubations at 37°C. Five animals per group were tested.

Spleen macrophage O_2^- production

Responses were examined in splenic macrophages from naive mice as well as mice treated with AEF alone, $AgNO_3$ alone, or AEF and $AgNO_3$ 3 days previously. Internal experimental positive controls consisted of proteose peptone (PP)–elicited peritoneal macrophages.

Spleen cells were flushed from isolated spleens with ice-cold RPMI 1640 supplemented with 0.3 mg/ml glutamine and 50.0 ng/ml gentamicin. Red blood cells were lysed by hypotonic shock on ice. Cell differentiation was determined on cytospin slides (Shandon, Sewickley, PA) stained with Giemsa (DiffQuik, Canlab, Montreal, Quebec). Each cell suspension was adjusted to 1.5×10^6 macrophages/ml in RPMI 1640 + 10% fetal calf serum. Macrophages were plated in 6- or 24-flat bottom well plates (Linbro, Flow Laboratories, McLean, VA) and purified by adherence overnight at 37°C, 5% CO_2. Each cell suspension was assayed in triplicate. In all instances, cell viability as established by trypan blue exclusion test was >90–95%. The cell monolayers were >90% macrophages as determined morphologically by Giemsa stain and phagocytosis of *L. monocytogenes*.

Production of O_2^- was measured following treatment with phorbol myristate acetate (PMA, 0.5 μg/10^6 cells) for 75 min by the method of Cohen and Chovaneic [20]. The protein content of each well was then determined following cell lysis with 0.5 N NaOH using the Bio-Rad protein assay kit [21]. Results are reported as nmol O_2^-/mg protein/75 min ± SEM. Statistical analysis was done by a two-tailed Mann-Whitney U test with a present probability level of $\alpha = 0.05$. Mean values represent data from three experiments.

Ia surface expression

After aseptic removal, each spleen was flushed with ice-cold RPMI 1640 supplemented with glutamine and gentamicin

washed by centrifugation (4°C) and resuspended in 30 ml of ice-cold RPMI 1640. Enumeration of cells and determination of viability (by stain exclusion) were accomplished using a hemocytometer and Turks or trypan blue stain respectively. Slides for cell differential counts were prepared as previously described. Following resuspension at a final concentration of 1–2 × 10^6 cells/ml with 0.5% bovine serum albumin (BSA) in PBS, 1 × 10^6 cells were aliquoted to 5 ml polystyrene round bottom tubes (Becton Dickinson Labware, Montreal) for immunofluorescent labeling. After incubation with goat immunoglobulin G (IgG) (Sigma, St. Louis, MO) to reduce nonspecific binding by blocking Fc receptors, cells were labeled successively with monoclonal antimurine Mac-1 antibody (clone M1/70, Boehringer Mannheim Biochemical, Laval, Quebec) phycoerythrin conjugated goat F(ab')$_2$ anti-rat IgG (Caltag Laboratories, San Francisco) and fluorescein isothiocyanate conjugated murine anti-Ia IgG (Cedarlane Laboratories). Each incubation with labeling antibody (at 1/20 dilution as determined optimal by serial titration) was carried out for 15 min on ice. The appropriate control labelings were prepared to correct for autofluorescence, nonspecific binding, and inhibition of binding due to steric hindrance for each experiment group. Labeled cells were fixed in ice-cold paraformaldehyde and kept at 4°C until analyzed on a FACScan flow cytometer with the Lysis II Program (Becton Dickinson) the following day. When indicated, interferon γ (IFN-γ) treatment consisted of 5000 units of rat recombinant IFN-γ (Amgen, Thousand Oaks, CA) administered ip on days 0 and 2 of the experimental design.

RESULTS

In vivo phagocytosis and bactericidal activity during AEF-induced secondary amyloidosis

To determine whether the ability of splenic and hepatic macrophages to phagocytose and kill *L. monocytogenes* is modified during AA fibril deposition, groups of mice were injected [illegible] mice injected with AEF or AgNO$_3$ alone.

Phagocytosis by spleen and liver cells was measured 30 min after the [illegible] infection. [illegible] development of transient neutropenia seen in AEF + AgNO$_3$ treatment was found not to [illegible] with increased bacterial uptake compared with naive animals or animals treated with AEF or AgNO$_3$ alone (Fig. 1). Approximately 5% of the bacterial inoculum was taken up by the spleen and liver in the first 30 min after infection.

The bactericidal [illegible] of macrophages [illegible] after the injection of [illegible] were not [illegible] and modulated in the liver of AEF + AgNO$_3$ treated animals despite marked depression after each of the [illegible] animals or animals treated with AEF or AgNO$_3$ alone (Fig. 1). All groups were shown to be capable of [illegible] approximately 85–90% of the bacterial load within the first 6 h following infection. No significant bactericidal activity was observed during the same time period in the [illegible] of both control and experimental animals.

O_2^- production in AEF induced secondary amyloidosis

To determine whether the [illegible] function of splenic macrophages is altered during AA fibril deposition due to AEF O_2^- production by cells isolated from mice 5 days after treatment with AEF, AgNO$_3$ or either agent alone was measured (Table 1). Splenic macrophages from mice injected with AgNO$_3$ alone (a non-amyloidogenic treatment) released a significantly higher concentration of O_2^- anion than those (following stimulation in vitro with PMA) from the cells obtained from the untreated control group. Period of 5 days when animals were treated with both AEF and AgNO$_3$, the ability of their spleen macrophages to release O_2^- was significantly decreased compared with that of cells isolated from the AgNO$_3$ treatment group. These results suggest that in an amyloid-inducing treatment AEF partially blocks AgNO$_3$-induced O_2^- production by spleen macrophages.

This pattern of partial inhibition also occurred at the level of H_2O_2 production. Again, the amyloidogenic treatment

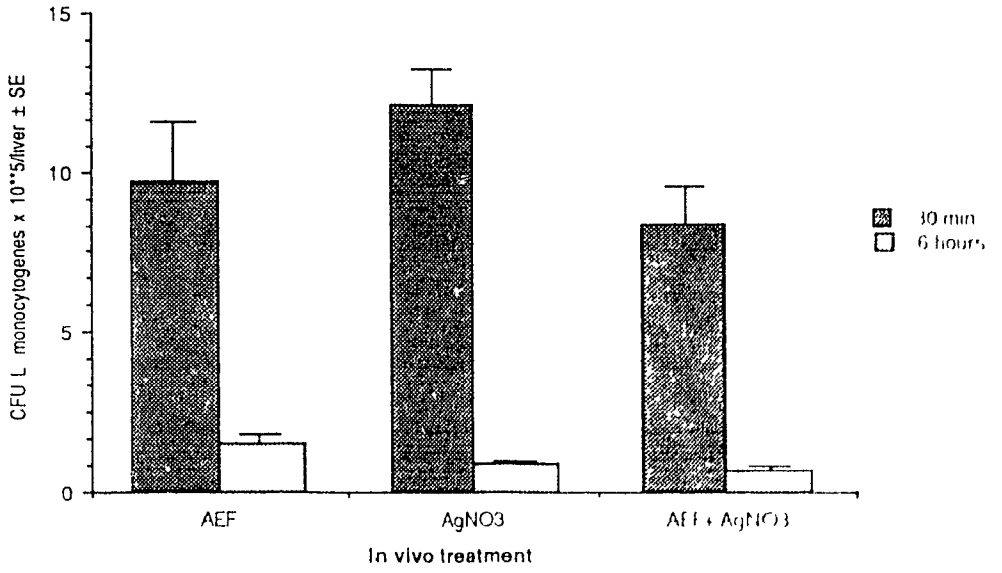

Fig. 1 In vivo phagocytosis and bactericidal activity measured in the liver of mice treated with AEF and/or AgNO$_3$ compared with mice treated with AgNO$_3$ or AEF alone. Mice were treated with AEF and/or AgNO$_3$ 3 days before being infected with 4 × 10^6 *L. monocytogenes*. The in vivo phagocytic ability of the liver cells was determined 30 min after infection and the in vivo bactericidal activity was measured 6 h later, as described in "Material and Methods". Normal mice displayed phagocytic and bactericidal activities similar to those observed in mice treated with AEF and/or AgNO$_3$.

TABLE 1 O_2^- Production by Macrophages in AEF-Induced Secondary Amyloidosis

Treatment group	O_2^- production[a] nmoles/ml per 45 min ± SEM
Control	92.47 ± 9.90
AEF alone	110.52 ± 17.90
$AgNO_3$ alone	506.26 ± 32.54
AEF and $AgNO_3$	217.83 ± 32.29

[a] O_2^- production was measured according to the method described by Cohen and Chovaniec [20]. Results obtained represent means ± SEM of three experiments (each done in triplicate).

[b] $P < 0.01$ (Student's t test) when value obtained with AEF alone was compared with value obtained with $AgNO_3$ alone.

was accompanied by a decrease in the $AgNO_3$-induced release of the relevant metabolite. Data obtained for H_2O_2 release, however, did not reach significance.

Ia surface expression during AEF-induced secondary amyloidosis

The injection of a combination of AEF and $AgNO_3$ (as an inflammatory stimulus) is known to induce amyloid fibril deposition in mice within 24–48 h [5]. $AgNO_3$ alone was injected into mice in a parallel group to provide a basis of comparison of the level of activation of splenic macrophages harvested from animals subjected to an inflammatory stimulus in the absence of AEF. Using a double immunofluorescence protocol, we determined the level of surface Ia antigen expression on Mac-1$^+$ spleen cells by flow cytometry, as a means of monitoring the activation state of the macrophages, 3 days after treatment of mice in vivo. Results presented in **Figure 2** reveal that maximal surface expression of Ia antigens by splenic macrophages occurred in response to the injection of $AgNO_3$ alone, which does not produce amyloid deposits in the tissues. When this treatment was combined with an injection of amyloid-inducing AEF, however, there was complete abrogation of the augmented macrophage Ia expression. Control preparations of AEF-free spleen extracts did not interfere with the inflammation-induced Ia expression, suggesting that the observed down-regulation in surface Ia is a specific effect of AEF. AEF treatment without concomitant $AgNO_3$ treatment caused a slight but insignificant increase in Ia expression compared with untreated animals (Fig. 2).

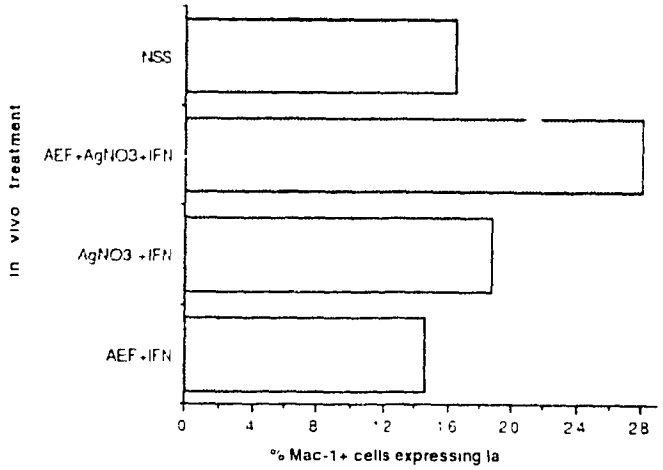

Fig. 3. Restoration of spleen macrophage Ia expression in mice treated with AEF and $AgNO_3$ by in vivo IFN-γ treatment. Spleen cells isolated from mice 3 days after in vivo treatments indicated in the figure (described in Materials and Methods) were prepared and analyzed as described in the legend of Figure 2. One to three groups (of at least three mice each) were tested for each treatment.

Having established that AEF may indeed down-regulate the normal macrophage response to activating agents, at least at the Ia level, we examined the possibility that the observed down-regulation may be related to a change in the degradation pattern of SAA leading to AA amyloid fibril deposition. By combining an injection of IFN-γ (in a dose previously determined to be sufficient to induce increased Ia expression by splenic macrophages in C57Bl/6 mice, unpublished results) with our AEF-$AgNO_3$ treatment protocol, we

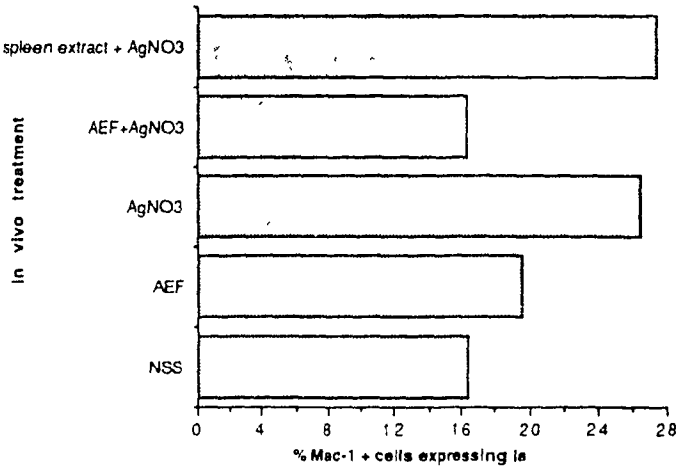

Fig. 2. Modulation of spleen macrophage Ia expression from normal steady state in mice rendered amyloidotic by treatment with AEF and $AgNO_3$ compared with mice treated with $AgNO_3$ or AEF alone, or a control, AEF-free spleen extract and $AgNO_3$. Spleen cells were isolated from mice 3 days after the indicated treatments. Cells were labeled with Ia and Mac-1 specific antibodies, as described in Materials and Methods, prior to analysis by flow cytometry. This double antibody label system distinguishes macrophages expressing Ia antigen on their surface (Mac-1$^+$Ia$^+$) from B cells (Mac-1$^-$Ia$^+$) and surface Ia negative macrophages (Mac-1$^+$Ia$^-$). Granulocytes and natural killer cells, which express only small quantities of Mac-1 antigen, are weakly Mac-1$^+$ relative to macrophages [22] and therefore do not stain intensely enough to be included in Mac 1$^+$ determinations. Scatter gates were set to exclude dead cells and debris. Results are expressed as a percentage of Mac-1$^+$ cells that are Ia$^+$. As indicated on the x axis, analysis of spleen cells from untreated, control mice revealed that 16.5% of Mac 1$^+$ spleen cells are Ia$^+$ under normal steady state (NSS) conditions. One to three groups (of at least three mice each) were tested for each treatment.

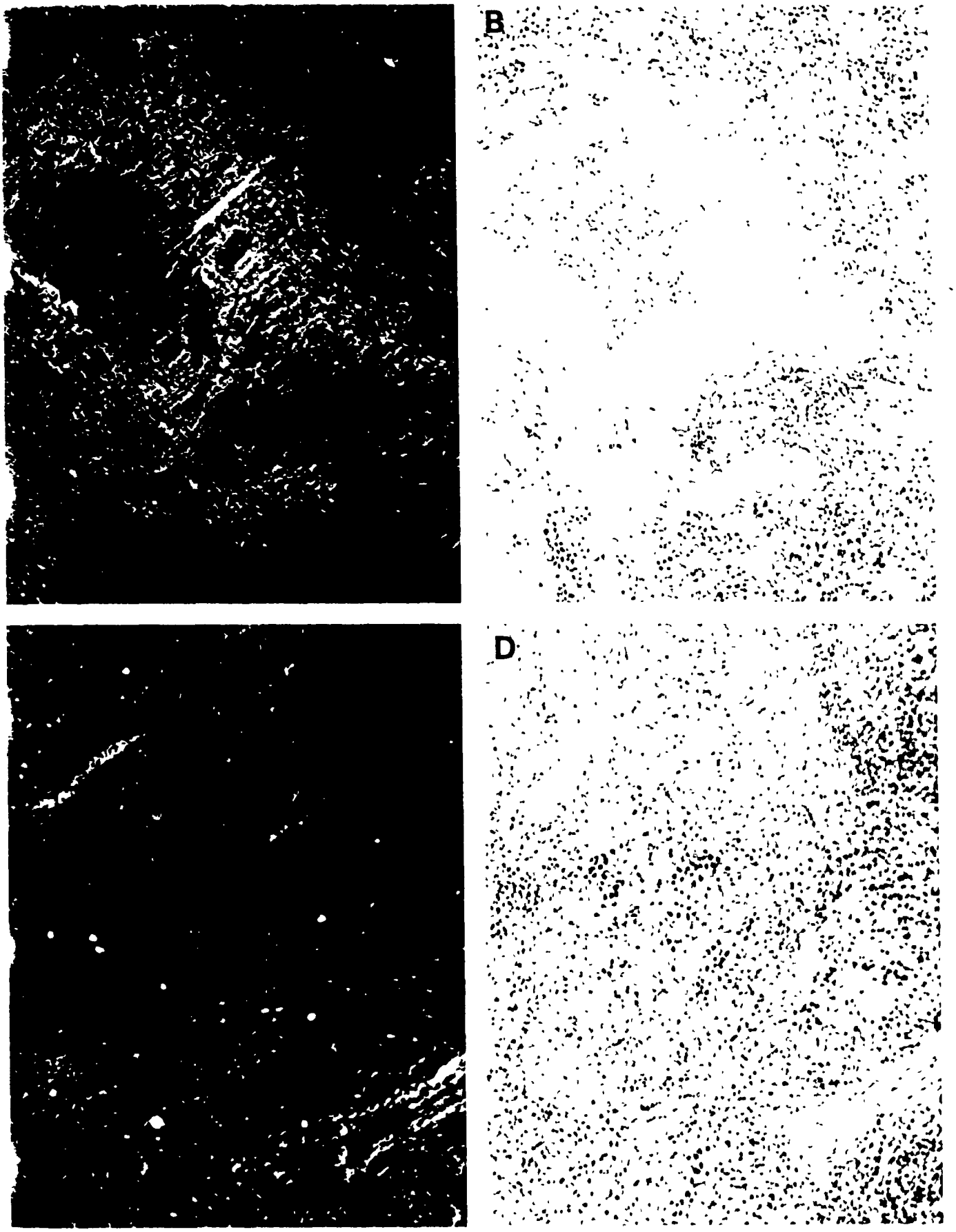

Fig. 4 Detection of amyloid deposition by Congo Red stain. Amyloid fibril deposits within spleen sections obtained from an AEF + $AgNO_3$ treated animal exhibit characteristic green birefringence under polarized light (A) when stained with Congo Red. Spleen sections from normal animals do not exhibit birefringence under polarized light (C). (B and D) Congo Red-stained spleen sections from amyloidotic and untreated mice, respectively, observed under a normal microscope. Spleen sections were prepared as described in Materials and Methods.

TABLE 2. Incidence of Amyloid Deposition in Spleen Sections from Mice Treated with AEF, $AgNO_3$, and IFN-γ

Treatment group	Amyloid deposition
AEF alone	0/3
None	0/4
AEF and IFN-γ	0/9
Control AEF plus $AgNO_3$	0/3
AEF and IFN-γ	0/3
$AgNO_3$ and IFN-γ	0/6
AEF, $AgNO_3$, IFN-γ	0/6

Treatment groups as described in Materials and Methods.
Sections were stained with Congo Red as described in Materials and Methods. Amyloid fibril deposition was detected by characteristic green birefringence under polarized light.

were able to restore Ia expression to levels seen with $AgNO_3$ alone (Fig. 3). Histological examination of portions of spleen retrieved from mice treated with all three agents, however, revealed that despite the IFN-γ-induced restoration of macrophage Ia expression in mice administered AEF and $AgNO_3$, splenic amyloid fibril deposition still occurred (**Fig. 4a, Table 2**). This finding suggests that AEF-induced down-regulation of splenic macrophage activation observed at the level of Ia expression is not related to aberrant SAA degradation leading to AA amyloid fibril deposition.

DISCUSSION

Based on studies reported in the literature [11, 13], we postulate that the degradation of amyloid precursors by macrophages is a critical event that prevents the deposition of amyloid fibrils in normal tissues. The process of macrophage activation involves a regulated series of changes in physiology that culminate in the ability to execute complex enzymatic functions [18–22]. The ability of a macrophage to perform certain specific functions, possibly including the degradation of SAA, may therefore be dependent on its activation state. In light of the fact that the appearance of endogenous AEF is directly correlated with the deposition of amyloid fibrils [5] and our preliminary observation that splenic macrophage surface Ia expression is decreased during the induction of amyloid fibril deposition (unpublished results), we suspected that AEF may indeed affect the ability of the macrophage to be appropriately activated for the degradation of the amyloid precursor, leading to the deposition of amyloid fibrils.

Because the mechanism by which SAA and macrophages interact remains unknown, we focused our studies on functions known to be enhanced in the activated macrophage. Macrophage phagocytic and microbicidal activity was monitored by the in vivo uptake and killing of the facultative intracellular pathogen *L. monocytogenes*, which has been shown to be processed by macrophages [14]. Our results indicate that AEF, whether administered alone or in conjunction with $AgNO_3$, does not affect either capacity.

Primed and activated macrophages exhibit increased release of reactive oxygen metabolites [23]. Treatment of animals with an inflammatory agent, such as $AgNO_3$, results in enhanced macrophage oxidative metabolism [15]. Our experiments measuring reactive oxygen metabolite production by splenic macrophages reveal that AEF damps O_2^- production by $AgNO_3$-induced macrophages and may similarly affect H_2O_2 production. At this point, one can only speculate about the mechanism by which AEF modulates O_2^- production. It is conceivable, however, that AEF inhibition occurs at the level of membrane components of the phagocyte superoxide-generating system, for example, by interfering with NADPH oxireductase cytochrome *c* activity. When AEF was administered in the absence of $AgNO_3$, no significant modification in metabolite release was observed as compared with steady state, suggesting that the down-regulatory action of AEF is dependent on a concomitant inflammatory stimulus. It is probable that AEF either acts in concert with other factors induced or released during an inflammatory response or exerts an inhibitory effect only on macrophages rendered responsive through inflammatory stimuli. This would explain why AEF triggers AA fibril deposition only when administered in conjunction with an inflammatory stimulus.

Having thus determined that AEF may selectively affect some macrophage activation-associated functions but not others, we chose to examine the effect of AEF on macrophage activation at the phenotypic level. Increased MHC class II (Ia) surface expression is considered a marker of macrophage activation [18, 24, 25] and is therefore useful for monitoring macrophage activation status. We first compared surface Ia expression by splenic macrophages harvested from mice subjected to amyloid deposit-inducing and -noninducing treatment protocols. Our data indicate that $AgNO_3$ treatment alone ineffective in causing amyloidosis, results in splenic macrophage activation as reflected by enhanced Ia expression over normal steady state. This increased expression is damped or blocked in the presence of AEF. The absence of any such "block" in Ia expression in mice treated with AEF-free spleen extract (obtained from control, untreated mice) in conjunction with $AgNO_3$ suggests that decreased Ia expression is attributable to AEF and not some contaminant in our AEF preparation. Furthermore, it is interesting to note that AEF treatment alone revealed a slight increase in Ia expression levels over normal steady state. As indicated by our respiratory burst studies, AEF seems to exert an inhibitory effect only in the event of a concomitant inflammatory stimulus. Again, this observation may be related to the fact that AEF is amyloidogenic only when administered in conjunction with an inflammatory stimulus.

Having thus linked decreased splenic macrophage Ia expression to AEF, we looked at whether the observed suppression of macrophage Ia expression in the presence of AEF played an integral role in altered SAA degradation leading to AA fibril deposition. By counteracting AEF-induced down-regulation of splenic macrophage Ia expression with IFN-γ, we were able to show that the effect of AEF on macrophage activation status during the induction of amyloidosis at the level of Ia expression is not necessary for the production and deposition of AA fibrils in the spleen. It is possible that IFN-γ increases Ia expression by a different pathway than that which may be blocked by AEF. Studies of the regulation of macrophage surface Ia expression suggest that at least four activation-associated transductional signal cascades (e.g., cAMP cascade, Ca^{2+}, and protein kinase C cascades) may be important for its regulation [18, 26]. It is therefore conceivable that Ia expression could be restored by IFN-γ despite continued inhibition of an alternative cascade by AEF. Should this alternative transduction cascade also mediate SAA degradation (i.e., by transcriptional regulation of SAA metabolic enzyme genes), the end result could be that AEF blockage of one macrophage activation pathway (causing aberrant SAA degradation) could persist despite replenished Ia expression due to IFN-γ.

These studies have established that, in the presence of an inflammatory stimulus, AEF selectively modifies certain aspects of the activated splenic macrophage. It is probable

hat AEF induces amyloid fibril deposition by altering the acrophage function responsible for normal or aberrant egradation of SAA Studies such as those described here will prove most revealing when the exact nature of this function has been characterized

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